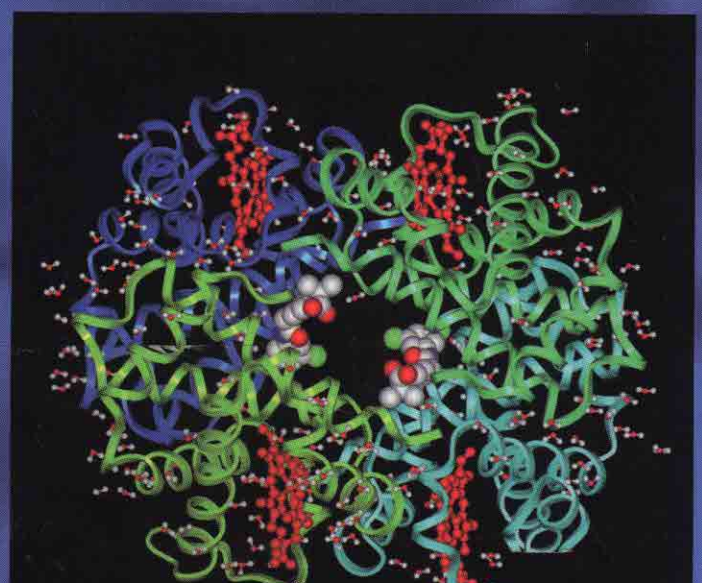
BURGER'S

Sixth Editi

Medicinal & Chemistry Drug Discovery

Edited by

Donald J. Abraham



VOLUME

Autocoids, Diagnostics, a

Drugs from New Biolo

BURGER'S MEDICINAL CHEMISTRY AND DRUG DISCOVERY

Sixth Edition

Volume 4: Autocoids, Diagnostics, and Drugs from New Biology

Edited by

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BURGER MEMORIAL EDITION

The Sixth Edition of Burger's Medicinal Chemistry and Drug Discovery is being designated as a Memorial Edition. Professor Alfred Burger was born in Vienna, Austria on September 6, 1905 and died on December 30, 2000. Dr. Burger received his Ph.D. from the University of Vienna in 1928 and joined the Drug Addiction Laboratory in the Department of Chemistry at the University of Virginia in 1929. During his early years at UVA, he synthesized fragments of the morphine molecule in an attempt to find the analgesic pharmacophore. He joined the UVA chemistry faculty in 1938 and served the department until his retirement in 1970. The chemistry department at UVA became the major academic training ground for medicinal chemists because of Professor Burger.

Dr. Burger's research focused on analgesics, antidepressants, and chemotherapeutic agents. He is one of the few academicians to have a drug, designed and synthesized in his laboratories, brought to market [Parnate, which is the brand name for tranylcypromine, a monoamine oxidase (MAO) inhibitor]. Dr. Burger was a visiting Professor at the University of Hawaii and lectured throughout the world. He founded the *Journal of Medicinal Chemistry, Medicinal Chemistry Research,* and published the first major reference work *"Medicinal Chemistry"* in two volumes in 1951. His last published work, a book, was written at age 90 *(Understanding Medications: What the Label Doesn't Tell You,* June 1995). Dr. Burger received the Louis Pasteur Medal of the Pasteur Institute and the American Chemical Society Smissman Award. Dr. Burger played the violin and loved classical music. He was married for 65 years to Frances Page Burger, a genteel Virginia lady who always had a smile and an open house for the Professor's graduate students and postdoctoral fellows.

PREFACE

The Editors, Editorial Board Members, and John Wiley and Sons have worked for three and a half years to update the fifth edition of Burger's Medicinal Chemistry and Drug Discovery. The sixth edition has several new and unique features. For the first time, there will be an online version of this major reference work. The online version will permit updating and easy access. For the first time, all volumes are structured entirely according to content and published simultaneously. Our intention was to provide a spectrum of fields that would provide new or experienced medicinal chemists, biologists, pharmacologists and molecular biologists entry to their subjects of interest as well as provide a current and global perspective of drug design, and drug development.

Our hope was to make this edition of Burger the most comprehensive and useful published to date. To accomplish this goal, we expanded the content from **69** chapters (5 volumes) by approximately 50% (to over **100** chapters in **6** volumes). We are greatly in debt to the authors and editorial board members participating in this revision of the major reference work in our field. Several new subject areas have emerged since the fifth edition appeared. Proteomics, genomics, bioinformatics, combinatorial chemistry, high-throughput screening, blood substitutes, allosteric effectors as potential drugs, COX inhibitors, the statins, and high-throughput pharmacology are only a few. In addition to the new areas, we have filled in gaps in the fifth edition by including topics that were not covered. In the sixth edition, we devote an entire subsection of Volume 4 to cancer research; we have also reviewed the major published Medicinal Chemistry and Pharmacology texts to ensure that we did not omit any major therapeutic classes of drugs. An editorial board was constituted for the first time to also review and suggest topics for inclusion. Their help was greatly appreciated. The newest innovation in this series will be the publication of an academic, "textbook-like" version titled, "Burger's Fundamentals of Medicinal Chemistry." The academic text is to be published about a year after this reference work appears. It will also appear with soft cover. Appropriate and key information will be extracted from the major reference.

There are numerous colleagues, friends, and associates to thank for their assistance. First and foremost is Assistant Editor Dr. John Andrako, Professor emeritus, Virginia Commonwealth University, School of Pharmacy. John and I met almost every Tuesday for over three years to map out and execute the game plan for the sixth edition. His contribution to the sixth edition cannot be understated. Ms. Susanne Steitz, Editorial Program Coordinator at Wiley, tirelessly and meticulously kept us on schedule. Her contribution was also key in helping encourage authors to return manuscripts and revisions so we could publish the entire set at once. I would also like to especially thank colleagues who attended the QSAR Gordon Conference in **1999** for very helpful suggestions, especially Roy Vaz, John Mason, Yvonne Martin, John Block, and Hugo

Kubinyi. The editors are greatly indebted to Professor Peter Ruenitz for preparing a template chapter as a guide for all authors. My secretary, Michelle Craighead, deserves special thanks for helping contact authors and reading the several thousand e-mails generated during the project. I also thank the computer center at Virginia Commonwealth University for suspending rules on storage and e-mail so that we might safely store all the versions of the author's manuscripts where they could be backed up daily. Last and not least, I want to thank each and every author, some of whom tackled two chapters. Their contributions have provided our-field with a sound foundation of information to build for the future. We thank the many reviewers of manuscripts whose critiques have greatly enhanced the presentation and content for the sixth edition. Special thanks to Professors Richard Glennon, William Soine, Richard Westkaemper, Umesh Desai, Glen Kellogg, Brad Windle, Lemont Kier, Malgorzata Dukat, Martin Safo, Jason Rife, Kevin Reynolds, and John Andrako in our Department of Medicinal Chemistry, School of Pharmacy, Virginia Commonwealth University for suggestions and special assistance in reviewing manuscripts and text. Graduate student Derek Cashman took able charge of our web site, http://www.burgersmedchem.com, another first for this reference work. I would especially like to thank my dean, Victor Yanchick, and Virginia Commonwealth University for their support and encouragement. Finally, I thank my wife Nancy who understood the magnitude of this project and provided insight on how to set up our home office as well as provide John Andrako and me lunchtime menus where we often dreamed of getting chapters completed in all areas we selected. To everyone involved, many, many thanks.

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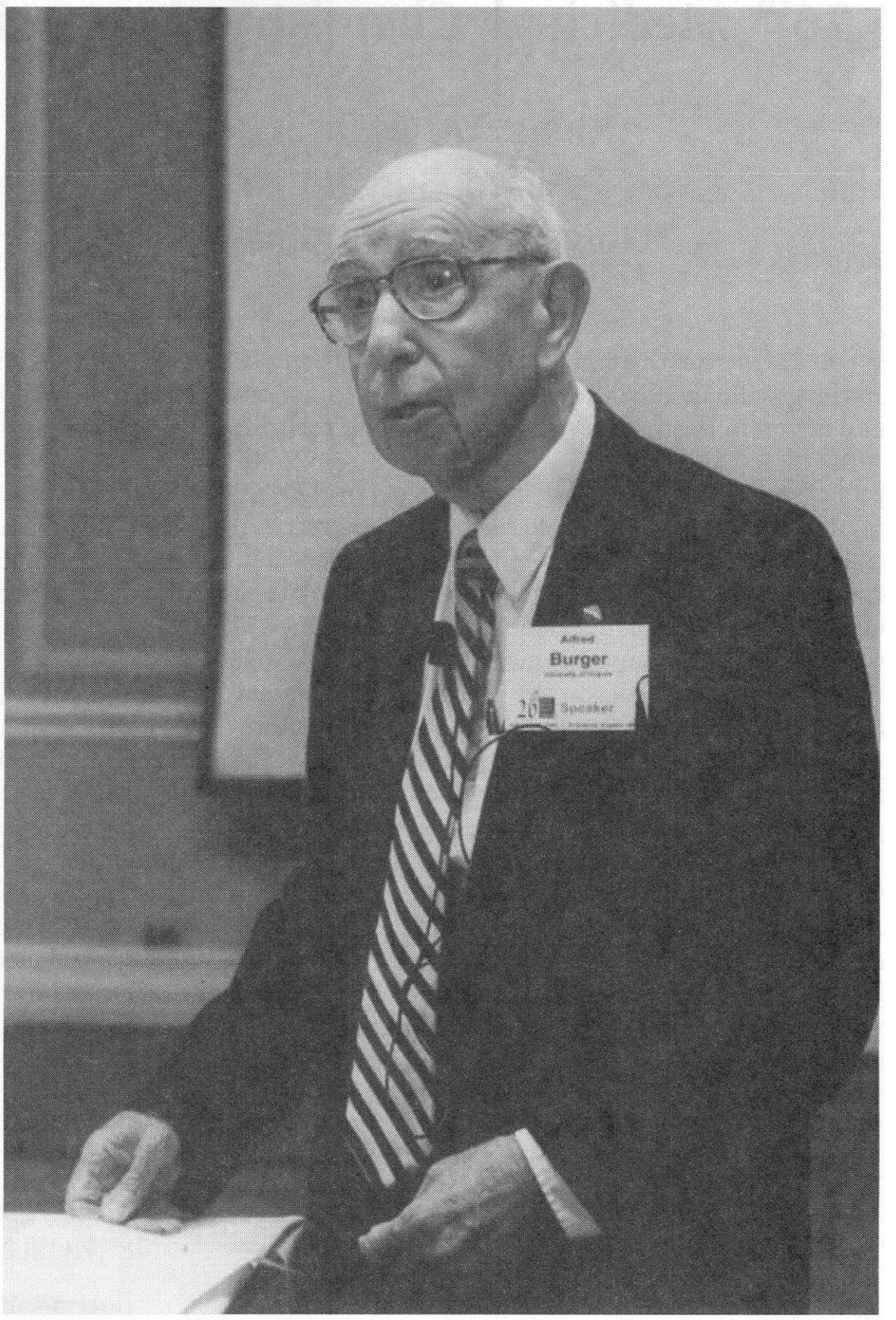
Midlothian, Virginia



Dr. Alfred Burger

Photograph of Professor Burger followed by his comments to the American Chemical Society 26th Medicinal Chemistry Symposium on June 14, 1998. This was his last public appearance at a meeting of medicinal chemists. As general chair of the 1998 ACS Medicinal Chemistry Symposium, the editor invited Professor Burger to open the meeting. He was concerned that the young chemists would not know who he was and he might have an attack due to his battle with Parkinson's disease. These fears never were realized and his comments to the more than five hundred attendees drew a sustained standing ovation. The Professor was 93, and it was Mrs. Burger's 91st birthday.

Opening Remarks

ACS 26th Medicinal Chemistry Symposium

June 14, 1998

Alfred Burger
University of Virginia

It has been 46 years since the third Medicinal Chemistry Symposium met at the University of Virginia in Charlottesville in **1952.** Today, the Virginia Commonwealth University welcomes you and joins all of you in looking forward to an exciting program.

So many aspects of medicinal chemistry have changed in that half century that most of the new data to be presented this week would have been unexpected and unbelievable had they been mentioned in 1952. The upsurge in biochemical understandings of drug transport and drug action has made rational drug design a reality in many therapeutic areas and has made medicinal chemistry an independent science. We have our own journal, the best in the world, whose articles comprise all the innovations of medicinal researches. And if you look at the announcements of job opportunities in the pharmaceutical industry as they appear in ***Chemical & Engineering News,*** you will find in every issue more openings in medicinal chemistry than in other fields of chemistry. Thus, we can feel the excitement of being part of this medicinal tidal wave, which has also been fed by the expansion of the needed research training provided by increasing numbers of universities.

The ultimate beneficiary of scientific advances in discovering new and better therapeutic agents and understanding their modes of action is the patient. Physicians now can safely look forward to new methods of treatment of hitherto untreatable conditions. To the medicinal scientist all this has increased the pride of belonging to a profession which can offer predictable intellectual rewards. Our symposium will be an integral part of these developments.

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BURGER'S
MEDICINAL CHEMISTRY
AND
DRUG DISCOVERY

CHAPTER ONE

Insulin and Hypoglycemic Agents

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Burger's Medicinal Chemistry and Drug Discovery
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1 INTRODUCTION

Insulin is a 58-kDa polypeptide hormone produced by p-cells in pancreatic islets of Langerhans regulating, in vivo, the storage, release, and utilization of nutrient energy, carbohydrate in the form of glucose, fat, and protein, in response to changing supply and demand. Pancreatic β-cells respond to an increase in circulating levels of glucose as occurs after ingestion of a meal by increasing the rate of insulin release. The major metabolic actions of insulin include the following: (1) promotion of uptake and storage of glucose in liver and muscle in the form of glycogen, (2) suppression of hepatic glycogenolysis and gluconeogenesis, (3) increasing the rate of glucose oxidation in muscle, (4) suppression of lipolysis and release of fatty acid from adipose tissue, (5) enhancing triglyceride synthesis and storage and de novo lipogenesis from carbohydrate in liver and fat, and (6) promotion of amino acid uptake and protein synthesis in muscle. Thus, in normal individuals, the postprandial increase in insulin suppresses the release and utilization of nutrient energy substrate (glucose, fatty acids, and amino acids) from hepatic and muscle glycogen, fat depots, and muscle protein, because these substrates are temporarily plentiful due to digestion and absorption of the meal. Insulin also stimulates the conversion of excess energy nutrient into storage forms (glucose into glycogen, fatty acids into triglyceride, amino acids into protein) and the sequestration of these in storage depots for later use. In addition, elevated postprandial insulin increases oxidation of glucose and decreases use of fatty acids in tissues (e.g., skeletal muscle) that can use either as metabolic fuel. Under fasting conditions when insulin levels are low, these processes are reversed, and metabolic fuel substrates are released from storage and used to provide the energy necessary for life processes.

Opposing the actions of insulin are the counterregulatory hormones, glucagon, epinephrine, growth hormone, and cortisol. In response to low blood glucose, the counterregulatory hormones increase hepatic glucose output by stimulating glycogenolysis and gluconeogenesis and also inhibit glucose uptake and utilization in peripheral tissue. This is critical because, while most peripheral tissue can utilize either glucose or fatty acids as fuel for energy metabolism, the brain relies almost exclusively on glucose, requiring 100–150 glday. For comparison, typical dietary intake of carbohydrate is about 300–400 glday, and total hepatic glycogen content is approximately 100 g. The brain has little capacity for glucose storage, and hypoglycemia, depending on degree and duration, can result in dizziness, seizures, coma, or death.

The term diabetes mellitus refers to a group of disorders that are characterized by hyperglycemia resulting from inadequate insulin secretion, failure of insulin to elicit normal level of response in insulin sensitive tissue (insulin resistance), or both. The vast majority of cases of diabetes mellitus fall into two main categories (1, 2), type 1 and type 2. In type 1 diabetes, which is also called insulin dependent diabetes mellitus or IDDM, there is an absolute deficiency in insulin secretion usually caused by autoimmune destruction of the insulin producing β-cells. Type 2 diabetes, also called non-insulin dependent diabetes mellitus or NIDDM, is characterized by both tissue insulin resistance and an insulin secretory deficit (3–5). Insulin resistance, particularly in liver and muscle tissue, plays a major role in the pathogenesis of type 2 diabetes. The elevated fasting plasma glucose levels used to diagnose the disease result primarily from failure of insulin, often at several times normal concentrations, to adequately suppress hepatic glucose output from gluconeogenesis and glycogenolysis (3, 6). Postprandial hyperglycemia is caused by both impaired suppression by insulin of hepatic glucose output and by the diminished ability of insulin to stimulate glucose uptake, storage and utilization, particularly in skeletal muscle, which is the primary site of postprandial glucose disposal (7). At the cellular level, insulin stimulates several processes required for glucose disposal including glucose transport and phosphorylation, glycogen synthesis, and glucose oxidation. Defects

in the insulin activation of these processes are present with insulin resistance in type 2 diabetes (3, 8–12). While the cause(s) at the molecular level for the impairment in glucose disposal are not completely understood, insulin resistance is highly correlated with obesity. There is good evidence supporting the hypothesis that abnormalities in fatty acid metabolism leading to elevated circulating free fatty acids and to nonadipose tissue accumulation of triglyceride and fatty acid metabolites contribute significantly to insulin resistance (8, 9, 13–16). Thus, insulin resistance is probably in part a consequence of action of excessive fatty acids and fatty acid metabolites on complex signaling pathways and feedback loops that regulate metabolic fuel utilization and storage.

Insulin resistance is relatively common, and it alone does not produce hyperglycemia. Obesity, for instance, is almost always accompanied by some degree of insulin resistance, but only a small proportion of obese individuals develop type 2 diabetes (8). This is because a compensatory increase in circulating insulin prevents hyperglycemia. In a population of nondiabetic individuals with normal blood glucose levels but a wide range of insulin sensitivity, insulin secretion is inversely proportional to insulin sensitivity and the product of these two parameters is a constant (4, 17–19). Thus, hyperinsulinemia compensates for insulin resistance and normal glycemic control is maintained. Hyperglycemia occurs when this compensation process fails, and further decline in p-cell function is associated with the progression of type 2 diabetes. Compensatory hyperinsulinemia is the result of increased β-cell mass (19, 20), reduced insulin clearance (19, 21, 22), and increased expression relative to the normal glucokinase of a low K_m p-cell hexokinase, which catalyzes the first rate-limiting step of glucose metabolism in the p-cell, lowering the set point for insulin secretion in response to glucose (19, 21). In type 2 diabetes, the insulin secretory deficit is generally believed to be due to both loss of p-cell mass and insulin secretory function, but the underlying causes are not well established. Chronic hyperglycemia (glucose toxicity) may be a factor in reducing p-cell function as type 2 diabetes becomes fully developed (4), and accumulation of triglycerides and fatty acid metabolites (lipotoxicity) in p-cells may also be important (4, 20).

Acute complications of hyperglycemia include thirst, polyuria, glucosuria, hunger, and with severe elevations, hyperosmolar hyperglycemic nonketotic coma, an often fatal result of osmotic diuresis and dehydration. Primarily in type 1 diabetes, ketoacidosis, caused by a near total lack of insulin to suppress excessive lypolysis, hepatic oxidation of the resulting fatty acids to acetoacetic and 3-hydroxybutyric acids, and accumulation of these substances in circulation, is also potentially fatal. Late complication with both type 1 and type 2 diabetes are classified as microvascular—retinopathy, nephropathy and neuropathy, and macrovascular—ischemic heart disease, peripheral vascular disease, and stroke. Prevention of late complications presents the greatest therapeutic challenge because it requires near normalization of the elevated blood glucose levels associated with the disease. Large randomized prospective clinical trials have established that therapeutic intervention to reduce hyperglycemia significantly decreases microvascular complications in both type 1 and type 2 diabetes (23). Although data from large interventional trials focused on postprandial glucose control are not available, epidemiological studies show a clear association between macrovascular risk and postprandial hyperglycemia (23, 24). Management of late complications of diabetes is a major health care problem. Diabetic retinopathy is the leading cause of blindness in the United States, and diabetic nephropathy is responsible for one-third of all patients requiring kidney transplants or dialysis (3, 25). Some 50,000 lower-extremity amputations performed annually in diabetic individuals, resulting from neuropathies and vascular insufficiency (26).

At the cellular and molecular level, hyperglycemia is believed to cause late complications in diabetes through a number of mechanisms (27). First, glucose is a low affinity substrate for aldose reductase, and hyperglycemia drives the reduction of glucose to sorbitol catalyzed by this enzyme. Excess flux through this pathway results in cellular depletion of reduced glutathione, rendering the cell

susceptible to damage from oxidative stress. Secondly, auto-oxidation of glucose is enhanced in hyperglycemia and results in the formation of reactive dicarbonyl compounds which in turn react with amino groups in proteins to form covalently bonded adducts termed advanced glycation end-products (AGEs). Modification of proteins alters their function, both intracellularly and in the extracellular matrix. Thirdly, hyperglycemia enhances glycolysis and increases intracellular diacylglycerol through increased precursor availability. Elevated diacylglycerol causes abnormal stimulation of various isoforms of protein kinase C, leading to decreased production of nitric oxide and other abnormalities affecting blood flow and capillary permeability. Finally, hyperglycemia enhances the flux of glucose through the hexosamine pathway increasing production of glucosamine and its derivatives. The hexosamine pathway is probably important in regulation of both carbohydrate and fat metabolism, and increased flux through this pathway may be an important contributor to insulin resistance (28). Through increased availability of cofactors, it is also believed to lead to modification of transcription factors and other proteins by *O*-acetylglucosaminylation, altering both gene expression and protein function. Inhibition of endothelial nitric oxide synthase by this process may be relevant to diabetic complications. Each of these four pathogenic mechanisms may reflect a single hyperglycemia-induced process, overproduction of superoxide by the mitochondrial electron transport chain (27).

The efficacy of therapeutic intervention in the treatment of diabetes can be assessed by monitoring plasma glucose levels in the fasted state, but postprandial and bedtime measures are also common. In normal individuals, plasma glucose concentrations are maintained within a narrow range of about 3.5–7.0 m*M* throughout the day (29). Fasting or preprandial plasma glucose concentrations are usually less than 6.1 m*M*. After a meal, plasma glucose levels rise and peak, usually within 30–60 min and return to basal concentrations within 2–3 h. It is rare for 2-h postprandial glucose concentrations to exceed 7.8 m*M* in nondiabetic individuals. For treatment regimens in both type 1 and type 2 diabetes, the American Diabetes Association (ADA) recommends goals of achieving average preprandial plasma glucose concentrations of 5.0–7.2 m*M*, and 6.1–8.3 m*M* at bedtime (30). Guidelines of the American College of Endocrinology (ACE) recommend targets of less than 6.1 m*M* for fasting plasma glucose and 2-h postprandial blood glucose concentrations of less than 7.8 m*M* (23). The utility of measures of plasma glucose in assessing the efficacy of treatment regimens is limited in that these measures reflect only the state of glycemia at the time of measurement. Hemoglobin is nonenzymatically glycosylated on the amino group of its terminal valine at a rate proportional to the concentration of glucose. Because 2–4 months are required for complete turnover of hemoglobin, determination of the fraction of hemoglobin glycosylated in this way (HbA_{1c} or A1C, usually expressed as percent) is used as a measure for assessing glycemic control over time. In normal individuals, HbA_{1c} is in the range of 4.0–6.0%. The goal for treatment recommended by the ADA is achievement of HbA_{1c} less than 7%, while the ACE targets less than 6.5%. The change in percent HbA_{1c} is the gold standard by which the efficacy of therapeutic agents and treatment programs are assessed. These therapeutic targets are difficult to achieve. In a large U.S. epidemiological study, mean HbA_{1c} values for diabetic individuals followed over a 10-year period and under a variety of treatment regimens exceeded 9% (31).

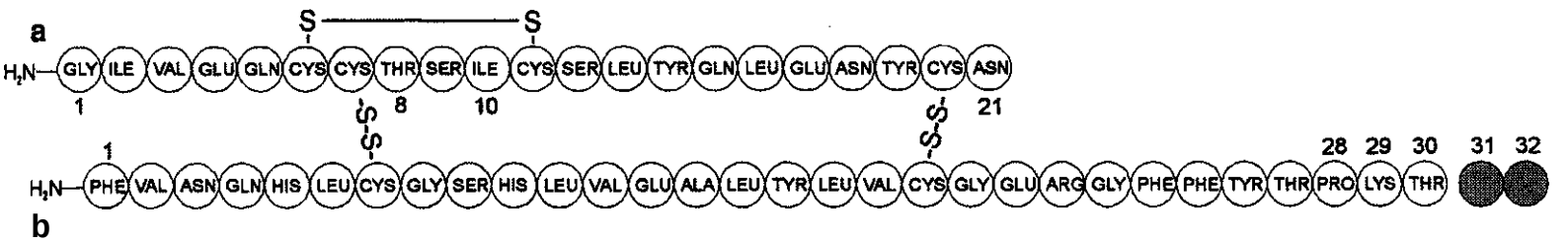
2 CURRENT DRUGS ON THE MARKET

Drugs currently available for treatment of hyperglycemia associated with diabetes mellitus fall into four classes:

1. Insulin and its analogs
2. Insulinotropic agents
3. Insulin-sensitizing agents
4. a-Glucosidase inhibitors

2.1 Insulin and its Analogs

Insulin replacement therapy is the only efficacious treatment for type 1 diabetes and is also useful for treatment of type 2 diabetes when



Animal Insulins – Amino acid residues differing from human insulin:

porcine	B30 Ala
bovine	A8 Ala, A10 Val, 830 Ala

Insulin Analogs – Amino acid residues differing from human insulin:

lispro	828 Lys, B29 Pro
aspart	828 Asp
glargine	A21 Gly, 831 Arg, 832 Arg

Figure 1.1. Amino acid sequence of human insulin and differences from the human sequence for animal insulins and marketed insulin analogs.

poor β-cell function limits the efficacy of oral medications. Insulin isolated from beef or pork was used for many years to treat diabetes mellitus. Animal insulins have largely been replaced by the human hormone, manufactured by recombinant methods. Porcine insulin is still available in the United States. Human insulin is a 51 amino acid polypeptide and differs from the porcine hormone by substitution of one amino acid. It is composed of a 21 amino acid A-chain and a 30 amino acid B-chain joined together by a pair of disulfide linkages. Three new insulin analogs with pharmacokinetic profiles considerably different than the natural hormone have recently become available. Amino acid sequences for human and porcine insulins along with marketed insulin analogs are given in Fig. 1.1.

2.1.1 Side Effects, Adverse Effects. Hypoglycemia is the major and potentially very serious side effect of therapeutic use of insulin and its analogs. This is particularly true in type 1 diabetes, where it is the major limiting factor in glycemic management (32). The frequency of severe hypoglycemia increases with intensive insulin therapy designed to maintain near normal plasma glucose concentrations. A meta-analysis of 14 clinical trials of at least 6 months duration that included 1028 type 1 patients intensively treated with insulin and 1039 patients treated with insulin in less intense conventional regimens found that intensive therapy increased the risk of a severe hypoglycemic episode by approximately threefold. A total of 846 of these patients suffered at least one episode of severe hypoglycemia (33). The incidence of severe hypoglycemia is lower in type 2 diabetes, due to both insulin resistance and less compromise of glucose counterregulation. After 10 years of treatment in the United Kingdom Prospective Diabetes Study (UKPDS) in type 2 diabetics, 2.3% of the patients receiving insulin experienced at least one hypoglycemic episode requiring the help of a third-party or hospitalization (3).

When administered acutely by intravenous infusion to type 1 diabetics, the newer insulin analogs elicit responses very similar to insulin with respect to hypoglycemia and hormonal counterregulation (34, 35). When administered subcutaneously in diabetes therapy, the pharmacokinetic profiles of insulin analogs facilitate their use in treatment regimens designed to closely mimic the natural variations circulating insulin—the pancreatic response to meals. As a consequence, some reduction in the risk of hypoglycemia would be expected, and this seems to be the case.

Insulin promotes the sequestration of fat, and weight gain is a common side effect. This is a concern particularly in type 2 diabetes in which obesity is common. In the UKPDS, obese patients on insulin therapy gained an average of 4 kg more than those treated by diet adjustment only.

2.1.2 Absorption, Distribution, Metabolism, and Elimination. The plasma half-life of insulin is quite short, 4–6 min, and the plasma concentration of the hormone after subcuta-

neous administration is largely determined by the rate of absorption from the subcutaneous depot. Depending on concentration, the presence of certain divalent metal ions, especially zinc, and pH, insulin may exist as a monomer or self-associate in dimeric or hexameric structures. Zinc is usually found in insulin pharmaceutical formulations to encourage hexamer formation and increase stability. Hexameric zinc containing insulin formulations can be prepared as solutions or as suspensions of solid material obtained by crystallization, precipitation as an amorphous solid, or co-crystallization with the basic protein protamine. Solutions have relatively rapid onset and short duration, while the rate of absorption of insulin from solid suspensions depends on the dissolution rate of the solid in the subcutaneous depot. There are about 180 different formulations of insulin marketed worldwide (29). Properties of the most widely used formulations are shown in Table 1.1.

Regular insulin (human) is clear solution of zinc insulin. Its time of onset is 30–60 min with peak activity at 2–3 h and duration of 3–6 h. The widely used insulin NPH (neutral protamine Hagedorn) is a suspension of zinc insulin co-crystallized with protamine and has a slower onset (2–4 h) with longer time to peak (4–10 h) and duration of action (10–16 h). Insulin lente is a suspension of mixed amorphous and crystalline zinc insulin, with onset time of 3–4 h and a duration of 12–18 h. Insulin ultralente, which is a suspension of crystalline insulin with a high zinc content, is the slowest onset (6–10 h) and longest duration (18–20 h) of these preparations. Peak plasma concentrations occur after about 10 h with insulin ultralente (36).

In recent years, results from large prospective clinical trials have shown the benefit of maintaining near normal glycemia with intensive insulin therapy in reducing diabetic late complications. Ideally, insulin formulations used in intensive therapy should be able to mimic the normal variations in circulating insulin levels. In nondiabetic individuals, changes in insulin closely follow glucose, rising rapidly to a peak 30–45 min after ingestion of food and then rapidly returning to basal levels within 2–3 h. Basal levels of insulin are relatively stable and sufficient to suppress excessive lipolysis and hepatic glucose output without inducing hypoglycemia. The pharmacokinetic profiles of human insulin preparations are not ideal for use in dosing regimens designed to provide constant basal circulating levels of the hormone and rapid, but relatively brief, increases associated with meals. Regular insulin has too slow an onset and too long a duration to match the natural mealtime response, and peaks in insulin levels with the longer duration preparations are undesirable in formulations providing the basal component. The insulin analogs lispro and aspart are more suitable than human insulin for use in rapid onset, brief duration formulations, while glargine is formulated for long duration and is nearly peakless (Table 1.1).

With soluble regular insulin, the transport of insulin hexamers from the subcutaneous depot into the microcirculation is sterically restricted, and a lag occurs as the hexamers slowly dissociate on dilution in the interstitial space into smaller species which can be absorbed (38). Insulins lispro and aspart are formulated with zinc and also form hexamers, but the slight amino acid sequence alterations in analogs promote more rapid dissociation and thus faster absorption. With insulin glargine, which is intended to provide a patient's basal requirements, the modified amino acid sequence of the peptide increases the iso-electric point from pH 5.4 for human insulin to 6.7. Because peptides are less soluble at a pH near their iso-electric point, glargine, which is formulated as a solution at pH 4, forms a microprecipitates after subcutaneous injection as the pH increases to the physiological level of 7.4. The slow dissolution of these microprecipitates provides a relatively constant delivery of insulin glargine into the circulation over an extended period of time. Comparative time-course parameters of the marketed insulin analogs in formulations for subcutaneous injection are shown in Table 1.1.

Insulin is rapidly removed from circulation and distributed into tissue by a process mediated by its receptor, and thus all insulin sensitive tissue take up the hormone (39). The primary site of clearance is the liver which removes about half of portal insulin during first-pass transit, but hepatic fractional ex-

Table 1.1 Insulin Formulations for Subcutaneous Injection (37)

USP or Nonproprietary Name	Trade Name	Manufacturer	Formulations	Onset	Peak (h)	Usual Effective Duration (h)	Usual Maximum Duration (h)
Insulin (human)	Humulin	Lilly	R (Regular)	0.5–1 h	2–3	3–6	6–10
	Novolin	Novo Nordisk	N (NPH)	2–4 h	4–10	10–16	14–18
			L (Lente)	3-4 h	4–12	12–18	16–20
			U (Ultralente)	6–10 h		18–20	20–24
Insulin (porcine)	Iletin II	Lilly	Regular	0.5–2 h	3–4	4–6	6–8
			NPH	4–6h	8–14	16–20	20–24
			Lente	4–6 h	8–14	16–20	20–24
Lispro	*Humalog*	Lilly		<15 min	0.5–1.5	2–4	4–6
Aspart	Novolog	Novo Nordisk		5–10 min	1–3	3–5	4–6
Glargine	Lantus	Aventis		1.1 h		24	24

traction is controlled to some degree by a variety of physiological factors. Absorption of glucose in the intestinal tract increases hepatic uptake of insulin, presumably mediated by release of signaling factors from the intestines since this does not occur when glucose is administered by other routes. Free fatty acids decrease insulin binding, degradation, and action in the liver. On binding, the insulin-receptor complex can dissociate and return insulin into circulation or it can be internalized by endocytosis. Most receptor bound insulin is internalized into endosomes and some insulin signal transduction processes may occur there. Endosomal insulin is either dissociated from its receptor and returned to circulation via retroendocytosis or partially degraded by insulin-degrading enzyme (IDE) and the fragments delivered to lysosomes for complete degradation. Another enzyme, endosomal acidic insulinase or EAI may also be important in this process (40). The kidney is also a major site for insulin clearance, removing about one-half of the insulin in the peripheral circulation by a receptor mediated process (39,411. Most endosomal insulin in the kidney is delivered to lysosomes where it is ultimately degraded. Insulin analogs, as well, are cleared and degraded in the kidney (41). On subcutaneous administration, renal clearance of insulin is a higher portion of the total than with the endogenously produced hormone because the normal process in which high concentrations of newly secreted insulin are delivered directly from the pancreas to the liver via the portal circulation is bypassed. Renal failure may significantly reduce insulin requirements.

2.1.3 Physiology and Pharmacology. Before the discovery and introduction of insulin as a therapeutic agent, the only available treatment for type 1 diabetes was a starvation diet and the life expectancy for a child diagnosed with the disease was 1.3 years (42). Judged against this standard, insulin therapy for type 1 diabetes is hugely successful. Intensive insulin therapy, usually three or more insulin injections per day of regular insulin in association with meals along with a longer acting insulin to supply basal requirements or continuous subcutaneous infusion both with frequent monitoring of blood glucose to adjust insulin dosage, dramatically decreases microvascular complications. In the landmark Diabetes Control and Complications Trial (DCCT) intensive treatment reduced the risk of retinopathy, nephropathy and neuropathy by 35–90% compared with conventional therapy of one or two insulin injections per day. A median HbA_{1c} level of 7.3% was obtained with intensive therapy while that for the conventionally treated group was 9.1% (43). However, the median HbA_{1c} level for intensive insulin therapy group shows that less than half achieved the ADA goal for HbA_{1c} in the rigorous environment of a clinical trial, and by 5-years poststudy, the mean HbA_{1c} for a subset of the intensively treated cohort was 8.1%.

Insulin is commonly used in the treatment of type 2 diabetes, but is most often introduced when oral agents fail to adequately control hyperglycemia. A decrease in HbA_{1c} of about 2%, similar to that found with the most efficacious oral agents, can be achieved with intensive insulin therapy, although a lesser reduction is probably the norm in general practice (3). After 6 years in the UKPDS, insulin treated type 2 diabetics, newly diagnosed at the beginning of the study, had lower fasting plasma glucose levels, but similar HbA_{1c} concentrations to patients treated with oral agents (44). The invasive nature of subcutaneous administration, greater weight gain and increased risk of hypoglycemia relative to oral agents may discourage the early use of insulin. Therapy for type 2 diabetes usually begins with lifestyle interventions, particularly changes in diet and exercise. In the UKPDS, only about 15% of newly diagnosed patients were able to reach target levels of glycemic control in 3 months of intensive dietary therapy, and there was further decline after 1 year (45). Oral agents are introduced as monotherapy if lifestyle adjustments are unsuccessful (46, 47). Type 2 diabetes is usually progressive with loss of β-cell mass and function, and oral agents that stimulate or rely on endogenous insulin production become less efficacious over time. Glycemic control with monotherapy in the UKPDS deteriorated such that after 3 years, only about one-half of the patients treated with a single agent were able to achieve HbA_{1c} less than 7% (48). After 9 years of monotherapy, only one-quarter were able to maintain this

level of control, and the majority of patients required multiple therapies. Ultimately insulin is almost always required to achieve optimal glycemic goals (46). Insulin can be introduced in place of or in combination with oral agents, but better results are often obtained with combinations of insulin and oral agents than with insulin alone (49–52).

The rapid onset, short acting insulin lispro and aspart are used in treatment of both type 1 and type 2 diabetes in place of regular insulin in intensive therapy regimens. The benefits of these newer agents used by injection at mealtime with optimized basal therapy in reduction of HbA_{1c} in the rigorously controlled environment of clinical trials are modest, 0.1–0.4% when compared with regular insulin (53). Reductions of up to 0.8% in HbA_{1c} compared with regular insulin have been obtained with insulin lispro administered continuously by infusion pump. When the long-acting insulin glargine is used as the basal component of insulin therapy, HbA_{1c} levels are usually very similar to those obtained with insulin NPH or ultralente (53), although less weight gain in type 2 diabetics and decreased frequency of nocturnal hypoglycemia have been reported with use of the analog. While the reductions in HbA_{1c} achieved with insulin analogs are modest when compared with human insulin therapy, even small improvements are believed to reduce the risk of microvascular complications in diabetes, and a survey of diabetic patients suggests that a reduction in hypoglycemic episodes, better glycemic control, and improved quality of life can often result from the use of these agents (53, 54).

At the cellular and molecular level, the binding of insulin to a specific membrane spanning receptor initiates a signal transduction cascade which ultimately produces the biological actions of the hormone. The insulin receptor is a tetrameric protein comprised of two *a* subunits that bind to insulin and two β subunits that are linked by disulfide bonds (55, 56), and belongs to a subfamily of receptor typrosine kinases which also includes the insulin-like growth factor I (IGF-I) receptor. The *a* subunits are extracellularly located, while β subunits span the membrane and have both intracellular and extracellular portions, with the tyrosine kinase domain located intracellularly in this subunit. Extracellular binding of insulin activates the receptor kinase activity, and the receptor is autophosphorylated on multiple intracellular β subunit tyrosine residues (13, 57). The insulin receptor shares about 50% amino acid sequence homology with the IGF-I receptor. Insulin and IGF-I bind and activate both types of receptors, but each has 2–3 orders of magnitude preference for own receptor (58). There are two isoforms of the insulin receptor differing by the presence or absence of 12 amino acids in the C-terminus of the α subunit. The smaller isoform has about a two-fold higher affinity for insulin than the longer, and this difference is paralleled in sensitivity for metabolic actions of insulin (59).

Ligand affinities for the insulin receptor can be measured in binding assays based on displacement of [^{125}I]-labeled insulin. The relative affinities for insulin and the insulin analogs used clinically are shown in Table 1.2. Many cell based assays can be used to assess the functional potency of insulin and its analogs. Primary adipocytes are exquisitely sensitive to insulin and are often used for this purpose. The relative potency of human insulin and analogs in stimulating lipogenesis in primary rat adipocytes is also shown in Table 1.2. The receptor affinities and functional potencies of these analogs are similar to insulin, with the exception that insulin glargine has about a sixfold higher affinity for the IGF-I receptor. To date, neither benefit nor risk has been clearly demonstrated to be associated with this higher affinity.

2.1.4 Structure-Activity. The structural basis and especially the role of specific amino acid residues in the interaction of insulin molecules in forming dimers and hexamers or binding to and activating insulin receptors have been studied extensively by comparing amino acid sequences from different species, in structure-function studies using insulin analogs, and with X-ray crystallography and molecular modeling. The insights gained have been used primarily to design therapeutic

Table 1.2 Relative Receptor Binding Affinities and Metabolic Potency for Human Insulin and Analogs Used Clinically

	Insulin Receptor Affinity % [mean (SE)]	IGF-I Receptor Affinity % [mean (SE)]	Lipogenesis Potency % [mean (SE)]
Human insulin	100	100	100
Lispro	84 (6)	156 (16)	82 (3)
Aspart	92 (6)	81 (9)	101 (2)
Glargine	86 (3)	641 (51)	60 (3)

Values are calculated as the ratio between EC_{50} for human insulin and EC_{50} for the analog. Binding affinities were determined using solubilized human insulin and IGF-I receptors, employing TyrA14[^{125}I]-human insulin and Tyr31[^{125}I]-IGF-I as radioligands. Potency in lipogenesis is based on stimulation of incorporation of the label from D-[^{3}H]-glucose into lipid in primary rat adipocytes. EC50 values for human insulin are 0.01 and 200 n*M* for insulin and IGF-I receptor binding respectively, and 45 p*M* in lipogenesis (60).

agents with improved pharmacokinetic profiles, but which mimic the natural hormone in other ways as closely as possible.

Insulin forms both dimers and hexamers, and dimerization occurs mainly through interactions of certain B-chain residues (Fig. 1.1), involving amino acids at positions B8, B9, B12, B13, B16, and B23–28 (61, 62). Hexamer formation is stabilized mainly through coordination of the six HisBlO residues (one from each monomer) to two zinc ions, but burial of a non-polar surface made up by amino acids at positions A13, A14, B1, B2, B14, B17, and B18 also contributes (62, 63). The amino acid residues that are considered most essential for high binding affinity of insulin to its receptor are at positions A1–A3, A16, A19, A21, B6, B12, B15, B23–B25, and perhaps A13 and B17. Minor contributions are made by residues at A8, B9–10, B13, and B16 (64, 65). Substitutions for residues at B26–30 are often possible while maintaining high affinity for the insulin receptor, but mainly effect IGF-I receptor binding.

X-ray crystallography has yet to provide detailed information at the atomic level for insulin bound to its receptor, due to difficulties in obtaining X-ray crystal structures of membrane-spanning ligand–receptor complexes. The three-dimensional structure of the complexed receptor has been obtained with a combination of electron cryomicroscopy using gold-labeled insulin to locate the binding domain and high resolution structures of insulin and individual subdomains of the receptor (66, 67). In this structure, insulin binds by bridging from a binding site on one extracellular *a* subunit to a dissimilar site in the second *a* subunit of the receptor dimer. Insulin residues at positions A4, A8, A17, A21, and B29 interact with polar amino acids in one *a* subunit primarily through electrostatic salt bridges, while there are both hydrophobic and polar interactions between insulin residues at positions B9, B10, B12, B13, B16, B17, B21, B22, B24, B26, A5, and A15 and the other α subunit. There is only a modest correlation between insulin amino acid residues found to contribute significantly to binding affinity based on structure-activity studies and those which closely interact with the receptor in the three dimensional structure. Not all close contacts observed in the structure necessarily make large contributions to binding energy, and factors such as change in conformation induced by substitution of residues not directly interacting with the receptor may also contribute to changes in binding affinity.

Recombinant methods have enabled the preparation of a very large number of insulin analogs, but relatively few have been extensively characterized pharmacologically. A single point mutation in the proinsulin gene identified as cause of a case of familial hyperproinsulinemia led to the synthesis of [AspB10]-insulin (68–70) and some related insulin analogs. Lacking the HisBlO residue which stabilizes hexamer formation through Zn^{2+} binding, [AspB10-insulin is absorbed about twice as rapidly as regular insulin. It exhibits a 3.5-fold increased binding affinity for the insulin receptor and is about twice as potent as insulin in metabolic assays. Surprisingly, [AspB10]-insulin is 10- to 20-fold more

potent than the parent in mitogenic assays (71, 72). While [AspB10]-insulin has increased affinity for the IGF-I receptor relative to insulin; its relative affinity for this receptor is about 1000-fold lower than for the insulin receptor, and enhanced mitogenic potency has been observed in a cell line lacking IGF-I receptors. The mitogenic/metabolic potency ratios of [AspB10]-insulin and several other insulin analogs are inversely correlated with their insulin receptor dissociation rate constants (71), suggesting that increased mitogenic potency results primarily from sustained activation of the insulin receptor tyrosine kinase through formation of long-lived ligand–receptor complexes. A dose-dependent increase in incidence of adenocarcinomas in laboratory animals treated with suprapharmacological doses of [AspB10]-insulin prevented development of this analog as a therapeutic agent (65). Thus, the pharmacologic actions produced on administration of a particular insulin analog may depend not only on insulin receptor affinity, intrinsic activity and selectivity, but also half-life of the ligand–receptor complex.

Modifications of the native insulin sequence at the C-terminus in the B26 to B30 region have led to all of the marketed insulin analogs. This region is not critical for binding to the insulin receptor (65, 73), but sequence modifications here often yield analogs with altered tendencies to form aggregates. A systematic investigation (61) of amino acid substitutions in the insulin B-chain with the intent of reducing dimer stability by introducing charge-charge or steric repulsion led to replacement of ProB28 with aspartic acid to afford [AspB28]-insulin (insulin aspart). Insulin aspart retains the biological profile of insulin, but is more rapidly absorbed. Synthesis of a series of analogs having LysB29 replaced with proline and varying the amino acid at B28 led to identification of [LysB28 ProB29]-insulin (insulin lispro) as a fully potent insulin analog with decreased association in solution and faster onset of action than the parent (38, 74).

Sequence modifications which increase the isoelectric point of insulin from 5.4 toward neutrality led to analogs which form microprecipitates and have extended duration of action (29, 75). Following this concept, addition of two basic arginine residues to the C-terminus of insulin and substitution of glycine for AspA21 gave the long acting [GlyA21 ArgB31 ArgB32]-insulin (insulin glargine) with an isoelectric point near pH7.0 (29, 76). Long acting insulin analogs also result from fatty acid acylation of the side-chain amino group of LysB29, increasing size and promoting binding of these analogs to albumin, thus delaying absorption from the subcutaneous injection site (29, 77, 78). [LysB29-tetradecanoyl des-B30]-insulin (insulin detemir) in which albumin binding is further enhanced by deletion of the terminal threonine at B30 is undergoing clinical evaluation.

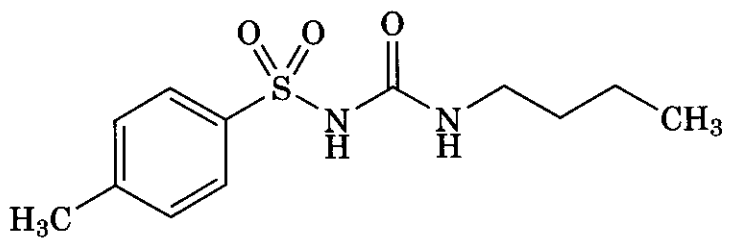
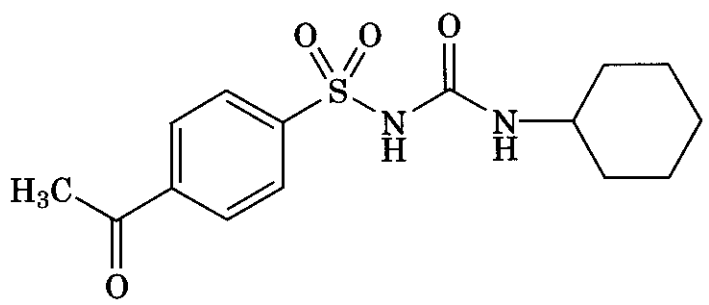
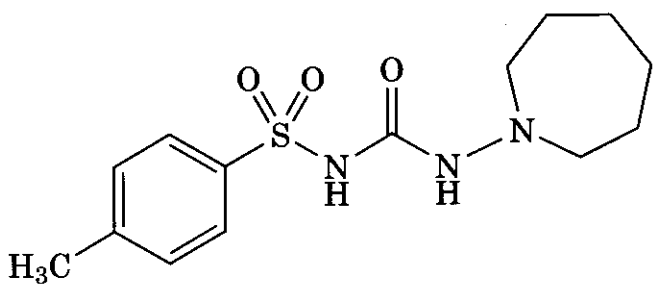
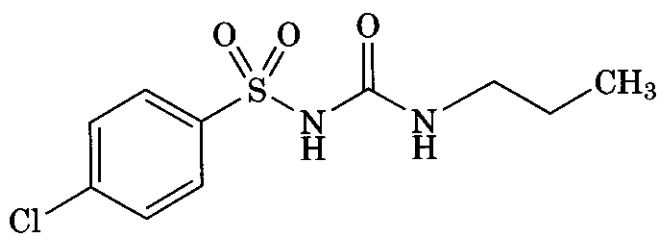
2.2 Insulinotropic Agents

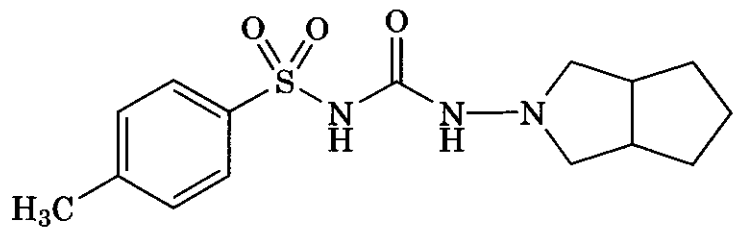
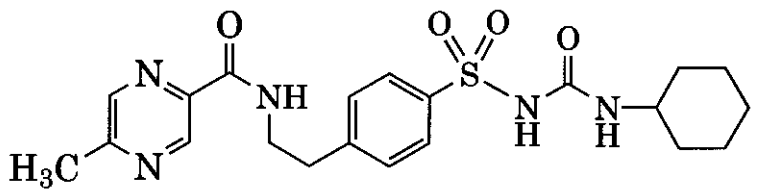
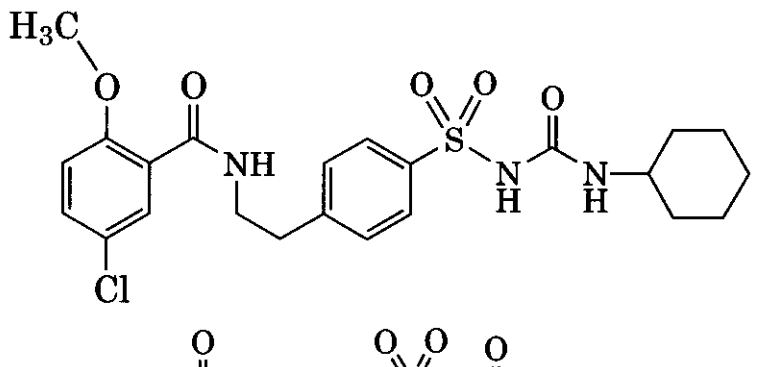
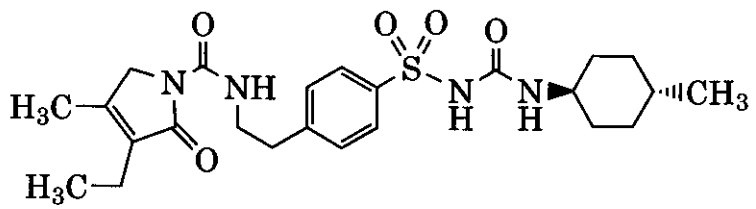
Insulinotropic agents directly stimulate the release of insulin from pancreatic β-cells, and are thus useful only in the treatment of NIDDM. The drugs in this class are often divided into the subclasses of sulfonylureas and glinides. These compounds are structurally related and share a common mechanism of action, and are described together.

Sulfonylureas have been used as hypoglycemic agents since the mid-1950s. They act by closing membrane-bound ATP-sensitive potassium (K_{ATP}) channels on the p-cell, causing depolarization and the opening of voltage-gated calcium channels. The resulting influx of Ca^{2+} triggers exocytosis of insulin. The structures and properties of the major marketed sulfonylureas are shown in Tablel.3, roughly in order of increasing potency. Compounds in the first generation of this class such as chlorpropamide, tolbutamide, and tolazamide are still in use, but are less potent than the more recently introduced second-generation drugs like glipizide, glyburide, and glimepiride. Treatment regimens with sulfonylureas provide more or less continuous hypoglycemic activity with once- or twice-daily dosing.

As with the rapid onset, short-duration insulin~two short-acting glinide insulin secretagogues, repaglinide and nateglinide (Table 1.4), have recently become available. Taken immediately before meals, these agents provide increased insulin secretion in the postprandial period. Like sulfonylureas, the compounds act by closing pancreatic β-cell K_{ATP}

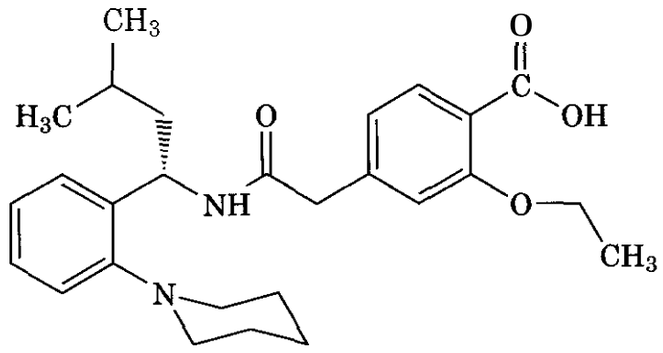
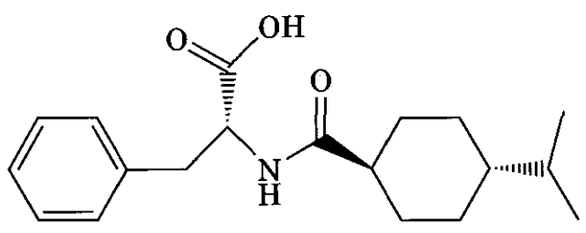
Table 1.3 Structures and Properties of Insulinotropic Agents: Sulfonylureas

USP or Nonproprietary Name	Trade Name	Manufacturer	Chemical Class	Structure	Daily Dose	t_{max}	$t_{1/2}$	Duration[a]
Tolbutamide	Orinase	Pharmacia and Upjohn	Sulfonylurea		250–3000 mg	3–4 h	4.5–6.5 h	6–12 h
Acetohexamide	Dymelor	Lilly	Sulfonylurea		250–1500 mg	1–2 h (parent) 2–5 h (metabolite)[b]	0.8–2.4 h 3.7–6.4 h	12–18 h
Tofazamide	Tolinase	Pharmacia and Upjohn	Sulfonylurea		100–1000 mg	3–4 h	7 h	12–24 h
Chlorpropamide	Diabinese	Pfizer	Sulfonylurea		100–750 mg	2–4 h	36 h	60 h

Gliclazide	Diamicron	Servier	Sulfonylurea		40–320 mg	4-6 h	10–12 h	
Glipizide	Glucotrol	Pfizer	Sulfonylurea		2.5–40 mg	1–3 h	2–4 h	12–24 h
Glyburide	Micronase	Pharmacia and Upjohn	Sulfonylurea		1.25–20 mg	2–4 h	4–10 h	16–24 h
	Diabeta	Aventis						
	Glynase	Pharmacia and Upjohn			0.75–12 mg			12–24 h
Glimepiride	Amaryl	Aventis	Sulfonylurea		1–8 mg	2–3 h	9 h	16–24 h

[a]Ref. 3.
[b]Refs. 79, 80.

Table 1.4 Structures and Properties of Insulinotropic Agents: Glinides

USP or Nonproprietary Name	Trade Name	Manufacturer	Chemical Class	Structure	Dose	t_{max}	$t_{1/2}$	Duration[a]
Repaglinide	Prandin	Novo Nordisk	Benzoic Acid		0.5–4 mg 2, 3, or 4× daily	1 h	1–1.4 h	4–6 h
Nateglinide	Starlix	Novartis	Phenylalanine		120 mg 3× daily	<1 h	1.5 h	

[a]Ref. 3.

channels, but have rapid onset after oral administration and short duration of action. Repaglinide is the more potent of the two, but nateglinide reportedly has somewhat faster onset and shorter duration of action (81).

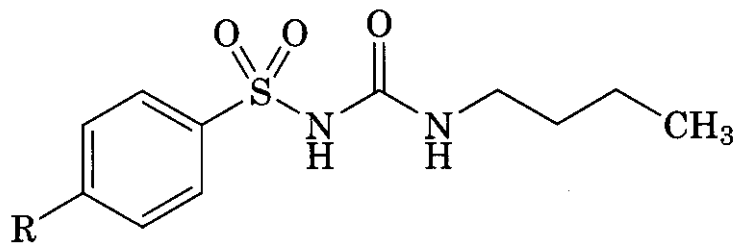
2.2.1 Side Effects, Adverse Effects. Sulfonylureas are generally well tolerated. The major safety concern is severe hypoglycemia, with the longer-acting agents carrying a greater risk. In the United Kingdom Prospective Diabetes Study (UKPDS), the proportion of patients experiencing major hypoglycemic episodes was 1.0 and 1.4% per year with chlorpropamide and glyburide, respectively, as compared to 0.7% with diet and 1.8% with insulin therapy (82). Sulfonylureas are for the most part subject to hepatic metabolism, yielding less active or inactive metabolites that are then eliminated through the kidney. Patients with impaired hepatic or renal function risk severe hypoglycemia because of accumulation of active drug in circulation. As with insulin, gain in body weight is common. The University Group Diabetes Program (UGDP) study found an increased risk of cardiovascular mortality associated with the treatment of type 2 diabetes with tolbutamide, although the methods used have been criticized. The UKPDS showed no increase in cardiac events with sulfonylurea treatment (3, 83). Less is known on long-term side effects with the newer glinide short-acting insulinotropic agents. Like sulfonylureas, weight gain is a side effect of repaglinide or nateglinide therapy. In year-long preapproval clinical trials with repaglinide, 13% of patients discontinued the use of the drug because of adverse events, most commonly hyperglycemia or hypoglycemia. In studies of 6 months or longer with nateglinide, 0.3% of patients discontinued because of hypoglycemia.

2.2.2 Absorption, Distribution, Metabolism, and Elimination. All of the marketed sulfonylureas are nearly completely absorbed, and the class has excellent oral bioavailability. A quantitative structure–activity relationship (QSAR) model for human drug oral bioavailability (84) has recently been developed, in which the descriptors A log D ($\log D_{6.5} - \log D_{7.4}$) and $\log D_{6.5}$ both proved to be important contributors in addition to a variety of structural descriptors. The $\log D_{6.5}$ descriptor is the log of the octanol–water distribution coefficient at pH 6.5 and a measure of lipophilicity at the pH of the small intestine. The A log D term is the difference between log D measured at pH 6.5 and that measured at pH 7.4, the pH of blood. It has a positive value for acids and is negative for bases. The optimum value for log $D_{6.5}$ is about −0.3 and the A log D weighting coefficient is positive, indicating that, all things being equal, there is better bioavailability for acids than for neutral or basic compounds. An interpretation of the A log D outcome is that a higher fraction of un-ionized compound at pH 6.5 aids absorption, whereas a higher fraction of ionized compound at pH 7.4 aids in preventing first-pass metabolism. Sulfonylureas are weak acids, with pK_a values in the range of 5.0 to 6.3 (85), and values (84) for log $D_{6.5}$ for glipizide, glyburide, and tolbutamide (1.31, 1.85, and 1.11, respectively) predict excellent oral bioavailability in the absence of metabolically labile functionality.

Although the marketed sulfonylureas are all well absorbed, they differ in the time required to reach maximum blood levels (Table 1.3) and in metabolic fate and in rate and mode of elimination. These differences can have important implications for safe use of the various drugs. Accumulation of these agents in *vivo* can lead to extended episodes of serious or fatal hypoglycemia through overstimulation of insulin release. Insulinotropic drugs, for instance. which are metabolized to active compounds that are eliminated solely by renal excretion, pose a serious risk of hypoglycemia in renally compromised individuals.

The volumes of distribution for the various sulfonylureas are similar, with V_d values in the range of 0.1–0.3 L/kg, indicating limited distribution beyond extracellular water (86). They are highly bound to serum protein (90–99%).

The older first-generation sulfonylureas are extensively metabolized and primarily excreted renally. Tolbutamide is transformed by oxidation of the benzylic methyl group, yielding a hydroxymethyl metabolite, (1a), which is further oxidized to the corresponding carboxylic acid, (1b). These metabolites have little activity.



(1a) R = CH_2OH
(1b) R = COOH

Tolazamide is more slowly eliminated than tolbutamide, with a half-life of about **7** h. Like tolbutamide, it is metabolized **(87)** by oxidation of the benzylic methyl group to a hydroxymethyl metabolite, which has some activity, and to the corresponding carboxylic acid. It is also hydroxylated on the 4 position of the hexahydroazepine ring and hydrolyzed to p-toluenesulfonamide. The metabolites are excreted largely in the urine along with about **7%** of the parent drug. Gliclazide is somewhat more slowly absorbed and has a longer half-life **(10–12** h) than that of tolbutamide and tolazamide. Excretion is **60–70%** in urine and 10–20% in feces. It is also metabolized (88) by oxidation of the benzylic methyl group to a hydroxymethyl compound and a carboxylic acid, which are the main metabolites in urine. Lesser amounts of five other metabolites, hydroxylated at various positions on the azabicyclooctyl ring, have also been identified. The metabolites are not believed to have significant hypoglycemic activity **(86).** Less than **1%** of the drug is found unchanged in urine.

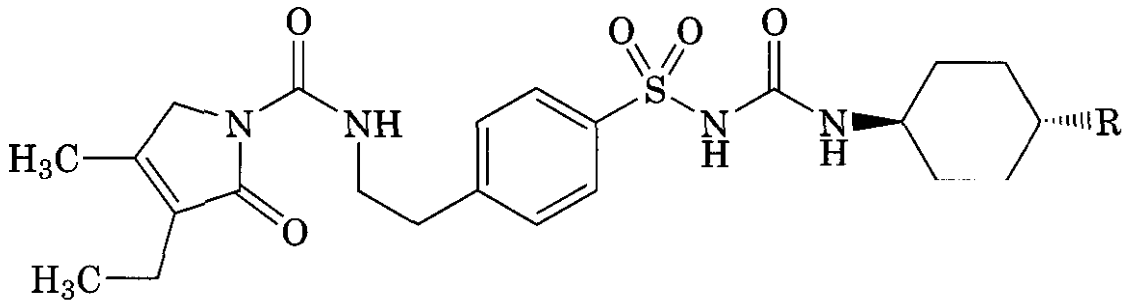
Acetohexamide is metabolized by reduction in the liver of its ketone function to an alcohol. hydroxyhexamide, which is pharmacologically active **(79, 86).** After oral administration, the plasma concentration of the parent compound peaks in **1–2** h, then falls rapidly. The active metabolite has a longer half-life and prolongs the duration of action. Hydroxyhexamide is excreted primarily in the urine with a small amount of the parent and other clinically unimportant metabolites. Chlorpropamide is quite long lived in circulation, with an elimination half-life of about **36** h. It is slowly metabolized by hydroxylation at the **2** or 3 position of the propyl substituent, yielding metabolites that retain some activity and by N-dealkylation to *p*-chlorobenzenesulfonylurea **(86, 89).** Urinary excretion is the main route of elimination from the system. About **20%** of the dose is excreted as unchanged drug.

With the newer second-generation compounds, glyburide is metabolized by hydroxylation on the cyclohexane ring, yielding **4-trans** and 3-cis-hydroxy derivatives. Both of these metabolites may contribute to the overall hypoglycemic activity **(90).** An elimination half-life of 15 h, on the basis of an improved analytical method, is probably more in line with the observed duration of action **(91).** Glyburide has been reported to accumulate in pancreatic islets, a finding'that was unique to this drug **(92).** It is eliminated as its metabolites with approximately equal amounts in urine and bile. Glipizide is similarly metabolized primarily by hydroxylation at the **3** and 4 positions of the cyclohexane ring, although the metabolites are not believed to contribute to the activity of the drug. It is eliminated somewhat more rapidly than either glyburide or glimepiride, mainly in the urine as hydroxylated metabolites and polar conjugates. Of the second-generation agents, glipizide has the most rapid onset, but also has a somewhat shorter duration than that of either glyburide or glimepiride.

Glimepiride is completely metabolized by oxidation of the pendant methyl substituent on the cyclohexane ring to a hydroxymethyl metabolite **(2a)** and a carboxylic acid (**2b**). The hydroxymethyl metabolite has about one-third of the hypoglycemic activity of the parent in an animal model, whereas the other was not active. Glimepiride is eliminated with a half-life of about **9** h, and metabolites, but no parent drug, are found in both urine **(60%)** and feces (40%), with (**2b**) the predominant species in urine and (**2a**) in feces.

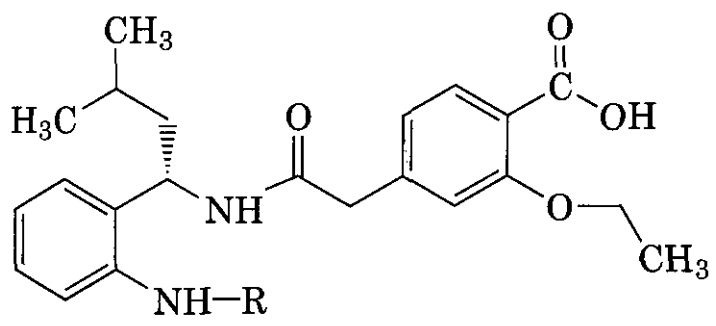
The short-acting insulinotropic agents repaglinide and nateglinide are rapidly and completely absorbed. After oral administration, both drugs reach peak plasma levels in about 1 h. Steady-state volumes of distribution (V_{ss}) after intravenous (i.v.) injection are **31** L for repaglinide and **10** L nateglinide, indicating little distribution beyond the plasma volume for the latter and a somewhat wider distribution for the former. Both drugs are >98% bound to serum protein.

Repaglinide is extensively metabolized in the liver by dealkylation and oxidation to a



(2a) R = CH_2OH
(2b) R = COOH

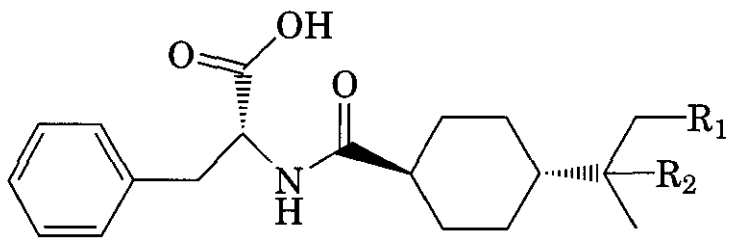
dicarboxylic acid, (**3a**), by further dealkylation of (**3a**) to amine, (**3b**), and by formation of an acyl glucuronide of the parent. Elimination is



(3a) R = CH_2-$(CH_2)_3$-COOH
(3b) R = H

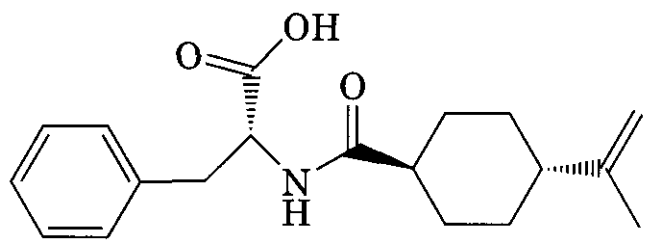
rapid, with a half-life of about 1 h. About **90%** of drug-related species are excreted in the feces in radiolabeled experiments, mainly **as** (**3a**) with less than **2%** of the parent **(93).**

Nateglinide is metabolized by the **mixed**-function oxidase system primarily by oxidation of the isopropyl side chain. It is eliminated mainly in the urine (94), primarily as (**4a**)



(4a) R_1 = H, R_2 = OH
(4b) R_1 = OH, R_2 = H
(4c) R_1 = OH, R_2 = OH

(33%) and unchanged parent (16%), along with lesser amounts of (**4b**) and (**4c**), acyl **glucuronides**, and dehydration or dehydrogenation product (5). Metabolite (5) is approximately equipotent with nateglinide, but only small amounts are formed.



(5)

2.2.3 Physiology and Pharmacology. Sulfonylureas and the carboxylic acid glinide hypoglycemic agents stimulate insulin secretion by a direct action on pancreatic islet **β-cells**, and are a mainstay in treatment of type **2** diabetes in patients with good β-cell function. The pharmacologic actions of these drugs are largely, if not entirely, mediated by increased insulin production, and thus are essentially the same **as** insulin. Sulfonylurea therapy typically has been found to reduce fasting **hyperglycemia** by **3.3–3.9 mmol/L** and HbA_{1c} by **1.5–2% (3).** There is less clinical experience with the short-acting glinides, but repaglinide appears to have a similar effect on HbA_{1c} and the very short acting nateglinide somewhat less as monotherapy.

Both sulfonylureas and glinides exert their action by binding to and closing an **ATP-sensitive** potassium channel (K_{ATP}) in the plasma membrane of the **β-cell (95, 96).** Glucose is transported into these cells predominately by the non-insulin-dependent GLUT2 transporter, and the rate of glucose transport into the cell and metabolism reflect plasma glucose concentration. At low glucose concentrations, the transmembrane potential of pancreatic **β-cells** is maintained at about **−70 mV** by an outward flow of K^+ ions through the K_{ATP} channel. After a rise in plasma glucose, the increase in glucose metabolism leads to a rise in the **ATP/ADP** ratio and closure of K_{ATP}

Table 1.5 Binding Parameters for Sulfonylureas and Glinides with HEK EBNA[Human SUR1] Cell Membranes

	K_i nM (SEM)	Hill Coefficient (SEM)
Glyburide	2.3 (0.4)	1.0 (0.05)
Glimepiride	4.5 (0.9)	1.1(0.13)
Glipizide	100 (24)	0.82 (0.12)
Repaglinide	240 (38)	0.90 (0.07)
Nateglinide	1300 (110)	0.80 (0.05)
Tolbutamide	270,000 (17,000)	1.05 (0.09)

channels, thus depolarizing the cell. The change in membrane potential results in the opening of voltage-gated Ca^{2+} channels and an increase in intracellular Ca^{2+}, which triggers insulin release. Sulfonylureas, by blockingpotassium current through the K_{ATP} channel, produce the same effect.

The K_{ATP} channel is composed of two protein subunits in a ratio of 4:4. One subunit, termed Kir6.2, is a member of the inward rectifying potassium channel family. The other regulatory subunit, SUR1, belongs to the ABC (ATP-binding cassette)-transporter superfamily. Sulfonylureas bind with the K_{ATP} channel at both a low affinity site on Kir6.2 and a high affinity site on SUR1, which confers the channel-blocking activity. The sulfonylurea binding site appears to be located on the intracellular side of SUR1. The number of bound sulfonylurea molecules required for K_{ATP} channel closure is not known. Because there are four SURl subunits in each K_{ATP} channel, binding ratios greater than unity may be required to block potassium current.

The relative potency of various sulfonylureas and glinides determined by direct competitive displacement of [^{3}H]glyburide from membrane preparations of recombinant human embryonic kidney cells expressing high levels of SURl is shown in Table 1.5 (97). Both sulfonylureas and the glinides displace [^{3}H]-glyburide from SUR1-containing membrane preparations. Moreover, they appear to occupy very similar sites on the receptor protein, given that neither repaglinide nor nateglinide has any effect on the dissociation kinetics of [^{3}H]glyburide.

The high affinity binding of sulfonylureas to SURl suggests the existence of an endogenous ligand. Two polypeptides isolated from porcine brain, *a*- and β-endosulfine, have been found to inhibit sulfonylurea binding to SUR1, although the functional significance is not clear (98, 99).

2.2.4 History. As early as 1942, Janbon (100) observed a high incidence of hypoglycemia in typhoid patients treated with a bacteriostatic isopropylthiadiazole derivative of sulfanilamide. Subsequently, during antibacterial clinical testing of N-(4-aminobenzenesulfonyl)-*N'*-butylurea, carbutamide (6) was also found to have similar hypoglycemic activity. Structure–activity studies at Boehringer Mannheim and at Hoechst led to the introduction of carbutamide and tolbutamide as antidiabetic agents in 1956.

2.2.5 Structure–Activity. The more recently introduced insulinotropic agents are complex, richly functionalized molecules, the optimized products of many extensive synthetic and pharmacological investigations (101–117) of structure–activity relationships (SAR). Common features found in potent compounds (102, 103, 106) of this class are summarized in Fig. 1.2, illustrated by the use of glyburide, repaglinide, and nateglinide as reference structures. The relationship to other compounds in Table 1.3 is readily apparent.

Hypoglycemic sulfonylureas and glinides contain an acidic functional group (A in Fig. 1.2) that is required for insulinotropic activity. In all of the marketed drugs of this class, the acidic group is attached to a phenyl ring (B in Fig. 1.2). The acidic group is generally a sulfonylurea, a propionate, or carboxylate, although compounds containing other acidic moieties like sulfonylsemicarbazides, sulfonylaminopyrimidines, sulfonylcyanoguanidines, and sulfonamidonitroethylenes are quite active.

Substitution on the acidic function with a pendant lipophilic group (C in Fig. 1.2) greatly enhances (103) affinity for the SURl and increases selectivity for SURl over related SUR2A receptors found in heart and skeletal muscle and SUR2B receptors found in smooth muscle. In the earliest sulfonylureas, this sub-

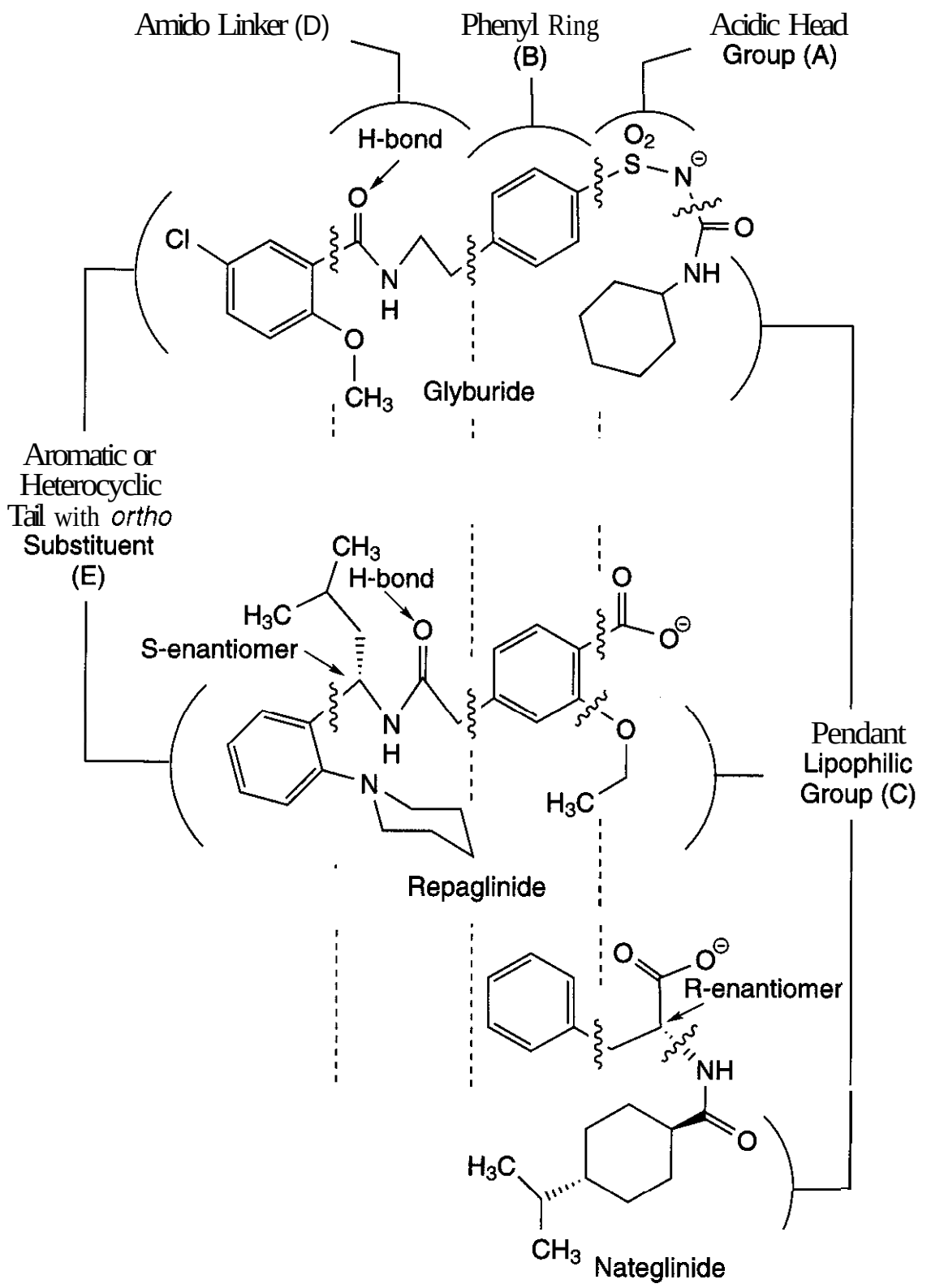
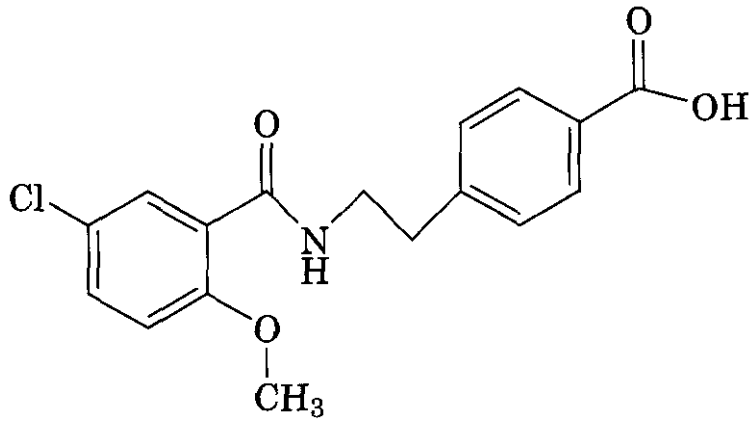


Figure 1.2. Common structural features found in sulfonylurea, benzoic acid, and phenylalanine derived hypoglycemic agents.

stituent is often an N-propyl or N-butyl group, whereas cycloalkyl groups are most common in later compounds. The pendant lipophilic group cannot be attached to benzoic acid derivatives like repaglinide, but it has been proposed that the alkyl group of the 2-ethoxy substituent on the phenyl ring (C) of this compound occupies a similar site on the receptor (106). Phenylalanine derivatives like nateglinide have a chiral center adjacent to the carboxylate. In these compounds, the R configuration at this center is required for activity (104, 105).

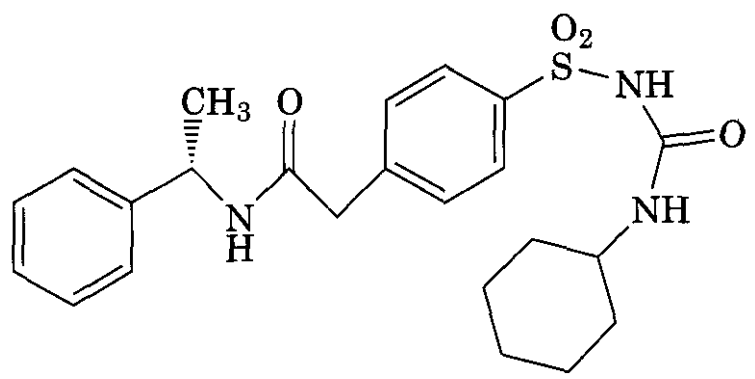
The acidic group in these agents is attached to a phenyl ring (C), which is most often substituted para to the acidic function. In first-generation sulfonylureas, the *para*-substituents are small groups like methyl, acetyl, or chloro. Introduction of larger groups composed of an amido linker (D) attached to an aromatic or heterocyclic tail (E) greatly increases the potency of the second-generation

compounds. In the amido linker, the carbon and nitrogen atoms of an amide are incorporated into a four-atom chain, with the amido nitrogen occupying the third position from the phenyl ring (C). In the sulfonylureas glyburide, glipizide, and glimepiride, the carbon atom of the amido carbonyl group is at the fourth position. Some benzoic acid derivatives like meglitinide (7), which has tolbutamide-



(7)

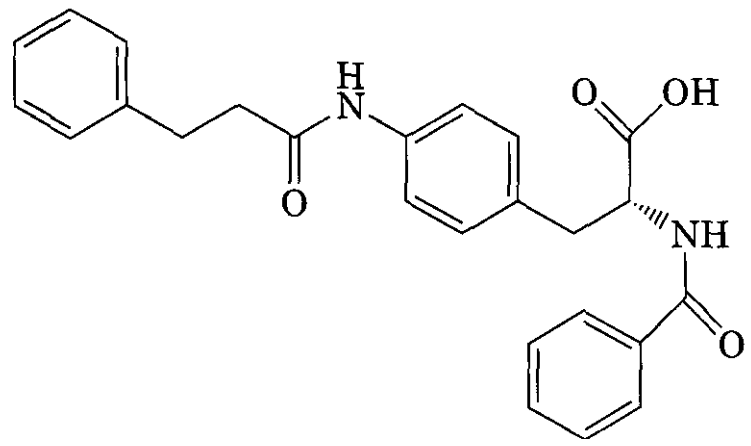
like potency (107, 110, 117), have a similar arrangement of the amido linker. The arrangement in which the carbonyl is at the second position as in repaglinide or the sulfonylurea (8) [active at 0.25 mg/kg p.o. in rabbits



(8)

(116)] also affords highly potent compounds. In this alternative, alkyl substitution of the carbon at the fourth position improves potency and activity resides in the *S*-enantiomers in both sulfonylurea and benzoic acid derivatives. Within the amido linker, the carbonyl group may be more important than the amide NH, and it has been suggested that each alternative chain arrangement positions its respective oxo function to accept a hydrogen bond from the same donor group in the SUR1 protein (106).

Nateglinide lacks an equivalent to the amido linker (D in Fig. 1.2) and the aromatic or heterocyclic tail (E). In related *N*-benzoylphenylalanine compounds (102), the SAR determined for substitution on phenyl ring C parallels that for sulfonylureas, and compound (9), containing a side chain somewhat



(9)

like those found in the second-generation sulfonylureas and repaglinide, was active ([^{3}H]-glyburide binding inhibition IC_{50} = 28 μM; inhibition of K_{ATP} channel activity EC_{50} = 3.5 μM).

The amido linker found in second-generation sulfonylureas and repaglinide terminates in an attachment to an aromatic or heterocyclic group (E in Fig. 1.2), which is often substituted *ortho* to the point of attachment. Substituents such as alkyleneimino, alkoxy, or oxo having an oxygen or nitrogen atom adjacent to the ring afford potent compounds (106). Glipizide lacks this *ortho* substituent, but the pyrazino nitrogen may serve a similar function in binding.

Conformational analysis of some second-generation sulfonylureas and repaglinide identified low energy conformations of these agents, in which the pharmacophoric groups derived by SAR studies could be best superimposed (106). The proposed binding conformations for glyburide and repaglinide are shown in Fig. 1.3.

2.3 Insulin-Sensitizing Agents

Two subclasses of insulin-sensitizing agents are currently available, the biguanides and the thiazolidinediones. They are described separately in this section because drugs in these

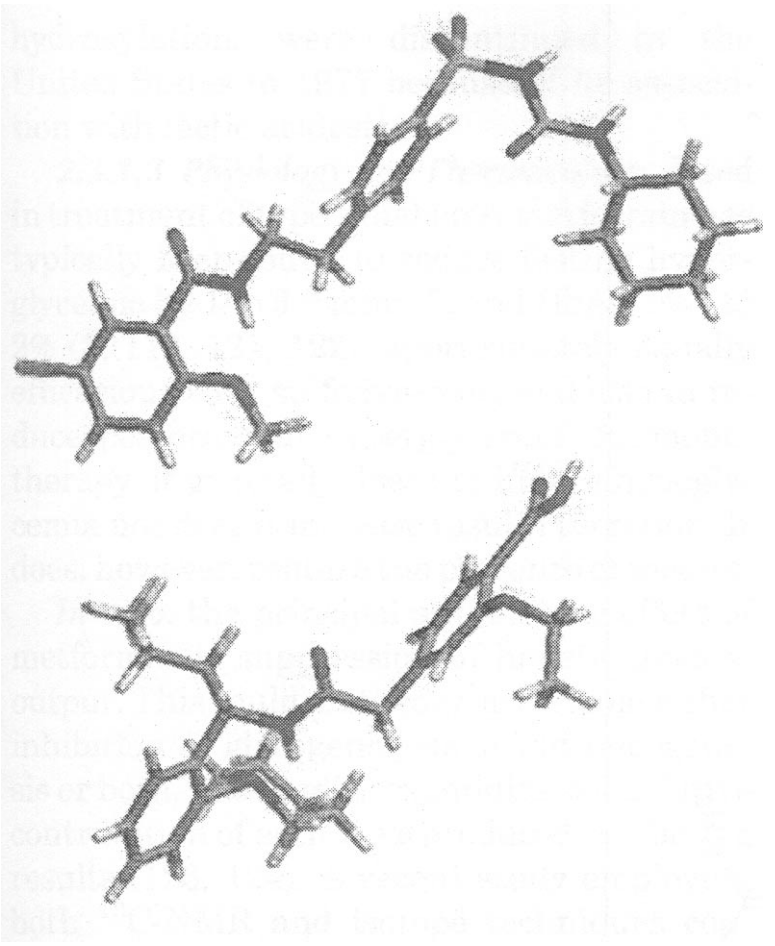


Figure **1.3.** Proposed binding conformations (106) for glyburide and repaglinide based on the selection of calculated low energy conformers, which allow approximate superimposition of the major pharmacophoric groups.

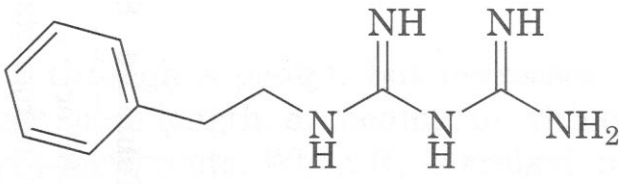
subclasses do not share a common mechanism of action and are not structurally similar.

2.3.1 Biguanides. First introduced in the late 1950, metformin (Table 1.6) is best described as an antihyperglycemic agent because it does not normally cause hypoglycemia. It enhances insulin sensitivity and is not effective in the absence of insulin (118). Metformin lowers blood glucose levels in NIDDM patients by suppressing hepatic glucose output and enhancing peripheral glucose uptake, but the underlying mechanism(s) is not completely understood. Other biguanides produce similar effects, but concern over potential to cause lactic acidosis has largely led to their removal from the market.

2.3.1.1 Side Effects, Adverse Effects. The major side effects associated with metformin therapy are gastrointestinal, including diarrhea, nausea, abdominal discomfort, and anorexia, which improve with dose reduction and can be minimized by slow dose titration (3, 118). Lactic acidosis, a serious often fatal side effect associated with biguanides, is rare with metformin, with incidence estimated at 0.01 to 0.08 per 1000 patient-years. With metformin therapy, lactic acidosis most often occurs in patients with renal insufficiency, problems with alcohol abuse, or liver and cardiopulmonary disease (3, 118, 119). Metformin is removed from the system almost exclusively by renal elimination of the unchanged drug. Thus, poor renal function may lead to accumulation. Hypoglycemia is rare with metformin monotherapy.

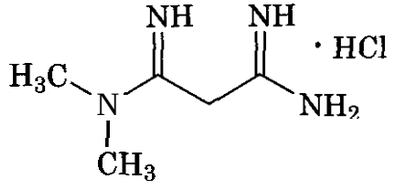
2.3.1.2 Absorption, Distribution, Metabolism, and Elimination. Biguanides are strongly basic, and metformin (pK_a = 11.5) is completely protonated and cationic over the physiologic pH range. Absorption of metformin from the GI tract after oral administration is incomplete, with 20 to 30% recovered in the feces (120). In single oral dosing studies, a lack of dose proportionality indicates decreased absorption with increasing dose. Peak blood levels are obtained in 2 to 4 h. The average volume of distribution after a single oral dose of 850 mg is 654 L. The pharmacokinetics of metformin have been described by use of a two-compartment model (120), with rapid distribution to a central plasma compartment followed by slow transfer to a deep compartment. Tissue concentrations higher than that in plasma are found in liver, kidney, salivary glands, and the intestinal wall. Metformin does not bind to plasma proteins, but does partition into erythrocytes. It is not metabolized and is excreted unchanged in the urine. After i.v. administration, renal clearance is about 3.5 times creatinine clearance, an indication of tubular secretion.

Metformin is slowly and incompletely absorbed and rapidly eliminated without hepatic metabolism. This pharmacokinetic profile may make drug accumulation and lactic acidosis less likely to occur with metformin than with other biguanides. Sales of the longer-acting biguanide phenformin (10), for instance, which is metabolized in the liver by aromatic



(10)

Table 1.6 Structure and Properties of Metformin

USP or Nonproprietary Name	Trade Name	Manufacturer	Chemical Class	Structure	Daily Dose	t_{max}	$t_{1/2}$	Duration[a]
Metformin	Glucophage	Bristol-Meyers Squibb	Biguanide		1500–2550 mg	2–4 h	**6.2** h	>3–4 wk

"**Ref. 3.**

hydroxylation, were discontinued in the United States in 1977 because of its association with lactic acidosis.

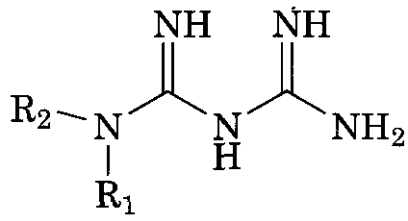
2.3.1.3 Physiology and Pharmacology. Used in treatment of type 2 diabetes, metformin has typically been found to reduce fasting hyperglycemia by 1 to 3.9 mmol/L and HbA_{1c} by 1 to 2% (3, 119, 121, 122), approximately equally efficacious with sulfonylureas, and also to reduce postprandial hyperglycemia. As monotherapy, it generally does not induce hypoglycemia nor does it increase insulin secretion. It does, however, require the presence of insulin.

In vivo, the principal antidiabetic effect of metformin is suppression of hepatic glucose output. This could, in theory, arise from either inhibition of glycogenolysis or gluconeogenesis or both, and studies examining the relative contribution of each have produced conflicting results (123, 124). A recent study employing both ^{13}C-NMR and isotope techniques concluded that suppression of endogenous glucose production with 3 months of metformin monotherapy in type 2 diabetics was completely attributed to a reduction in gluconeogenesis, although hepatic glycogen content did increase (125). Metformin enhances insulin sensitivity and glucose disposal in peripheral tissue (3, 118). At the cellular level, this appears to be attributable in part to increased glucose transport (119, 125–129). Metformin treatment is often associated with an improved plasma lipid profile (121, 130), lowering plasma triglyceride and LDL cholesterol.

At the molecular level, precise target receptor(s) have not been identified. Metformin has been reported to activate the AMP-activated protein kinase system (AMPK) in primary hepatocytes (131). AMPK has been proposed as a key regulatory enzyme of carbohydrate and fat metabolism (132). This kinase is activated through an allosteric mechanism by binding 5'-AMP and also by phosphorylation. Activation of AMPK by phosphorylation is 5′-AMP dependent because the binding of AMP to the enzyme makes it a better substrate for AMPK kinase, which phosphorylates AMPK, and a poorer substrate for protein phosphatase 2C, which dephosphorylates it. AMPK kinase is also activated by 5'-AMP. These actions of 5'-AMP are antagonized by high concentrations of ATP, and thus the system responds to the AMP/ATP ratio. Metformin has no direct effect on the activity of partially purified AMPK, but in primary hepatocytes it increases the phosphorylation and activity of AMPK in a time-dependent manner. This may occur through modulation of AMPK kinase or PP2C, or through a change in the AMP/ATP ratio. At relatively high concentrations, metformin inhibits respiratory chain complex I in intact hepatocytes through an unknown signaling pathway (133), but not in isolated mitochondria. Such fnhibition might lead to an increase in the AMP/ATP ratio and thus account for increased phosphorylation of AMPK. In any case, activation of AMPK would be expected to increase fatty acid oxidation and glucose uptake in muscle, while decreasing hepatic gluconeogenesis and lipogenesis, a pattern of activity that is similar to that observed with metformin.

In Xenopus oocytes (126, 134), treatment with metformin has been shown to increase insulin receptor tyrosine kinase activity associated with activation of phospholipase C and increased levels of IP_3 and intracellular Ca^{2+}. Thus metformin, through increasing receptor tyrosine kinase activity, may facilitate IRS protein phosphorylation and activation of phosphatidylinositol-3-kinase, leading to increased glucose uptake.

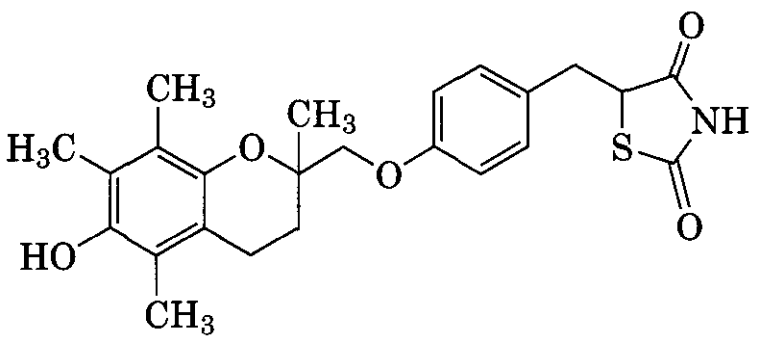
2.3.1.4 Structure–Activity. The available data on the relationship of structure to hypoglycemic activity for substituted biguanides result from a very limited number of studies, generally in healthy animals (135–137). Alkyl substitution (structure 11) at R_1 increases activity through n-pentyl, but decreases with longer chain length, branching, or with cyclic alkyl substituents. When R_1 is aralkyl, phenethyl is the most potent. Potency is highest with a hydrogen or methyl substituent at R_2.



(11)

2.3.2 Thiazolidinediones. The thiazolidinediones (Table 1.7) are the most recently introduced class of oral agents for treatment of type 2 diabetes, improving insulin sensitivity and lowering blood glucose, free fatty acid, and triglyceride levels. The thiazolidinediones are PPARγ (peroxisome proliferator-activated receptor γ) agonists. The PPARγ receptor is a member of the nuclear hormone receptor family of ligand-activated transcription factors that regulates gene expression of several genes involved in fatty acid and carbohydrate metabolism and adipocyte differentiation (138).

2.3.2.1 Side Effects, Adverse Effects. Because of their recent introduction, long-term side effects are less well known than with the other classes of hypoglycemic agents. Both rosiglitazone and pioglitazone are generally well tolerated. Weight gain and minor edema are associated with thiazolidinedione therapy, and their use in patients with moderate to severe chronic heart failure is not advised. Decreases in hemoglobin levels and hemocrit have also been found. The first marketed thiazolidinedione, troglitazone (**12**), was withdrawn because of increased risk of hepatotoxicity. Approximately 1.9% of patients treated with troglitazone in preapproval controlled clinical trials showed an increase in plasma of the liver enzyme alanine aminotransferase (ALT) greater than three times the upper limit of the normal range. In trials with rosiglitizone or pioglitizone, the incidence of significantly elevated ALT was low and similar to that of placebo. However, regular monitoring of liver enzymes is recommended when these drugs are used (139,140).



(12)

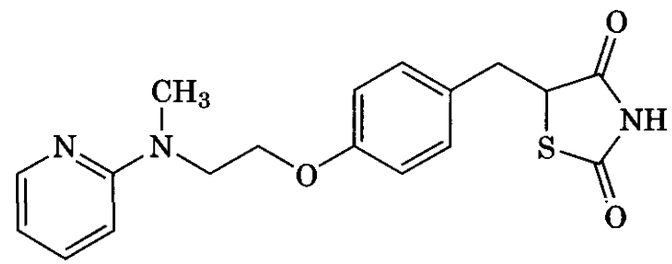
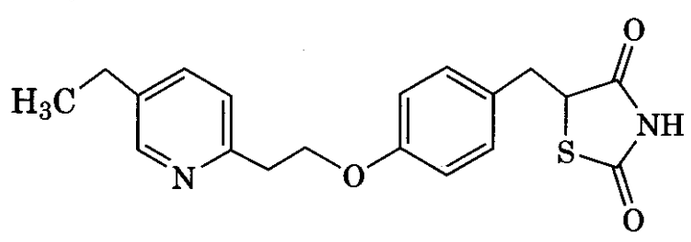
2.3.2.2 Absorption, Distribution, Metabolism, and Elimination. Rosiglitazone is well absorbed, with absolute bioavailability of 99%. After oral dosing, peak blood levels are observed in about 1 h. Rosiglitazone is highly bound to plasma protein (99.8%), with a steady-state volume of distribution of approximately 17.6 L. Pioglitazone is also well absorbed, reaching maximum blood levels in about 2 h. Binding to plasma proteins is greater than 99%, with a single dose volume of distribution of 0.63 L/kg.

Rosiglitazone is metabolized in the liver primarily by N-demethylation and by hydroxylation of the pyridine ring at the 5 position (*para* to the side chain), followed by conjugation (141, **142**), but the metabolites are not believed to contribute significantly to clinical effect. The drug is eliminated, with a half-life of 3 to 4 h, primarily in the urine (64%), with lesser amounts in the feces (23%). Pioglitazone is extensively metabolized (143, **144**) by O-dealkylation to (**13**), by benzylic oxidations of methylene groups adjacent to the pyridine ring (**14a**, **14b**, **14c**, and **14e**) and by ω oxidation of the pyridine ethyl substituent (**14d**).

Metabolites (**14a**), (**14b**), and (**14c**) have hypoglycemic and hypolipidemic effects in Wistar fatty rats, and may contribute significantly to the pharmacological activity of pioglitazone in humans. Serum half-life of the parent drug is 3–7 h, whereas that for total pioglitazone-related species is 16–24 h. From 15 to 30% of an oral dose is excreted in the urine, primarily as metabolites and their conjugates. The balance is presumably excreted in the bile.

2.3.2.3 Physiology and Pharmacology. Rosiglitazone and pioglitazone have only recently been introduced for the treatment of type 2 diabetes, and evaluations of the efficacy of these drugs over years of therapy are not yet available. In large preapproval clinical trials of 24–26 weeks in type 2 diabetics (145), pioglitazone treatment (45 mg, once daily) as monotherapy reduced fasting blood glucose by 3.6 and 3.8 mmol/L compared with placebo and HbA_{1c} by 1.5 and 1.6%, respectively. In two similar 26-week preapproval studies (146), rosiglitazone monotherapy (4 mg, twice daily) resulted in decreases in mean fasting plasma

Table 1.7 Structures and Properties of Thiazolidinediones

USP or Nonproprietary Name	Trade Name	Manufacturer	Chemical Class	Structure	Daily Dose	t_{max}	$t_{1/2}$	Duration[a]
Rosiglitazone	Avandia	SmithKline Beecham	Thiazolidinedione		4–8 mg	1–1.75 hr	3-4 hr	>3–4 wk
Pioglitazone	Actos	Takeda/Lilly	Thiazolidinedione		15–45 mg	2–4 hr	3–7 hr	>3–4 wk

[a]Ref. 3.

glucose levels of 3.4 and 4.2 mmol/L relative to a placebo-treated group, with a mean decrease of 1.5% in HbA_{1c} in both. In a 52-week glyburide-controlled study, rosiglitazone at 2 or 4 mg twice daily was initially less efficacious than glyburide in reducing hyperglycemia, but by the end of the study, both drugs produced a similar response (change in HbA_{1c}: −0.7% with glyburide and −0.5% with rosiglitazone). This might suggest that the hypoglycemic activity of rosiglitazone and perhaps other thiazolidinediones, although initially less than that of sulfonylureas, is more durable over time. In addition to hypoglycemic activity, thiazolidinediones reduce insulin levels and improve insulin resistance, markedly reduce plasma free fatty acids, increase the storage of fat, and often improve the blood lipid profile (139. 147). The thiazolidinediones also increase adipose tissue cell differentiation. Rosiglitazone promotes the differentiation of human subcutaneous preadipocytes into adipocytes, but has little effect on preadipocytes from visceral adipose tissue depots (147, 148). As discussed later in this section, the effects of thiazolidinediones on carbohydrate metabolism are probably at least in part a result of the action of these compounds on the storage and metabolism of fat.

Thiazolidinediones bind to and activate peroxisome proliferator-activated receptor γ (PPARy) (149–152). PPARy is a member of the PPAR family of nuclear receptors, which are ligand-activated transcription factors regulating storage and metabolism of fatty acids. The three members of the PPAR family, termed a, 6, and γ, are activated by fatty acids and fatty acid metabolites. PPARa is the target receptor for the fibrate class of hypolipidemic agents. Activation of this receptor in rodents, but not humans, leads to an increase in the number and size of hepatic peroxisomes, organelles involved in β-oxidation of fatty acids, a property unique to PPARα, from which the name of the receptor family was derived. Less is known about the function of PPARδ, although a PPARδ-selective agonist has been reported to increase reverse cholesterol transport (153).

PPARy and transcription factors of the CCAAT/enhancer-binding protein family (C/EBPα, -β, and -6) are integral members of the genetic cascade leading to adipogenesis (154), the process of adipocyte differentiation. Activation of PPARy, which is highly expressed in adipose tissue (155–159), leads to upregulation of the expression of genes regulating fatty acid transport, storage, and oxidation. There are two isoforms of PPARy, termed PPARyl and PPARγ2, differing in that PPARγ2 has 28 additional amino acids at the N-terminus in humans (157). Both PPARyl and PPARγ2 are abundant adipose tissue, with mRNA for PPARγ2 generally about 10 to 15% of that of PPARyl. Lesser amounts of PPARγ2 have been found in liver, whereas PPARyl appears to be more widely distributed (152, 156, 158–160). PPARy activation also leads to decreased expression of gene-encoding enzymes required for gluconeogenesis in the liver and suppressing glucose oxidation in muscle (150, 161).

Comprehensive mRNA profiling has been used to identify genes regulated directly or indirectly by PPARy in various tissues in Zucker diabetic fatty rats (161). Proteins for which PPARy activation leads to increased mRNA in white adipose tissue include enzymes acetyl-CoA carboxylase, fatty acid synthase, and dihydrolipoamide acyltransferase [a component of the pyruvate dehydrogenase (PDH) complex] required for lipogenesis, and phosphoenolpyruvate carboxykinase (PEPCK), glycerol-3-phosphate acyltransferase, and long-chain acyl-CoA synthetase for triglyceride biosynthesis. In muscle, expression of pyruvate dehydrogenase kinase 4 (PDK4) is decreased. PDK4 phosporylates and deactivates PDH complex, limiting oxidative glucose disposal. Thus, suppression of PDK4 gene expression would lead to an increase in muscle glucose oxidation. In contrast to adipose tissue, liver expression of PEPCK is suppressed by PPARy activation along with other enzymes required for gluconeogenesis, including pyruvate carboxylase and glucose-6-phosphatase.

In common with other PPARs, PPARy forms an obligate heterodimer (152, 162) with 9-cis retinoic acid receptors (RXR) and regulates gene expression through binding of the heterodimer to DNA response elements, containing two copies of the sequence AGGTCA, separated by a single nucleotide (DR1 motif).

Specificity is likely a result of small differences in optimal DNA-binding sites and differences in tissue distribution (150). Agonist binding allows recruitment of coactivator proteins such as cAMP response element binding protein (CBP) or steroid receptor coactivator 1 (SRC-1), which mediate the transcriptional activation of target genes. Coactivator proteins contain one or more leucine-rich LxxLL motifs known as nuclear receptor (NR) boxes, which bind to hydrophobic pockets formed on agonist binding to PPARy and other nuclear receptor proteins. The nature of the xx amino acid residues as well as residues flanking the NR box appears to determine the coactivator-receptor specificity (163).

The affinity of particular agents for binding to PPARy can be assessed with conventional binding assays (149) employing radioligands such as [^{3}H]rosiglitazone. A scintillation proximity assay (164) amenable to high throughput screening has also been developed. Variations of functional transactivation assays are used that measure expression of PPARy target genes in mammalian cells transfected with both a chimeric receptor expression construct, composed of the ligand-binding domain of PPARy coupled with the DNA-binding domain of the yeast transcription factor GAL4, and a construct containing a GAL4 response element and reporter gene combination (151, 152, 165). Reporter genes such as chloramphenicol acetyltransferase (165), secreted placental alkaline phosphatase (SPAP) (151), or firefly luciferase (149) whose products can be conveniently assayed have been used. Because GAL4 is not found in mammalian cells, activation of other nuclear receptors does not interfere with these assays. Comparative binding affinities (152) and PPARy agonist activity (151) in a PPARγ–GAL4/SPAP assay for troglitazone, pioglitazone, and rosiglitazone are shown in Table 1.8. These compounds have no significant activity in PPARα- or PPARδ-specific assays, and the relative potencies of these thiazolidinedione agents in PPARy assays compares well with in *vivo* hypoglycemic potency in humans.

PPARy has a significant role in adipocyte differentiation and in the regulation of fat storage and utilization. However, thiazolidinediones are used clinically as hypoglycemic agents and to improve insulin sensitivity. The mechanism(s) by which PPARy activation produces these clinically useful effects is not completely established. Although at lower levels than those in adipose tissue, PPARy is present in skeletal muscle and liver, tissues primarily responsible for glucose disposal and gluconeogenesis. Hence, direct regulation by PPARy of expression of key enzymes controlling these processes cannot be ruled out. On the basis of comprehensive mRNA profiling, it has been estimated that administration of a PPARy agonist in a diabetic rodent model altered the expression level directly or indirectly of approximately 10% of all genes in white or brown adipose tissue, whereas about 2% of the genes in liver and 1% in skeletal muscle were regulated (161). The alteration in specific enzyme levels predicted by the observed changes in mRNA in liver and muscle (for instance, PEPCK and PDK4) should lead to suppression of hepatic gluconeogenesis and increased glucose oxidation in muscle. The administration of the PPARy agonist troglitazone to mice engineered to lack adipose tissue improved insulin sensitivity (166), and preincubation with troglitazone increased glucose uptake, glycogen synthase fractional velocity, and fatty acid oxidation in cultured human myotubes (167). Thus, PPARy agonists like the thiazolidinediones may have a direct action on muscle and liver that is independent of their action on fat.

Alternatively, improved insulin sensitivity and lowered blood glucose with these agents in treatment of NIDDM may be an indirect effect of their action on adipose tissue. Signaling through a number of adipocyte-derived factors, including TNF-a (168), leptin (169–172), resistin (172) and adiponectin (173), is altered by PPARy activation in ways that could lead to

Table 1.8 Thiazolidinedione *PPARy* Binding Affinity and Cell-Based *PPARy* Agonist Activity

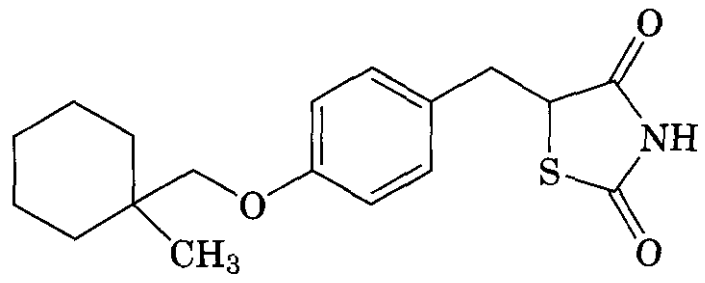
	PPARγ Binding Potency (n*M*)	Human PPARγ Agonist Activity EC_{50} (μM)
Troglitazone	7900	0.55
Pioglitazone	5500	0.58
Rosiglitazone	40–200	0.043

enhanced insulin sensitivity. In addition, free fatty acids, triglycerides, and products of fatty acid metabolism regulate insulin sensitivity. An inverse relationship between insulin sensitivity and fasting plasma free fatty acid concentrations has been observed in offspring of type 2 diabetic parents (8). Increased plasma concentrations of free fatty acids contribute to insulin resistance by inhibition of glucose uptake, glycogen synthesis, and glucose oxidation in muscle (8, **9,** 16, 174) through regulatory interaction of fatty acid metabolites with components of the insulin signal transduction cascade and enzymes controlling glucose metabolism.

In normal individuals, skeletal muscle has the capacity to switch from the predominant uptake and oxidation of fatty acid as metabolic fuel under fasting conditions to greater uptake, storage, and oxidation of glucose under insulin stimulation. In obesity, which is linked strongly with type 2 diabetes, metabolic inflexibility in the form of lower fasting rates of lipid oxidation and failure of insulin to suppress fatty acid uptake and utilization is believed to lead to triglyceride accumulation in muscle (9, 175). The amount of triglyceride in skeletal muscle is significantly associated with insulin resistance. Thiazolidinediones promote the storage of fat and also redistribution of intracellular triglyceride from insulin-responsive organs into adipose tissue (175), accounting, perhaps, for improved insulin sensitivity with the use of these drugs. Although increasing the fat store might intuitively seem more likely to increase than to decrease insulin resistance, this is thought to be depot specific. Visceral adipose tissue volume, rather than general obesity or subcutaneous adipose tissue volume, appears to correlate with insulin resistance (147). After 6 months of rosiglitazone therapy, a modest but significant increase in subcutaneous fat mass was observed, with no change in visceral fat.

Elevated free fatty acids and intracellular accumulation of triglycerides in pancreatic β-cells may also play a role in loss of β-cell mass and insulin secretory function, which occur in NIDDM as the disease progresses (176, 177). In short-term studies, rosiglitazone prevented loss of p-cell mass in Zucker Diabetic Fatty rats (178), suggesting utility in slowing or preventing the progression of diabetes.

2.3.2.4 ***History.*** In the course of investigations on the fibrate class of hypolipidemic agents at Takeda (179, 180), a series of 5-(4-alkoxybenzyl)-2,4-thiazolidinediones were shown to reduce insulin resistance in genetically diabetic and obese animals. Ciglitazone (15), which became the prototype for this class



(15)

of drug, was found to reduce hyperglycemia, hyperlipidemia, and hypertriglyceridemia in insulin-resistant animal models, but not in normoglycemic animals (181, 182). Ciglitazone was taken into human trials in NIDDM subjects, but was withdrawn because of low potency and the appearance of cataracts in animals receiving long-term exposure to the drug. It was replaced in development with the more potent pioglitazone (183), which has subsequently received marketing approval in the United States and much of the world.

2.3.2.5 ***Structure-Activity.*** The relationship of structure to hypoglycemic activity and activation of the various PPARs with substituted thiazolidinediones and related compounds has been the subject of intensive investigation (179, 180, 183–202). Some general structure–activity relationships are apparent (Fig. 1.4) from a comparison of the common features of the more potent compounds identified in these studies. Thiazolidinedione hypoglycemic agents can be viewed as being composed of an acidic head group connected to a lipophilic tail by a phenoxyalkyl linker. The pK_a value for thiazolidinediones is about 6.8, and thus these compounds are partially ionized at physiological pH. This appears to be important, given that removal of the acidic function by *N*-methylation leads to loss of activity. Other acidic moieties, heterocyclic groups like oxazolidinediones and particularly α-substituted carboxylic acids, can also

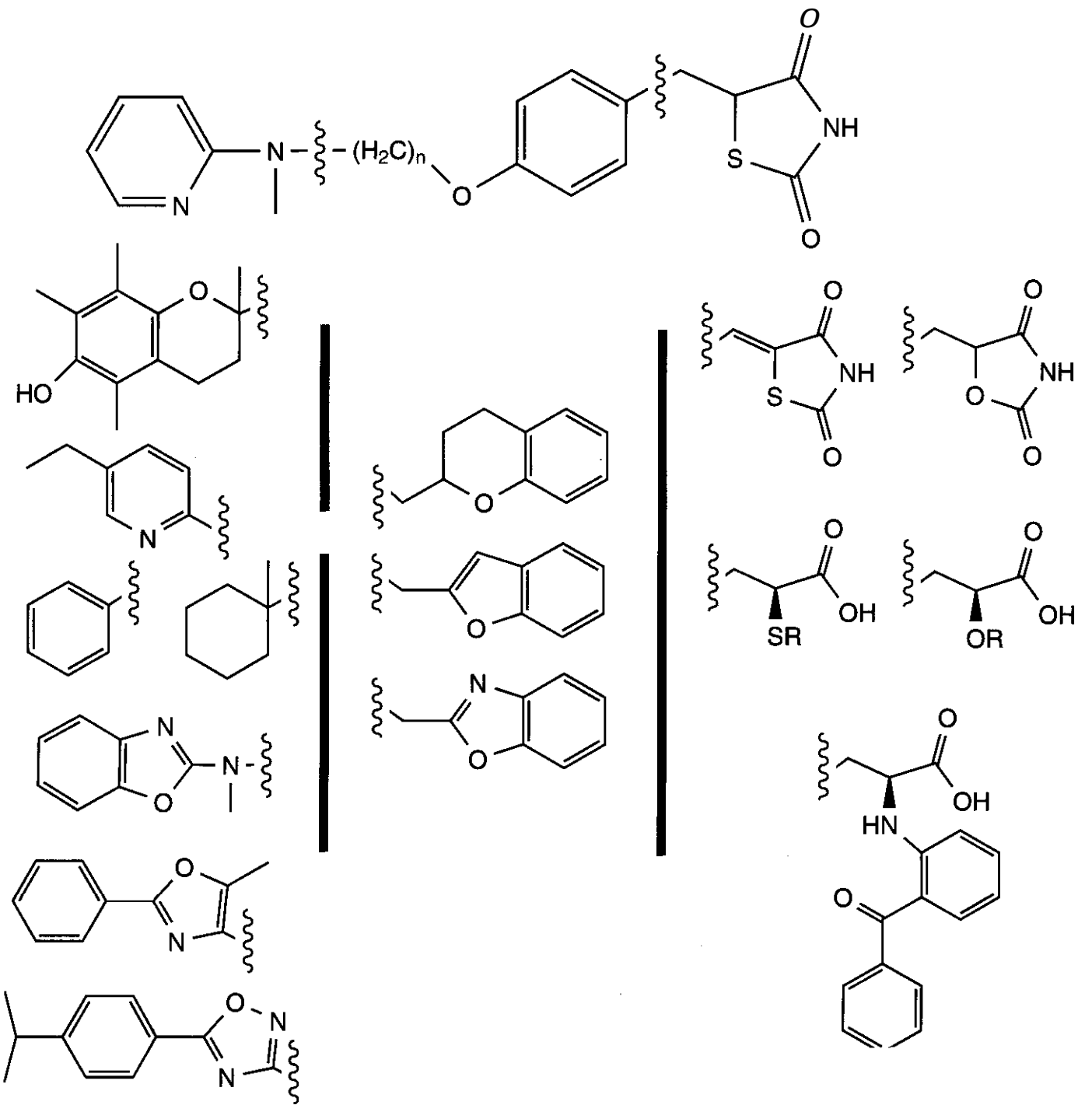
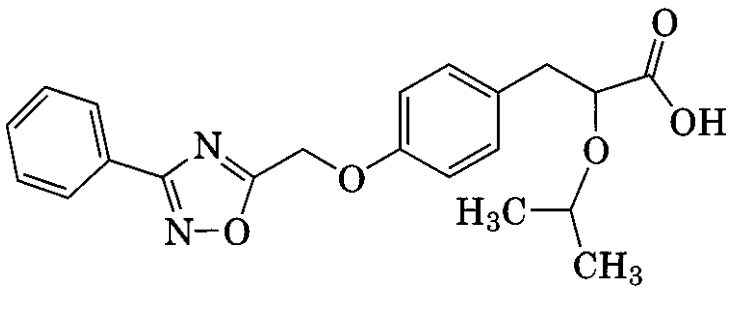


Figure 1.4. Common structural features found in thiazolidinedione PPARγ agonists and related compounds.

replace the thiazolidinedione ring. The α-substituted carboxylic acids are often highly potent, but may not be selective for PPARγ. Thus compound (16) has similar potency (195) in functional transactivation assays for both PPARα and -γ (EC_{50} PPARα 13 n*M*, PPARγ 4 n*M*), whereas farglitazar (17) is highly selective (190) for PPARγ (EC_{50} PPARα 450 n*M*,



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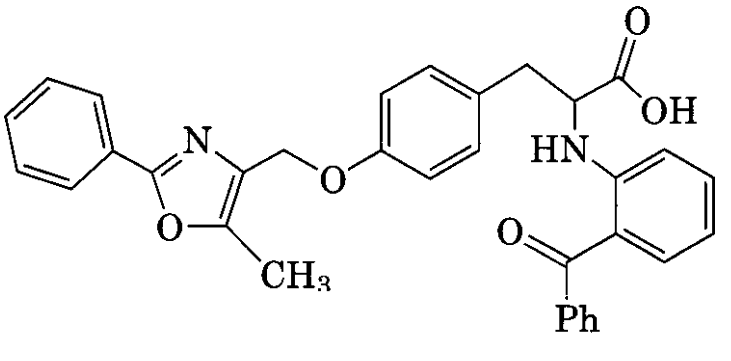
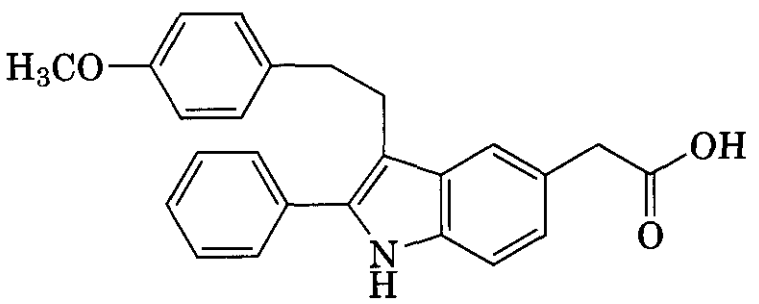


(17)

PPARy 0.35 n*M*). Neither (16) nor (17) has significant PPARS activity.

There is a chiral center at the 5 position of the thiazolidinedione ring, but this is not configurationally stable under physiological conditions. For analogous a-substituted carboxylic acids, the PPARy activity resides in the *S*-enantiomer. These compounds, including the thiazolidinediones, can be viewed as derivatives of phenylpropionic acid by combining the acidic head group with the phenyl group of the linker. Some arylacetic acids (18 and 19)



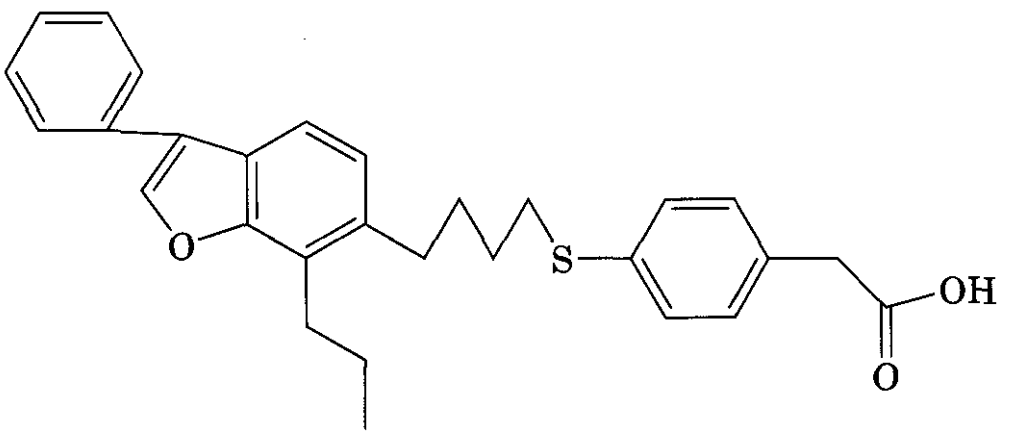
(18)

are also fairly potent PPARy agonists (201, 202), although the SAR has not been as thoroughly explored. Compound (18) is highly selective for PPARy, whereas (19) activates both PPARS and -y.

A phenoxyethyl group (Fig. 1.4, n = 2) as the central phenoxyalkyl linker is commonly found to yield highly active compounds in SAR studies of hypoglycemic thiazolidinediones. Often shorter chain lengths (Fig. 1.4, $n = 1$) or inclusion of the phenoxyethyl group into a heterocyclic ring also leads to active compounds. In the lipophilic tail, incorporation of a wide variety of mostly aromatic and heteroaromatic groups has produced active agents. In a very limited study (186), the hypoglycemic potency in a series of oxazolidinediones with variations in this lipophilic tail was found to increase with increasing log P.

A small number of 3D-QSAR studies (203, 204) on the thiazolidinediones have been reported, but these agents and the PPAR receptors are not particularly well suited for this type of analysis. 3D-QSAR studies are highly dependent on alignment, which can be difficult with very flexible molecules that bind to a receptor principally by large regional diffuse hydrophobic interactions.

X-ray crystallographic studies (205–210) of thiazolidinedione and related ligands cocrystallized with various PPAR ligand-binding domains (LBDs) and often a fragment of the coactivator protein SRC-1 have provided the basis for a detailed understanding of binding conformation, PPAR receptor specificity, receptor shape, and the ligand-activation process. Rosiglitazone (205) binds to the PPARy LBD in a roughly U-shaped conformation (Fig. 1.5) in a large (−1440 Å^3) convoluted pocket. The thiazolidinedione ring binds in a polar site, making hydrogen bonds with groups in the side chains of His-449, Tyr-473, His-323, and Ser-289. Each of these amino acids forms part of a different helical component of the protein. Agonist binding results in the stabilization of a conformation in which the terminal carboxylate group of the AF-2 helix at the C-terminus of the PPARy LBD is positioned to form hydrogen bonds with elements of the RXR protein in the heterodimer. In addition, it places the LxxLL motif of the coactivator in position to bury its Leu residues in a hydrophobic cleft of the LBD. NMR studies (211)



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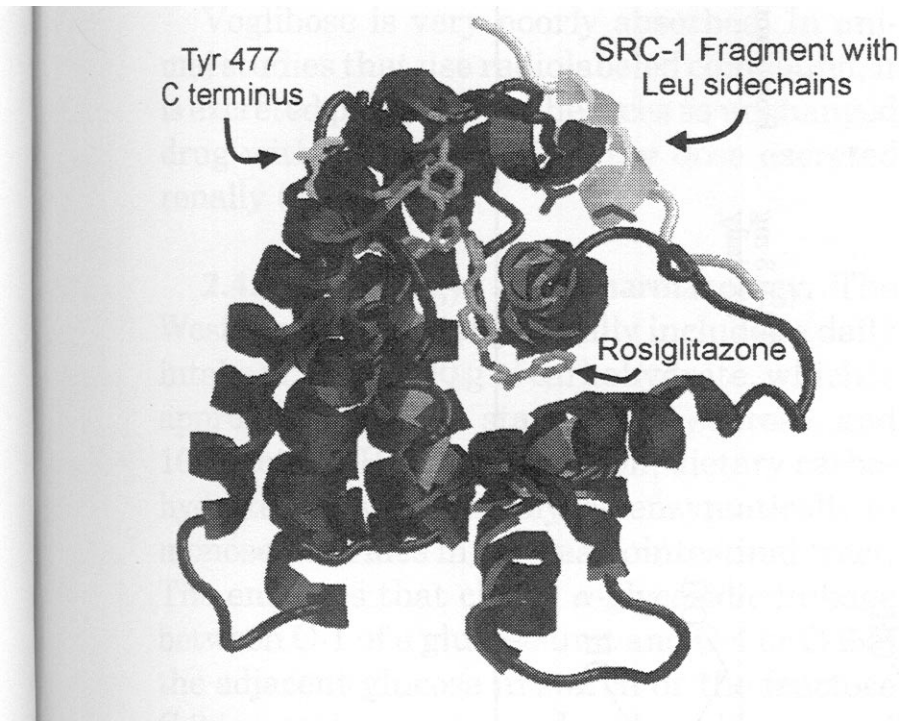


Figure 1.5. A portion of the X-ray structure (205) of rosiglitazone co-crystallized with the PPARγ ligand-binding domain (LBD) and a fragment of the coactivator protein SRC-1, showing the bound conformation of rosiglitazone. Coordinates were obtained from the Protein Data Bank **(212)** and displayed with RasMol.

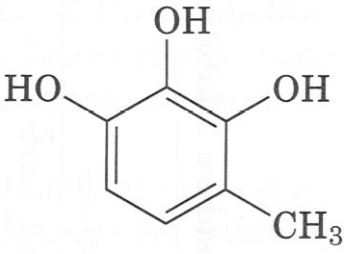
suggest that agonist ligand binding results in a population shift in a dynamic ensemble of conformations rather than a switch between single active and inactive conformations. PPARα, -δ, and -γ are structurally quite similar, and the agonist-binding conformations of the LBD are nearly identical. The PPARγ selectivity of the thiazolidinediones manifests subtle differences (206, 207). The binding pocket of *PPARS* is narrower in the region of AF-2 and unable to accommodate the thiazolidinedione ring or large substituents *a* to the carbonyl in carboxylic acid head groups. In PPARα, substitution of a larger tyrosine for the histamine (His-323)in *PPARy* involved in hydrogen bonding to the thiazolidinedione prevents binding.

2.4 α-Glucosidase Inhibitors

a-Glycosidase inhibitors (Table 1.9) delay the digestion of dietary carbohydrate in the form of starch and sucrose into monosaccharides, which can be absorbed from the small intestine. By delaying absorption, these agents lower postprandial blood glucose and insulin levels and are used for this purpose in the treatment of type 2 diabetes.

2.4.1 Side Effects, Adverse Effects. Gastrointestinal disturbances in the form of flatulence, abdominal discomfort, and, to a lesser extent, diarrhea are common side effects of therapy with a-glucosidase inhibitors. Use of acarbose at higher doses (100 mg or greater) has been associated with a low incidence of elevated serum transaminase levels, most often in patients weighing less than 60 kg.

2.4.2 Absorption, Distribution, Metabolism, and Elimination. a-Glucosidase inhibitors exert their action in the intestinal tract, and systemic pharmacokinetics do not directly indicate the availability of the drugs at their site of action. Acarbose is extensively degraded in the intestinal tract by digestive enzymes or intestinal microorganisms, and in humans, less than 2% of an orally administered ^{14}C-labeled dose was absorbed as the intact drug. An average of 51% of the radioactivity was excreted in the feces, whereas 35% was found in urine as at least 13 metabolites. These metabolites appear to be various O-methylated, O-sulfated, and O-glucuronidated derivatives of 4-methylpyrogallol (20) (213). Peak plasma

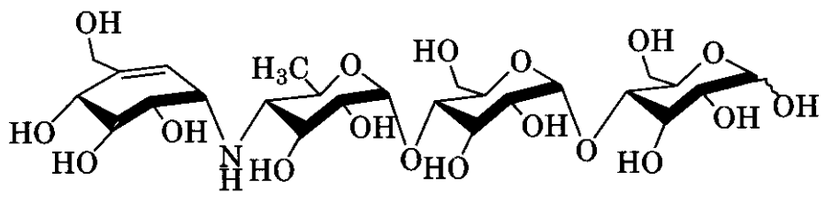
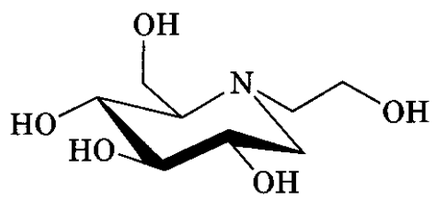
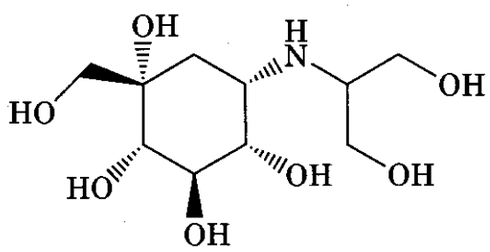


(20)

concentrations of radioactivity were found 14 to 24 h after dosing, whereas the plasma concentration of active drug reached a maximum in about 1 h. On i.v. administration, 89% of the dose is excreted in the urine as active drug within 48 h.

Low doses (25 mg) of miglitol are completely absorbed, but absorption is saturable and is incomplete at higher doses. Peak plasma concentrations occur in 2–3 h. The volume of distribution, 0.18 L/kg, is consistent with distribution primarily into extracellular water and binding to plasma proteins is negligible. Miglitol is renally excreted as unchanged drug, with a plasma elimination half-life of 2 h.

Table 1.9 Structure and Properties of α-Glucosidase Inhibitors

USP or Nonproprietary Name	Trade Name	Manufacturer	Chemical Class	Structure	Dose	Duration
Acarbose	Precose	Bayer	**Pseudotetrasaccharide**		25–100 mg **3**× daily	Postprandial
Miglitol	Glyset	**Pharmacia** and **Upjohn**	**Deoxyno**jirimycin derivative		25–100 mg **3**× daily with meals	Postprandial
Voglibose	**Basen**	**Takeda**			0.2–0.3 mg **3**× daily	Postprandial

Voglibose is very poorly absorbed. In animal studies that use radiolabeled compound, it is excreted primarily in the feces as unchanged drug with less than 5% of the dose excreted renally (214).

2.4.3 Physiology and Pharmacology. The Western human diet generally includes a daily intake of about 400 g of carbohydrate, which is approximately 60% starch, 30% sucrose, and 10% lactose. Before absorption, dietary carbohydrates must be hydrolyzed enzymatically to monosaccharides in the gastrointestinal tract. The enzymes that cleave a-glycosidic linkage between C-1 of a glucose unit and C-4 or C-6 of the adjacent glucose in starch or the fructose C-2 in sucrose are termed a-glucosidases, and temporary inhibition of these enzymes delays the formation of absorbable glucose from larger carbohydrate species. Competitive inhibitors of a-glucosidases are used in the treatment of type 2 diabetes to reduce the rate of appearance of glucose in circulation after a carbohydrate-containingmeal and thus to reduce postprandial hyperglycemia. Clinically, acarbose reduces fasting plasma glucose by 1.4–1.7 mmol/L, postprandial glucose levels by 2.2–2.8 mmol/L, and HbA_{1c} values by 0.7–1% (3). Miglitol appears to be rather similar. Thus these agents, although less efficacious than sulfonylureas or metformin, reduce fasting as well as postprandial hyperglycemia, probably through an indirect mechanism.

Several different a-glucosidases are required for digestion of dietary carbohydrate. Salivary and pancreatic a-amylases are endoglycosidases that hydrolyze interior $\alpha(1,4)$ glycosidic linkages in the glucose chains of the two major types of starch: (1) amylose, a linear $\alpha(1,4)$-linked glucose polymer; and (2) amylopectin, a branched glucose polymer containing $\alpha(1,6)$ linkages at branch points in addition to the $\alpha(1,4)$. Pancreatic a-amylase does not cleave amylopectin chains at or near the $\alpha(1,6)$-linked branch points, and neither sucrose nor lactose is hydrolyzed. The action of pancreatic α-amylase on dietary carbohydrate thus yields a mixture composed mainly of oligosaccharides containing small linear $\alpha(1,4)$-linked glucose polymers (maltose, maltotriose, etc.), the a-limit dextrins {oligosaccharides containing 5 to 9 glucose units terminating at or near a glucose attached to the chain by the a-amylase inert $\alpha(1,6)$ glycosidic linkage), sucrose, and lactose.

These oligosaccharides are hydrolyzed to absorbable monosaccharides by the action of a group of enzymes on intestinal brush border epithelial cells. Hydrolysis of sucrose and starch-derived oligosaccharides is accomplished by two homologous enzyme complexes, sucrase–isomaltase and maltase–glucoamylase, which have overlapping substrate specificities (215). Both are exoglycosidases, hydrolyzing the terminal glucose from the nonreducing end of an oligosaccharide. Sucrase–isomaltase cleaves both $\alpha(1,4)$ and $\alpha(1,6)$ linkages in small gluco-oligosaccharides, debranching the a-limit dextrins and hydrolyzing $\alpha(1,4)$-linked maltose and maltotriose. The sucrase subunit of the complex hydrolyzes sucrose to fructose and glucose. Maltase–glucoamylase also hydrolyzes maltose and larger linear $\alpha(1,4)$-linked glucose oligosaccharides. In vivo, the maltase–glucoamylase complex is believed to account for almost all glucoamylase activity, 20% of maltase, and 1% of isomaltase activity, whereas the sucrase–isomaltase complex accounts for all of the sucrase, most of the isomaltase, and about 80% of the maltase activity. Lactose is cleaved by a third enzyme, lactase, to glucose and galactose.

Therapeutically useful a-glucosidase inhibitors delay glucose absorption by temporarily inhibiting one or more of these digestive enzymes. Complete inhibition of extended duration is not desirable because this leads to unacceptable symptoms of carbohydrate malabsorption (cramps, flatulence, abdominal distension, diarrhea) resulting from carbohydrate fermentation by colonic bacteria. For in vitro assessment of a-glucosidase activity, inhibition of the hydrolytic activities of intestinal brush border membrane preparations or pancreatic homogenates toward various substrates (maltose, isomaltose, sucrose, etc.) are used in quantitative assays (216). Table 1.10 lists K_i values (24, 216) for sucrase, isomaltase, and glucoamylase inhibition by acarbose and miglitol. Perhaps because of its oligosaccharide-like structure, acarbose is most potent at inhibition of glucoamylase activity, whereas monosaccharide-like miglitol is most active

Table 1.10 α-Glucosidase Inhibition by Acarbose and Miglitol[a]

	Acarbose K_i (μM)	Miglitol K_i (μM)
Sucrase	0.086	0.99
Isomaltase	0.36	46.3
Glucoamylase	0.21	0.009

[a]Refs. 143, 144.

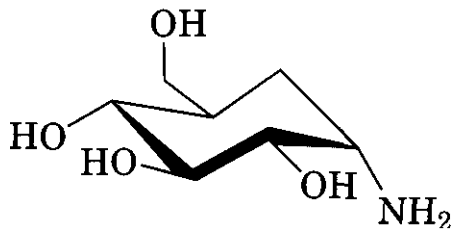
against sucrase. Acarbose also inhibits pancreatic a-amylase (68% inhibition at 4 μM), whereas miglitol inhibits this enzyme only at very high concentrations (216). Voglibose is a very potent inhibitor of maltase and sucrase activity (K_i values of 3.8 and 2.0 nM, respectively), but also has little effect on pancreatic a-amylase (217). Lactase is inhibited only at very high concentrations by miglitol, and not inhibited by acarbose.

In addition to delaying glucose absorption by retardation of carbohydrate digestion, the antidiabetic activity of a-glucosidase inhibitors may be partly mediated by alterations in the release of incretins. These intestinal hormones, such as glucagon-like polypeptide 1 (GLP-1) and gastric inhibitory polypeptide (GIP), are released in response to nutrients and have insulinotropic activity as well as many other regulatory effects. Both acarbose and voglibose increase circulating levels of GLP-1, whereas a-glucosidase inhibition appears to suppress GIP (218–222).

2.4.4 Structure–Activity. The structural requirements for a-glucosidase inhibition, particularly in series of compounds related to those in Table 1.9, have been extensively investigated, and much of this work has been summarized in an excellent review (223). Analysis is complicated, however, by a number of factors. First, several a-glucosidases that have differing but somewhat overlapping substrate specificities and differing affinities for various inhibitors are involved in intestinal carbohydrate digestion. Thus, no single K_i value describes "a-glucosidase" inhibition. Second, the inhibition kinetics of the enzymes are complex (224, 225). The K_i values determined for competitive inhibitors of glucoamylase–maltase, for instance, were substrate (maltose or maltooligosaccharides) dependent, contradicting the classical model of competitive inhibition and suggesting the existence of subtrate-induced binding modes. Finally, a therapeutically useful a-glucosidase inhibitor must delay, but not block completely, digestion and absorption of dietary carbohydrate. For this reason, favorable on–off rates and pharmacokinetics are as important as high affinity of the inhibitor for the target enzymes in the drug optimization process.

Enzymatic cleavage of the glycosidic linkage in the substrates for a-glucosidases is believed to proceed through a pair of high energy oxonium ion intermediates (illustrated with maltose in Fig. 1.6, Intermediates I and II), which must be stabilized by interaction with the enzyme to increase the rate of the reaction. Similar interactions with protonated amine functions of a-glucosidase inhibitors occupying positions normally filled by the oxonium ions stabilize the binding of the nonhydrolyzable inhibitor to the enzyme (223,226). Historically, the lead compounds in the series that led to the marketed a-glucosidase inhibitors in Fig. 1.6 were identified through random screening, but can be seen as pseudoglucosylamines related to high energy intermediate I (inhibitor type I) or as 1-deoxynorjirimycin derivatives related to high energy intermediate II (inhibitor type II).

Compounds (**21**), (**22**), and (23) are moderate to highly potent inhibitors of intestinal sucrase and maltase (217,223) and fall into the category of inhibitor type I pseudoglucosylamines. Valiolamine (**23**), which can be recognized as a structural component of voglibose, is the most potent of the three, suggesting that the non-glucose-like 5-hydroxyl substituent greatly enhances affinity for sucrase. Valienamine (**22**), a component of acar-



(21) validamine
IC_{50} sucrase 7.5×10^{-6}
IC_{50} maltase 1.1×10^{-4}

Pseudoglucosylarnines

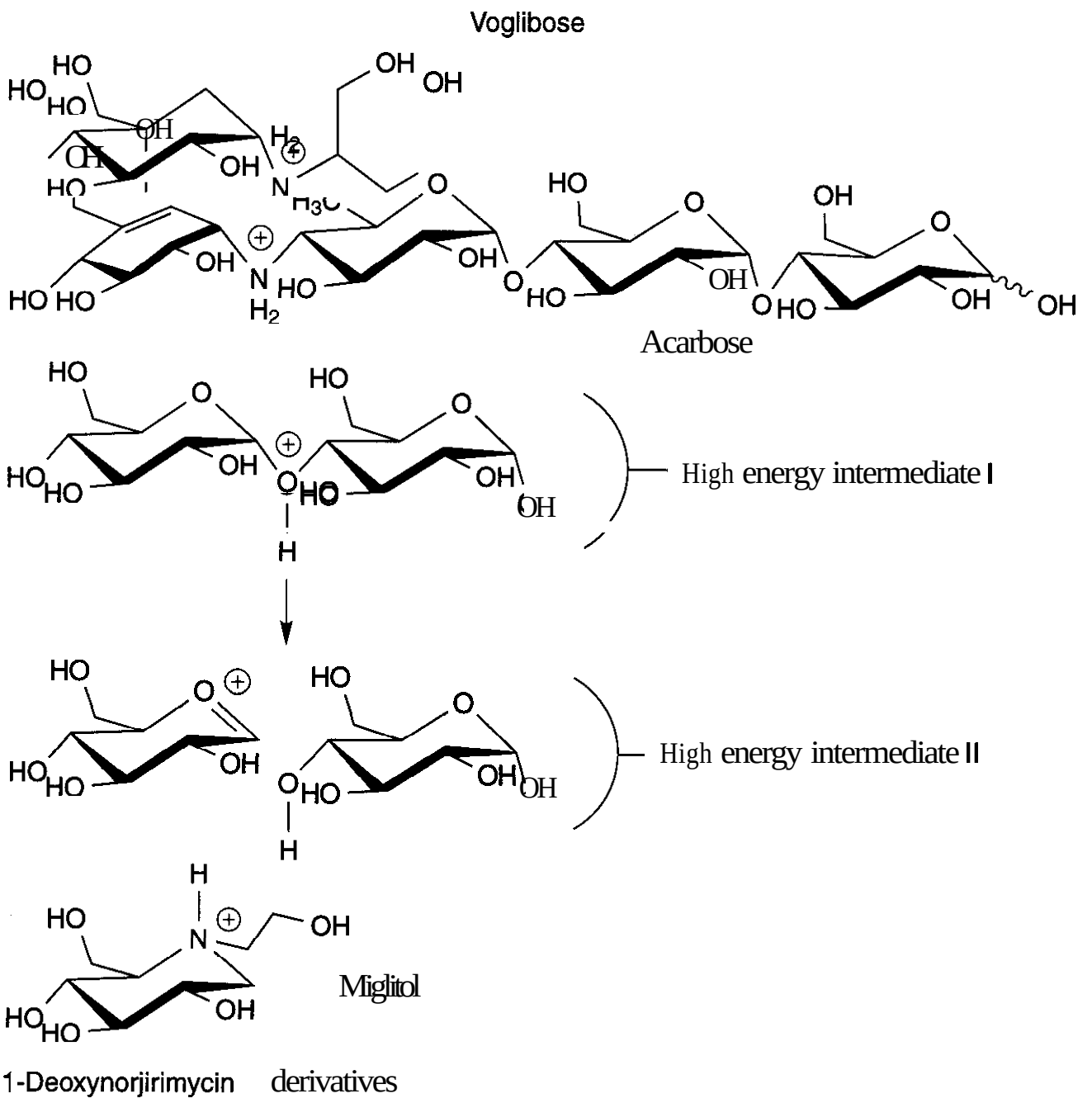
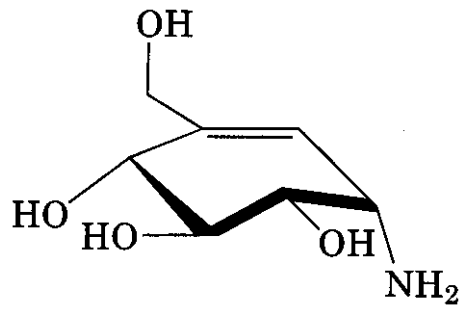
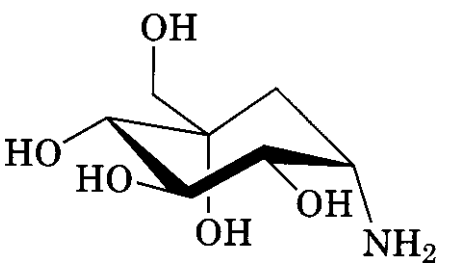


Figure 1.6. Relationship of α-glucosidase inhibitors to high energy intermediates in hydrolysis of maltose.



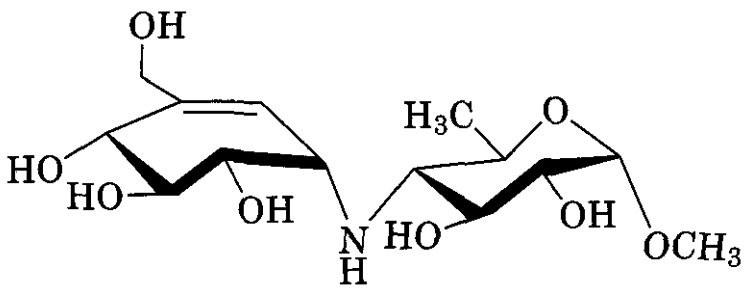
(22) valienamine
IC_{50} sucrase **5.3** x 10^{-5}
IC_{50} maltase **3.4** x 10^{-4}



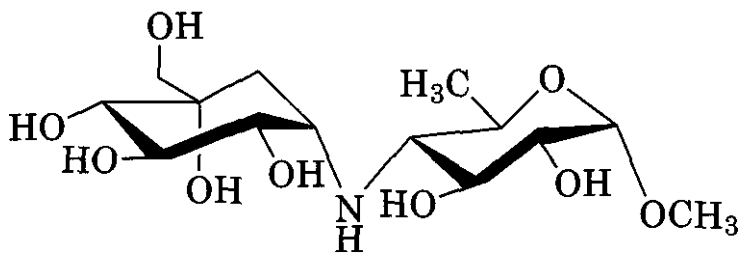
(23) valiolamine
IC_{50} sucrase **7.5** x 10^{-8}
IC_{50} maltase 1.1 **x** 10^{-6}

bose, is the least potent. SAR studies that use derivatives of these compounds in intestinal sucrase and maltase inhibition assays have shown that both the position amine function and the presence of glucoselike **hydroxyl substituents** are essential for good sucrase inhibition.

Compounds (24) and (**25**) are disaccharides incorporating the methyl glycoside of the second sugar unit in the acarbose tetrasaccharide structure into the inhibitor structures. Addition of the second sugar unit yields a modest improvement in IC_{50} for sucrase inhibition with a much greater improvement in that for maltase.

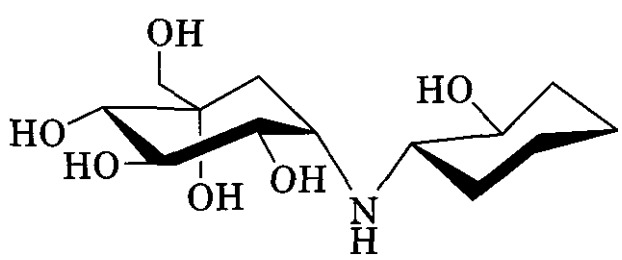


(24) a-methylacarviosin
IC_{50} sucrase 1.6 x 10^{-7}
IC_{50} maltase **3.2** x 10^{-6}



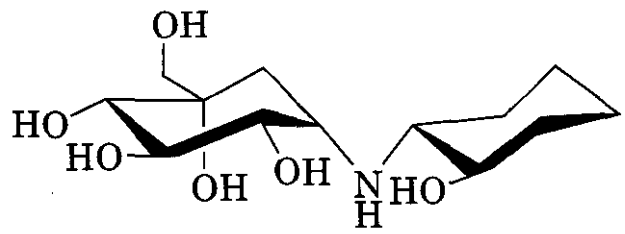
(25)
IC_{50} sucrase 1.0 x 10^{-8}
IC_{50} maltase 4.9 x 10^{-9}

Interestingly, compounds incorporating a glucose-like 6'-hydroxyl group were slightly less potent than (24) and (25). In fact, the high potency of (27) for both sucrase and maltase inhibition suggests little contribution to inhibitor-binding affinity from groups other than the 3'-hydroxyl, which, comparing (26) and (**27**), must be correctly oriented, and relatively simple groups lacking a ring structure

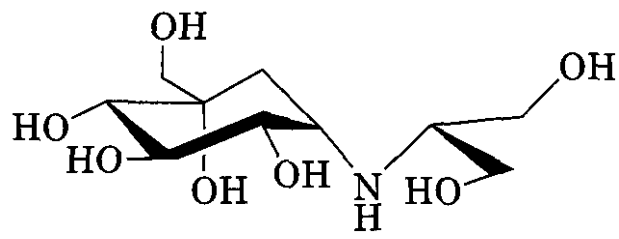


(26)
IC_{50} sucrase 1.6 x 10^{-7}
IC_{50} maltase 1.6 x 10^{-6}

but incorporating this feature (e.g., voglibose, 28) are quite potent.



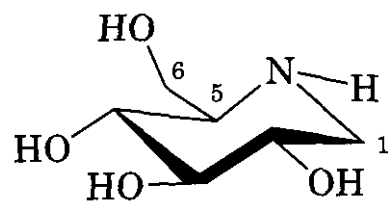
(27)
IC_{50} sucrase 5.2 x 10^{-9}
IC_{50} maltase 6.1 × 10^{-9}



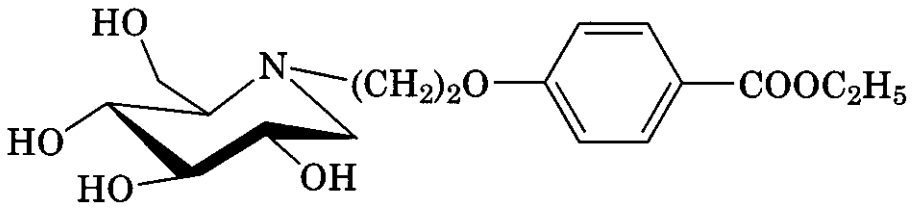
(28) voglibose
IC_{50} sucrase 4.6 x 10^{-9}
IC_{50} maltase 1.5 x 10^{-8}

Acarbose, in *vitro*, is somewhat less effective than a-methylacarviosin (24) in sucrase inhibition (223), and thus the two additional glucose units in acarbose compared with (**24**) contribute little to the affinity of the inhibitor for this enzyme. This is in agreement with kinetic studies showing two saccharide unit binding subsites at the sucrase catalytic center of intestinal sucrase–isomaltase (225). Glucoamylase–maltase appears to have four such sites (227), whereas pancreatic α-amylase has five glucose unit binding subsites (228). Acarbose derivatives with additional glucosyl substituents are potent inhibitors of pancreatic a-amylase, but have not shown therapeutic utility.

Miglitol is an inhibitor type II derivative of 1-deoxynorjirimycin (29). The parent compound has potent inhibitory effects in porcine intestinal mucosal preparations on α-glucosi-



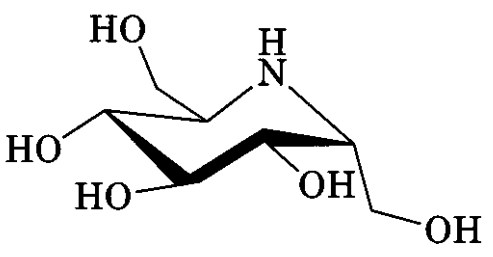
(29) deoxynorjirimycin



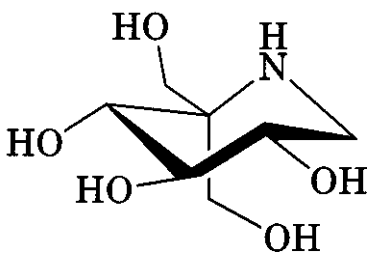
(30) emiglitate

dase enymes. Concentrations in a narrow range of 9.6×10^{-8} to 2.2×10^{-7} M inhibited 50% of the sucrase, maltase, isomaltase, and glucoamylase activity.

Many derivatives of (29) have been prepared and tested, particularly for inhibition of porcine intestinal sucrase activity (223). A small alkyl nitrogen substituent is allowed, but activity decreases as chain length is increased from methyl to propyl, increasing again for very long 11- or 12-carbon alkyl chains. Introduction of a double-bond allylic to the nitrogen increases the inhibitory effect. Polar subtituents, with the exception of hydroxyethyl (miglitol) and aryloxyalkyl as in (30), generally decrease sucrase inhibitory effect. In *vivo*, compound (30) (emiglitate, rat sucrase IC_{50} value of 0.41 μM) produces long-lasting inhibition. Substitutions at the 1 or 5 -position with polar substituents like hydroxymethyl (compound 31, mouse sucrase IC_{50} value of 0.08 μM, and 32) also affords strong inhibitors.



(31)



(32)

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CHAPTER TWO

Peptide and Protein Hormones, Peptide Neurotransmitters, and Therapeutic Agents

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1 INTRODUCTION

It is interesting to contemplate how ignorance and misunderstanding can affect what people learn or do not learn in science, and how this influences what gets done in science. No where is this more evident than in the medicinal chemistry, organic chemistry, and biochemistry of peptide and protein hormones and neurotransmitters and their relationships to chemistry, biology, and medicine. Despite the fact that at least 50% of current drugs have, as their therapeutic target, receptors whose natural ligands are amino acids, peptides, and proteins, most chemists know little about peptide chemistry in its many manifestations (structural, synthetic, biochemical, biological, etc.). As a result what they believe, as shown in Table 2.1, is often an inaccurate reflection of current reality. This is not to say that there are still not many problems to solve in the areas of peptide, protein, and peptidomimetic drug design and development. Indeed there are problems, and they will require state-of-the-art scientific investigations by people knowledgeable about what has been accomplished and what is the nature of the problems in the larger context of health and disease.

However, if the current attitudes persist and most scientists are not educated in the peptide sciences, and do not know how to design, synthesize, and evaluate peptides and peptidomimetics with desirable chemical and biological properties, the current ignorance and prejudice will become a self-fulfilling prophecy. Considering that Emil Fisher, perhaps the greatest organic chemist, was the father of peptide and protein chemistry, and that two peptide hormones, insulin and oxytocin, had such a dramatic affect on twentieth century medicine, with millions of people having taken these two hormones, often in life-saving or enhancing situations, and with minimal toxicity compared to that of most other drugs, it is strange that so many medicinal chemists, natural products chemists, and organic chemists have paid so little attention to this area. Nonetheless, peptide science has the potential to produce hundreds of new drugs that can affect all of our major diseases, including cancer, pain, addictive behaviors (drugs, food, cigarettes, alcohol, sex, etc.), cardiovascular disease, mental illness, autoimmune diseases, and prion diseases to name some of the most important.

In this chapter we provide a few examples of the use of peptide science for examining peptide and protein hormones and neurotransmitters. Some of the compounds we could have included (e.g., insulin) are discussed elsewhere in these volumes. We will concentrate on examining four examples from the literature that we hope will illustrate both the potential of peptide and protein chemistry to solve therapeutic problems, and the important considerations in design, synthesis, conformational/topographic-biological activity relationships, and other factors that have to be considered in peptide and peptide mimetic design. In our considerations we will concentrate, because of space limitations, on a few bioactive peptide hormones and neurotransmitters that have led to therapeutic agents or that show great promise for doing so. It should be emphasized that many important and useful reviews and overviews about this subject have been written (Refs. 1–25 are 25 such references that have been useful to us in prepar-

Table 2.1 Commonly Held Beliefs about Peptide Drugs

Belief	Comments
1. Peptides are unstable	Many are because they are biological switches or substrates, but they can easily be made stable.
2. Peptides are readily attacked by protease	Many are for the reason in 1, but again this can easily be remedied.
3. Peptides are not bioavailable	Most peptides and proteins are not orally bioavailable, but many are >80% bioavailable, given in the lungs, through the eyes, transdermally, i.v., end so forth.
4. Peptides are too expensive	The cost of manufacturing peptides has decreased dramatically in the last 20 years and can be expected to decrease even more in the future.

ing this chapter), and should be referred to by the interested reader. Many of these provide important additional insights and information regarding the design, synthesis, and conformational structure-biological activity aspects of this research. In addition a few books/monographs have appeared that provide much useful information (27, 28), including the nine-volume series *The Peptides* (29–37), which, although somewhat out of date, still provides much essential information on synthetic, structural, and molecular pharmacological/medicinal aspects of this area.

2 BACKGROUND

The discovery of peptide and protein hormones and peptide neurotransmitters began in the late nineteenth century with the discovery of the pituitary principles (now referred to as oxytocin, vasopressin, ACTH, α-melanotropin, etc.). However, at the time it was not thought that these "principles" were peptides or proteins. That they were was first firmly established by duVigneaud and colleagues with the sequence of oxytocin (38) followed shortly thereafter by its total synthesis (39). A few years later Sanger et al. (40) determined the primary structure of insulin. These two seminal discoveries ended all the controversies, and established that peptides and proteins could be hormones, and later, peptide neurotransmitters were similarly discovered. Since then hundreds of peptide and protein hormones and neurotransmitters have been identified and their structures determined.

After duVigneaud's proof of the biologically active structure of oxytocin by total synthesis and the demonstration of its equivalence to natural oxytocin by chemical, biological, and clinical studies, many thought the next task was to continue synthesis of the ever more complex peptides and proteins whose primary structures were being determined. However, duVigneaud had other ideas. He thought that his structure determination and synthesis were only a beginning, and set out immediately to modify the structure of oxytocin by total synthesis (e.g., see Ref. 41). Thus began what we call today *de novo* ligand-based drug design. Important early contributors to this approach were Joseph Rudinger (42) and Robert Schwyzer (2). Work from their laboratories and many others led to the concepts of structure-activity relationships, which we take for granted today. Over time it has become clear that not only structural considerations (functional groups; acid/bases; hydrophobes/hydrophiles; methyl, ethyl, propyl, isopropyl, etc.) were important, but that conformational considerations also were critical components of biological activity, especially with regard to molecular recognition, transduction, stability against proteases, and bioavailability. This has led to the development of a strategy of conformational constraints that continues to be further developed today. Because many of the constraints make use of some kind of cyclization, it is critical to consider the synthesis of medium size rings and macrocyclic rings as part of a strategy for developing peptide and peptidomimetic drugs. Despite considerable success, there are still many unexplored areas involving design and synthesis of these cyclic structures. In this chapter we outline how

these considerations have been used to design bioactive peptides that can lead to therapeutic agents.

3 METHODOLOGY

The successful development of peptide, protein, and peptide mimetic biologically active hormones and neurotransmitters depends critically on five chemical aspects:

1. Robust synthetic methods, including asymmetric synthetic methodology.
2. Considerations of peptide conformation.
3. Considerations of side-chain conformations, particularly of pharmacophore moieties (topography).
4. Critical use of conformational and topographical space.
5. Proper choice and use of biological assays.

We briefly discuss each of these with particular reference to peptide, protein, and peptide mimetic hormone and neurotransmitter ***de novo*** design.

3.1 Peptide Synthesis

The development of peptide synthesis methodology during the past 40 years has been spectacular, to the point where today it is perhaps the most robust organic synthetic methodology, particularly through the use of the 20 amino acids commonly used by genes to construct proteins. It is now possible, using optimized chemistry, to construct a peptide of 20 to 40 amino acids by stepwise synthesis with an average yield at each coupling step of >99%. Although one could draw attention to many important synthetic developments to account for this robust chemistry, perhaps three major developments were most important. First was the introduction of the N^{α}-*tert*-butyloxycarbonyl group for temporary protection of the a-amino group of the incoming amino acid during assembly of a polypeptide chain (43, 44). Second was the development of the solid-phase synthetic method by Merrifield (45). Initially, the solid-phase method was severely criticized by most organic chemists, including most synthetic peptide chemists, because "intermediates" were not purified. This stimulated Merrifield and others to optimize every step in solid-phase synthesis to today's levels of nearly 100% completion in many cases (for a few excellent reviews, see Refs. 46–49; for an excellent book, see Ref. 50). Ironically, nowadays many organic and medicinal chemists use solid-phase organic synthesis to make their favorite organic molecules on solid supports. Seldom, however, do they optimize their syntheses. This leads to significant impurities that need to be removed. Finally, the third major reason for the excellent progress in peptide synthesis was the introduction and development of high pressure liquid chromatography (HPLC). The thousands of theoretical plates on HPLC columns allow rapid purification of peptides after synthesis of the polypeptide chain and removal of the side chain protecting groups. Reverse-phase HPLC has been particularly useful for the purification of most peptides.

3.2 Peptide Conformation

A central dogma of molecular biology is that the biological activity of a peptide or protein is directly related to its three-dimensional structure. In the postgenomic era, this dogma will take center stage as we seek a more critical understanding of the chemical/physical basis for life and the underlying chemistry related to diseases. A clear understanding of the relationships of peptide structure to conformations in biological systems is critical for future progress. It is often stated that small (<10 residues) linear and cyclic disulfide-containing peptides have "random" conformations in solution. This is a misunderstanding of the nature of peptide and protein conformations. In this regard, the prescient insights of Ramachandran and coworkers regarding the energy of peptide and protein backbone conformations stands out as perhaps the greatest insight to date into peptide and protein backbone conformations (51–53). By simple steric considerations and evaluation of the energy landscape of backbone phi (ϕ) and psi (ψ) angles (the bond omega angle was considered to be a "rigid" ***trans*** conformation, except for X-Pro where it could be cis) (Fig. 2.1), they determined that a good deal of ϕ-ψ torsional space was of high energy and therefore not

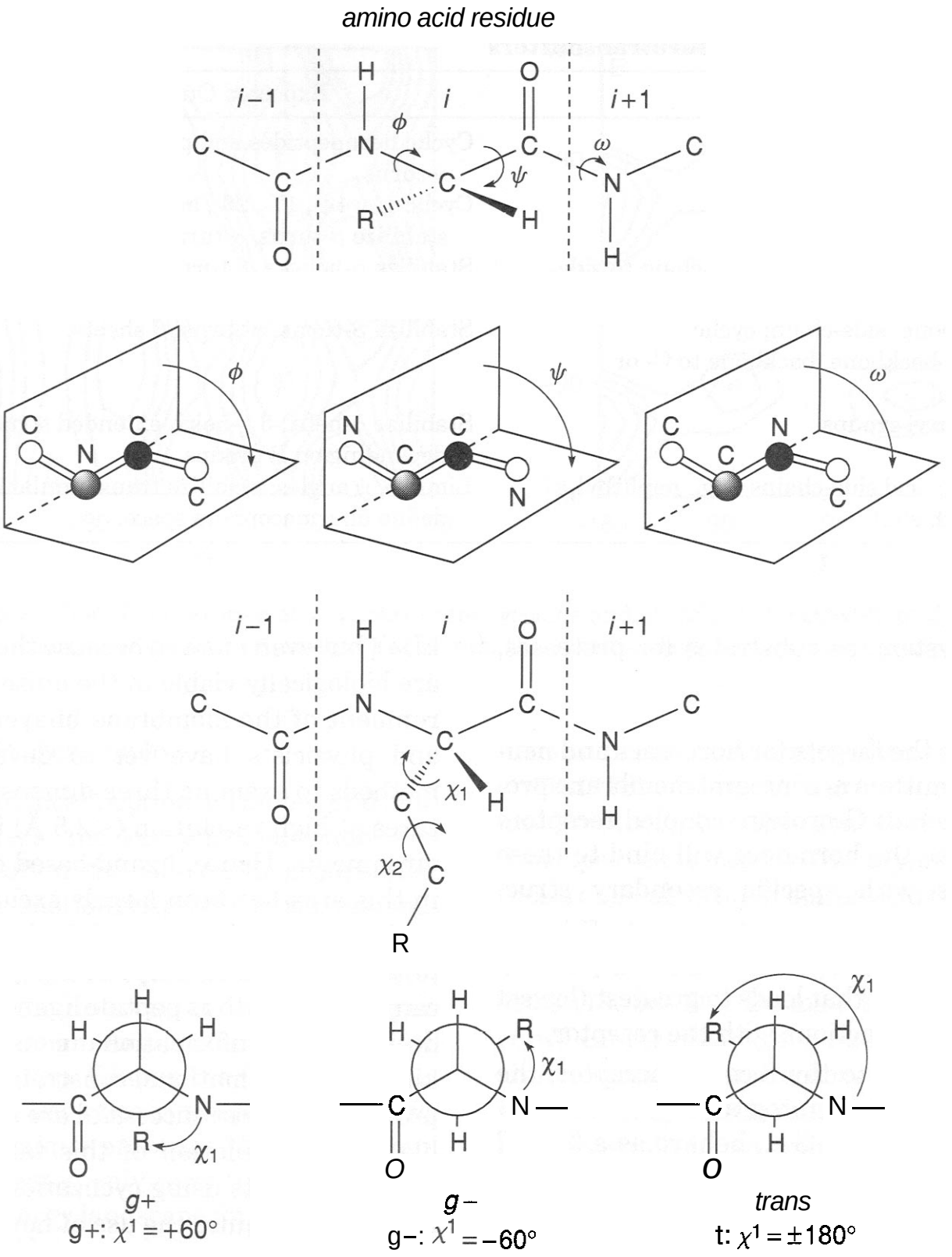


Figure 2.1. Definitions of the conformations of phi (ϕ), psi (ψ), omega (ω), and chi-1 [$g(-)$, $g(+)$, *trans*] conformation.

accessible to most amino acid residues in a peptide (Gly is an exception). For L-amino acids, a relatively small number of low energy conformations were available, including α-helix (right-handed and left-handed), β-sheets (parallel and antiparallel), extended conformations, and β-turns [for definitions of different types see Lewis et al. (54,5511. At the times of these predictions, the high resolution X-ray analyses of peptide and protein structures were minimal. Since then, thousands of X-ray structures of peptides and proteins have been determined, and essentially all residues in polypeptides (Gly excepted) are found near the low energy conformations found on a Ramachandran plot (a plot of ϕ and $ torsional angles as a function of energy). From this insight several tentative hypotheses can be considered in the design of peptide hormones and neurotransmitters [and for many other peptides that are either part of a larger protein or function in other ways (e.g., as epitopes for the

Table 2.2 Kinds of Conformational Constraints Leading to Specific Secondary Structures for Peptide Hormones and Neurotransmitters

Structural Constraint	Expected Outcome/Comments
N-terminal to C-terminal cyclics	Cyclic hexapeptides and pentapeptides β-turns, y-turns.
Cyclic disulfides	Cyclic 11-, 14-, 17-, 20-, and 23-membered rings: stabilize p-turns, y-turns, β-turns/β-sheets.
Cyclic lactams and lactones: side-chain to side-chain, terminal to side-chain, and so forth	Stabilize α-helices, β-turns.
Cyclic backbone: side-chain; cyclic backbone–backbone, backbone to C- or N-terminal	Stabilize β-turns, y-turns, β-sheets.
C^{α}-alkyl or aryl groups	Stabilize a-helix, 3_{10}-helix, extended structure depending on R groups.
Bulky amino acid side-chains (e.g., naphthyl, adamantyl, etc.)	Limit ϕ, ψ angles; stabilize transannular interactions; define pharmacophore space.

immune system, as substrates for proteases, etc.)]:

1. Because the targets for hormones and neurotransmitters are integral membrane proteins such as G-protein–coupled receptors (GPCRs), the hormones will bind to these proteins with specific secondary structures.
2. The solution conformation will adapt to the conformation that leads to greatest (lowest energy) interactions with the receptor.
3. When bound to the receptor/acceptor, the peptide will be an integral part of the receptor conformation (i.e., behave as a "mini" protein).
4. Agonists, competitive antagonists, and competitive inverse agonists bind in the binding pocket of the receptor differently (have different structure-activity relationships and different pharmacophores), which leads to different structures for the receptor-ligand complex in each case.

A way to test these hypotheses directly would be to examine by X-ray crystallography or high resolution nuclear magnetic resonance (NMR) spectroscopy, the three-dimensional structure of the peptide-protein complexes. However, examination of the high resolution structure of integral membrane proteins has turned out to be very difficult, not only because of their reasonably large size (40–80 kDa), but even more so because they primarily are biologically viable in the anisotropic environment of the membrane bilayer. Chemists and physicists have yet to develop robust methods to examine three-dimensional structures at high resolution (<2.5 Å) in these environments. Hence, ligand-based drug design in this area has been largely excluded. Nonetheless, a highly successful alternative approach has been developed for peptides, which can be referred to as peptide ligand-based drug design with conformational constraints. The kinds of conformational constraints that have proved to be most successful are shown in Table 2.2. Examination of this table indicates that constraints using cyclizations of various kinds often require the use of novel amino acids, C^{α}-substituted amino acids, and/or the use of novel bulky side-chain amino acids for success. Importantly, proper choice of conformational constraints can lead to small peptide analogs whose three-dimensional structure in solution can be determined by state-of-the-art two- and three-dimensional NMR methods, with insight from circular dichroism spectroscopy, FT-infrared spectroscopy, and Raman spectroscopy. X-ray crystallography also has provided important conformational information. In addition, modern computational chemistry methods with appropriately modified force fields can provide predictive conformational insights that are helpful in the design of biologically active ligands.

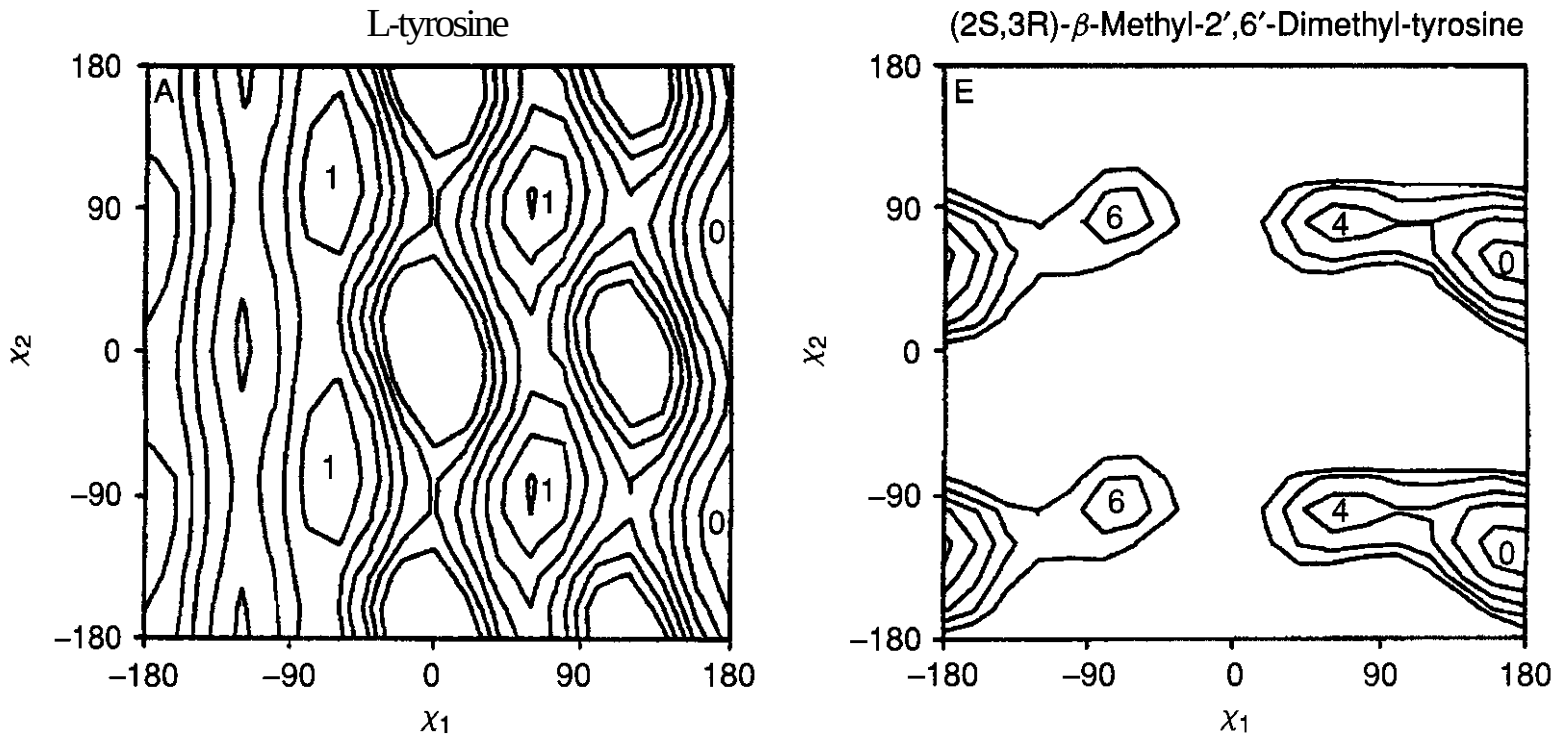


Figure 2.2. Plot of energy map of χ^1 versus χ^2 for tyrosine and of (2*S*,3*R*)β-methyl-2′,6′-dimethyl-tyrosine, where each contour represents 1 kcal from the lowest energy conformation.

3.3 Peptide Topography

In the case of most peptide hormones and neurotransmitters, the key pharmacophore elements are often the side-chain groups that make up the peptide structure. In the vast majority of cases aromatic residues of Phe, Tyr, Trp, and His; amino residues from the *N*-terminal; the ϵ-amino group or Lys and the guanidyl group from Arg; and/or the carboxyl groups from Asp, Glu, or the C-terminal carboxylate play key roles in the pharmacophore. This poses unique problems that have begun to be investigated only recently. The problem is that the energy landscape for the side chains of amino acid is much flatter than that in phi-psi space. As shown in Fig. 2.1 each chi angle starting with χ_1 has three possible side-chain conformations: gauche(−), trans, and gauche(+); and, as shown in Fig. 2.2, although indeed the g(−), trans, and *g*(+) conformations are the low energy conformations, the energy difference between them is small, and the energy barriers between them are small and easily accessible at 300°C. Thus it is very difficult to predict which side-chain conformation the receptor/acceptor prefers, or whether it makes any difference in terms of molecular recognition in binding affinity, transduction, or biological activity in general. To investigate this problem Hruby et al. initiated a comprehensive program of topographical design constraint (18) of side-chain groups in amino acids. To accomplish this goal they and others developed methods for the asymmetric synthesis of β-substituted amino acids (e.g., β-Me, -Et, -*i*-Pr-Aryl, etc.) and in the case of Phe and Tyr also 2'- and 6'-methyl substituted amino acids (18, 56–60). Because there is a second chiral center created for most of these novel amino acids, we and others have had to develop novel synthetic methodology to prepare the desired designed amino acids (see Fig. 2.3 for some examples). Constraints in chi space (χ^1 and χ^2) of various degrees are found in the derivatives of phenylalanine (1, 2, 3, and 4; Fig. 2.3), tyrosine (1, 2, 3, and 4; Fig. 2.3). Because many of the novel amino acids have chiral centers at the α- and β-carbons, they exist as four isomers: (*S*,*S*), (*S*,*R*), (*R*,*S*), and (*R*,*R*). We have shown by both NMR (e.g., see Ref. 61) and computational chemistry (18) that significant constraints are introduced between the g(−), trans, and g(+) conformations, and that there are significant energy differences between them in many cases (18). We provide specific examples of the use of such topographically constrained amino acids in de *novo* peptide design in Section 4 of this chapter. Suffice it to say at this point, that such constraints can provide unique insights into

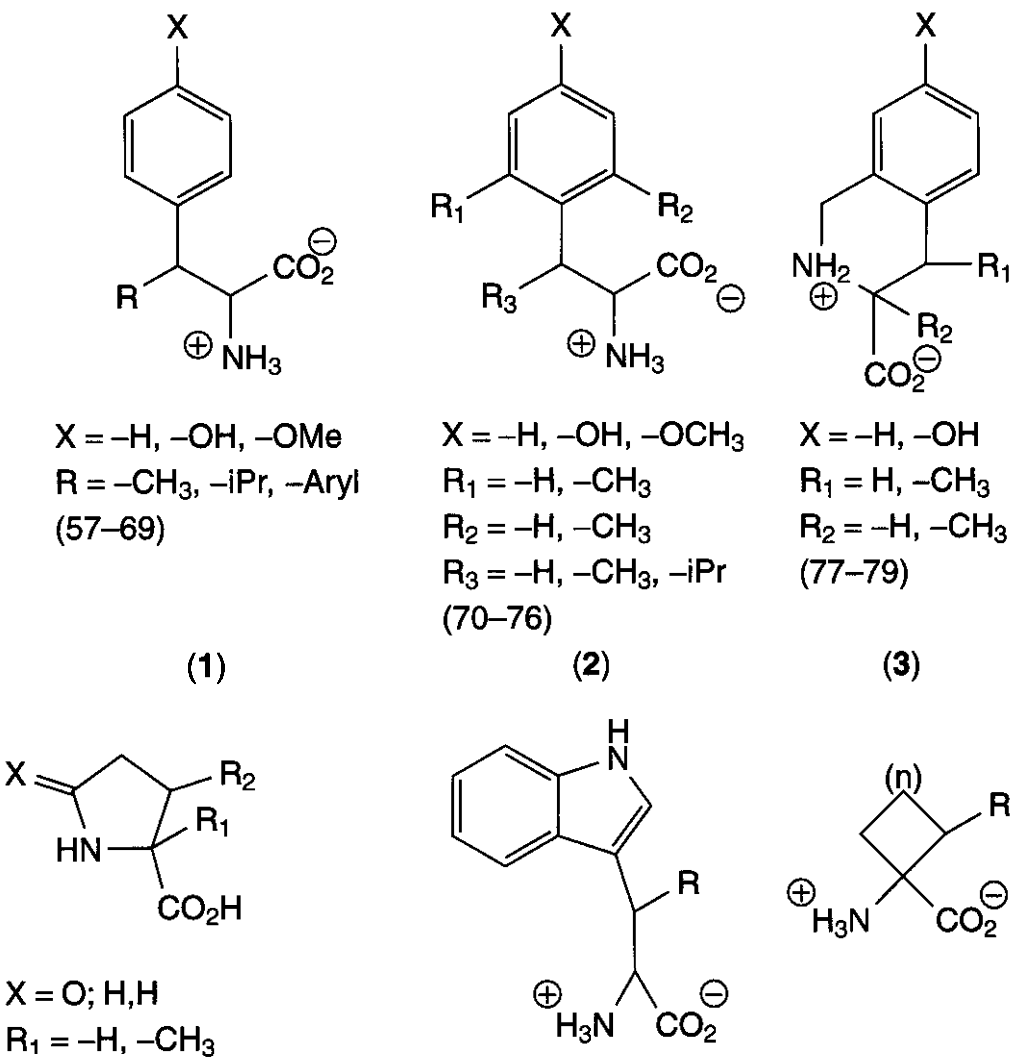


Figure 2.3. Some examples of novel chi-constrained amino acids for topographical design (related lead references).

the topographical requirements of pharmacophore side-chain groups (for reviews, see Refs. 18, *56)*.

3.4 Topographical and Conformational Constraints

From the above discussion it is clear that optimal design of biologically active peptides requires the use of both conformational and topographical constraints in the same molecule to obtain insights about the physiologically active conformation. Because only a limited number of topographically constrained amino acids are commercially available, studies that make use of both types of constraint simultaneously have been rather limited. However, in Section 4 we illustrate the power of this methodology to provide peptide and peptidomimetic analogs with unique biological activities. As work proceeds on the proteome and with the observation that molecular recognition is critical in virtually all life processes, we believe that the use of conformational and topographical considerations will be an integral part of any drug or ligand design project. It seems to be particularly important for the *de novo* design of a nonpeptide ligand based on a peptide pharmacophore (e.g., see Refs. 18, 92, 93).

3.5 Bioassays

Central to any program in ligand-based drug design or in the development of novel conformationally and/or topographically constrained biologically activity peptides is the need for robust bioassays. Currently, high throughput screening is touted as the best method for discovering drug leads. Often this involves only binding affinity studies, and sometimes a second-messenger assay as well. Although this is often a good starting point to get a micromolar binding lead compound, it has become increasingly clear that for treatment of diseases, especially those involving peptide hormones and peptide neurotransmitters, the adaptive changes that occur in the

Table 2.3 Biochemical and Biological Assay for Peptide Hormones and Neurotransmitters

Assay	Considerations
Binding affinity	For potency and selectivity must use multiple receptor types and subtypes, membranes or cells or pure receptor.
Second-messenger assays	cAMP, GTPγS, and so forth: membranes, cells, or tissues; agonist vs. antagonist vs. inverse agonist.
Bioassays	Tissues: provide functional information (e.g., smooth muscle; heart; liver; brain; etc.).
In vivo assays	Assess functional or behavioral effects (e.g., blood pressure; diuresis; pain; heart rate; respiration; pigmentation).
Behavioral assays	Assess changing behaviors (e.g., pain; addiction; feeding; sexual; aggression; learning; etc.).

endocrine system or in the central nervous system render the relationships of hormone or neurotransmitter bioactivity different in normal and diseased states. As a result more extensive assays that address the adaptive changes that have occurred as a result of disease are needed (e.g., see a discussion of changes that occur in pathological pain states in Ref. 94). Thus more extensive and perhaps different assays will be needed. In Table 2.3 we outline some of the assays that should be used and some considerations related to these assays.

4 EXAMPLES

4.1 Parathyroid Hormone

4.1.1 Structure and Function. The parathyroid hormone (PTH) is a linear polypeptide of 84 amino acids (Fig. 2.4) and plays a critical physiological role in bone growth, renal functions, and calcium homeostasis (95–98). This peptide, along with the active forms of vitamin D, constitutes the principal regulator of calcium homeostasis in humans. PTH increases the concentration of extracellular (blood) calcium through a concerted effect primarily on bone, intestine, and kidneys. Lowering of blood calcium levels stimulates production of PTH, which consequently works to maintain appropriate levels of blood calcium through three distinct mechanisms: (1) increased rate of dissolution of the bone mineral; (2) reduced clearance of calcium by the kidneys, by returning more of the calcium filtered by the glomerulus back to blood; and (3) increasing the efficiency of calcium absorption in the intestine through an indirect mechanism involving the activation of vitamin D (98). In addition, PTH plays a role in maintaining the concentrations of inorganic phosphates in the body.

PTH is biochemically synthesized as a prohormone, which differs from PTH by the fact that it has six additional residues at the amino-terminus. The exact function of the prohormone sequence is unclear but it has been hypothesized that the production of PTH as a prohormone is important for the efficient packaging of the hormone. Although calcium ion concentrations in blood appear to play a significant role in the regulation of PTH, other agents such as magnesium, epinephrine, and dopamine also play a role in regulating PTH secretions *in vivo*.

Recently, a related peptide, the parathyroid hormone-related protein (PTHrP) containing 140 amino acids was identified as a secretory product of tumors associated with the clinical syndrome of humoral hypercalcemia of malignancy. This peptide hormone is also produced by a variety of normal cells and acts as a paracrine and/or autocrine regulator in development (99, 100). PTHrP appears to play a key role in early skeletal development in the embryo, in cell differentiation (99), and also facilitates the transport of calcium across the placenta and during lactation. Both PTH and PTHrP have high affinity for the parathyroid hormone receptor (PTH1R), a G-protein–coupled, seven-transmembrane receptor (GPCR) with nearly equivalent potency. The fact that PTH and PTHrP, despite sharing only limited sequence homology, binds to and activates the same receptor (PTH1R) suggests that both

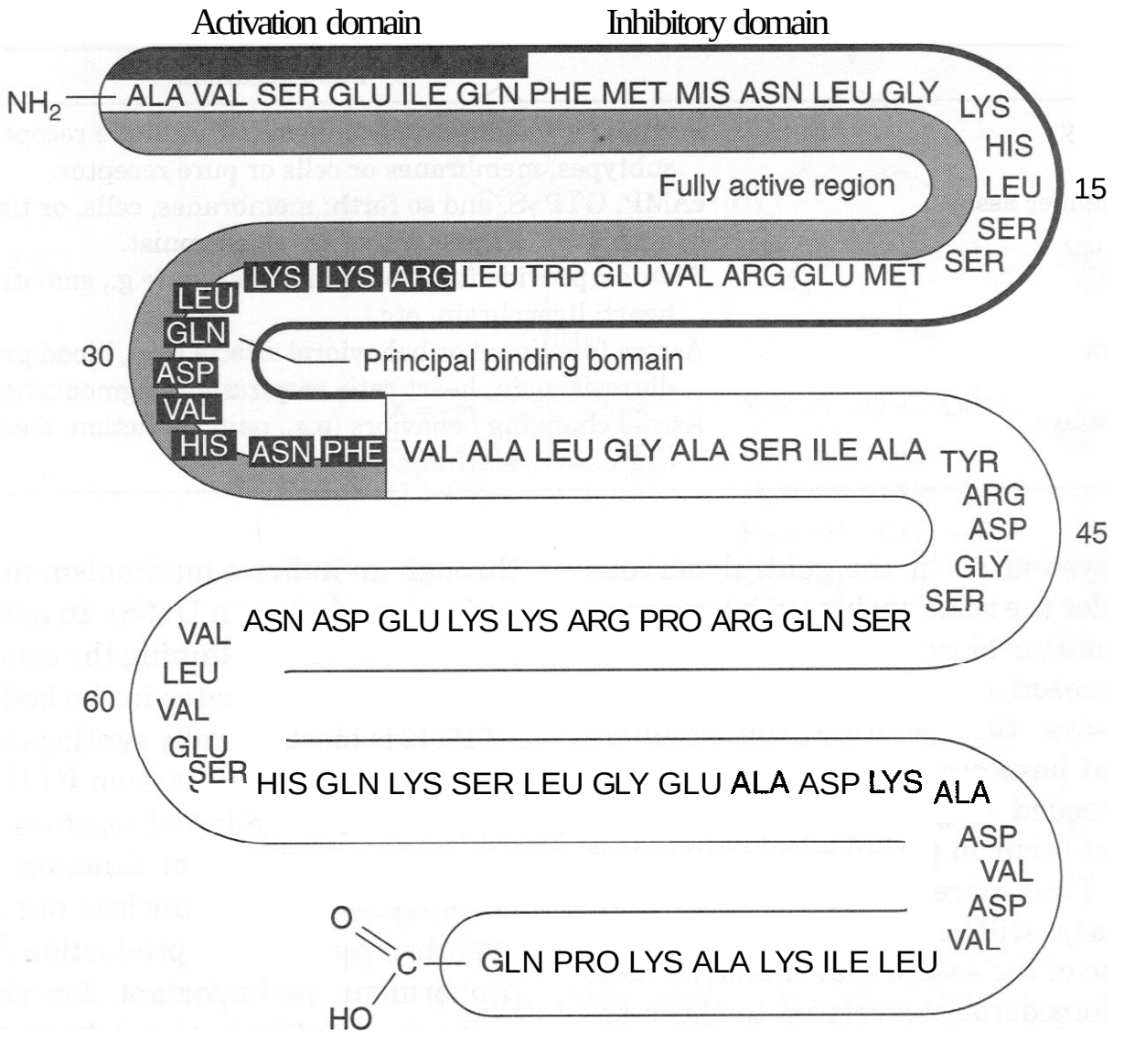


Figure 2.4. Schematic representation of the functional domains of parathyroid hormone molecule.

peptide hormones assume very similar bioactive conformations when bound to the receptor.

The importance of both peptide hormones in controlling various physiological processes, and the observation that their malfunction can result in disease states such as hypercalcemia, osteoporosis, cancer, renal and cardiac problems, as well as problems associated with skeletal development has stimulated research in the design and development of stable and potent synthetic analogs of these hormones as therapeutics. Hence, extensive structure-activity relationship (SAR) studies aimed at elucidating amino acid residues in PTH or PTHrP that are important for binding and potency have been undertaken. The SAR studies have shown that the N-terminal portion, consisting of the first 34 amino acids, possesses full activity in both PTH and PTHrP (100a).

This review focuses on SAR studies that have helped in understanding the molecular basis for ligand-receptor interactions and illustrates key linear, truncated, or cyclic analogs that agonize or antagonize the PTH receptor. Both classes of compounds have been used to study the mechanism of action of the hormone in normal and pathophysiological states. Some specific potential therapeutic applications of these compounds might include treatments for acute hypercalcemia arising from parathyroid adenoma, hyperplasia, and carcinoma. Highly selective and potent antagonists of PTH also have been proposed as therapeutic agents for the treatment of, for example, chronic hyperparathyroidism, osteoporosis, and fibrous dysplasia.

4.1.2 Overall View of the Structure of PTI

Parathyroid hormone is an 84 amino acid, li ear polypeptide secreted by the parathyro gland. Early structure activity studies showe that the amino-terminal region consisting 34 residues was sufficient for full biologic activity (96, 97). This fragment could furth be divided into three distinct regions: (1) Tl

N-terminal region which is helical in nature and plays a key function in hormone stimulated adenylate cyclase activity in vitro and receptor activation in *vivo*; (2) The middle 'hinge' region which is possibly involved in a beta-turn and is responsible for the proper orientation of the truncated peptide in the receptor pocket; and (3) A carboxyl terminal domain which is also helical and contains residues important for binding and receptor recognition (101b, 102).

4.1.3 Importance of Ala[1] and Val[2] in Signal Transduction. SAR studies have implicated the C-terminus domain to be important for maintaining potent receptor-binding affinity. The more flexible N-terminus region appears to play a critical role in signal transduction. Deletion of the first two amino-terminal residues results in a loss of all, or virtually all, biological activity but not in a loss of binding affinity to the PTH1 receptor in vitro. Thus there appears to be an apparent structural separation of binding and activation regions in PTH. Deletion of Ala^{1} was accompanied by a marked decrease in adenylate cyclase activity, implying that the minimum sequence required for biological activity starts at the second amino acid.

4.1.4 Importance of Arg[20] and Arg[25] in Receptor/Ligand interactions. Early SAR studies had indicated that conserved structural modifications in the central portion of the active fragment of PTH-(1–34) were remarkable for their relative lack of effect on biological activity. To evaluate the biological role of Arg^{20} and Arg^{25} in PTH-(1–34), fragment-selective postsynthetic modifications of these two residues in hPTH-(1–34) active fragment were done by use of 1,2-cyclohexanedione (100). 1,2-Cyclohexanedione reacts specifically, completely, and reversibly with the guanidino group of arginine, to give a single product, N^{7},N^{8}-(1,2-dihydroxycyclohex-1,2-ylene)arginine, [DHCH-Arg^{20},DHCH-Arg^{25}]hPTH-(1–34) (103). This modified analog showed, at most, 16% of the activity of the unmodified hormone hPTH-(1–34), based on in vitro renal adenylate cyclase assays using borate buffer. Reversal of arginine modification completely restores biopotency, indicating that the potency decline observed after 1,2-cyclohexanedione treatment is not caused by nonspecific alterations of structure. The near total loss in biopotency of the modified hormone may be further explained by the increase in steric bulk of the $Arg^{20,25}$ side chains or by a change in the formal charge of the guanido group from a +1 to zero in a putative arginine-borate complex. Whether these two effects individually or in combination may cause a dramatic decrease in biopotency through adverse effects on hormone-receptor interactions or through the induction of a conformational change in the peptide is not clear. However, these results illustrate the importance of the positively charged guanidino group in Arg on binding and bioactivity.

4.1.5 Importance of Gly[12] in Ligand-Receptor Actions. Glycine, the simplest amino acid, plays a very important role in proteins and peptides. The absence of a side-chain functional group renders Gly a high degree of conformational flexibility in peptides. In addition, this amino acid can also access the phi and psi space of D-amino acids. Hence, glycine is generally found in the region where proteins and peptides undergo reverse-turn structures (55, 104,105). Glycine is the 12th residue from the N-terminus in the PTH sequence (106, 107). This residue is highly conserved in PTH and PTHrP isolated from all species to date. Structural studies that use the Chou-Fasman analysis and both CD and NMR studies have predicted that the α-helical N-terminal domain is followed by a β-turn at positions 12–15 in PTH and 9–12 in PTHrP. Thus, the biological consequences of single-residue changes at position 12 were assessed in vitro, to determine the role of this residue in hormone-receptor interactions and its contribution toward the bioactive conformation of the peptide. In the human hormone [Tyr^{34}]hPTH-(1–34)-NH_2 substitution of Gly^{12} by Ala^{12}, D-Ala^{12} and Aib^{12} gave agonists with binding affinities similar to that of [Tyr^{34}]hPTH-(1–34)-NH_2 in bovine renal cells (K_b [Tyr^{34}]hPTH-(1–34)-NH_2 = 0.7 d ; K_b for substituted analogs between 0.7 and 1.0 d). The Pro^{12} analog binds approximately 840-fold less tightly than [Tyr^{34}]hPTH-(1–34)-NH_2. The K_m values for adenylate cyclase activity are also similar to

Table 2.4 Binding and Cyclase Activity of Selected Antagonist Analogs of PTH with Bone-Derived ROS17/2.8 Cells

Analog	Binding, K_b (n*M*)	Cyclase K_m (n*M*)	Ref.
[Tyr^{34}]hPTH-(7–34)NH$_2$ (**1**)	700	2700	109
[D-Trp^{12},Tyr^{34}]hPTH-(7–34)NH$_2$ (**2**)	120	210	109
[Trp^{12},Tyr^{34}]bPTH-(7–34)NH$_2$ (**3**)	400	1360	110
[Nle^{8,18},Tyr^{34}]bPTH-(7–34)NH$_2$ (**4**)	960	1550	110
[Nle^{8,18},D-Trp^{12},Tyr^{34}]bPTH-(7–34)NH$_2$ (**5**)	180	70	110
[Nle^{8,18},Trp^{12},Tyr^{34}]bPTH-(7–34)NH$_2$ (**6**)	410	220	110

that of [Tyr^{34}]hPTH-(1–34)-NH$_2$ for the Ala^{12}, D-Ala^{12}, and Aib^{12} analogs, whereas the K_m value for the Pro^{12} analog is 3500-fold lower (107).

To design potent and selective antagonists (108) of PTH or any other peptide ligand, it is necessary to identify the sites within the hormone where structural modifications can be made that maintain high receptor affinity, but without inducing agonist properties. In principle these goals can be achieved by two different routes: (1) stabilize the antagonist in a conformation favored by the receptor for molecular recognition but not transduction; and (2) introduce new structural moieties that will add binding elements that interact with the receptor at sites different from or in addition to those present in the native agonist. These binding elements should retain the "bioactive conformation" of the ligand required for receptor binding that does not lead to information transduction.

Previous SAR studies with human and bovine PTH have shown that removal of the first six amino-terminal residues gives an analog with potent antagonistic activity in *vivo*, such as [Tyr^{34}]hPTH-(7–34) NH$_2$ (109) (Table 2.4). Substitution of Gly^{12} in this fragment by Ala^{12} and D-Ala^{12} gives analogs that are about twice as potent as the parent peptide [Tyr^{34}]hPTH-(7–34) NH$_2$ in both binding and cAMP assays, whereas the Pro^{12} analog is approximately twofold less active vs. the parent peptide. Furthermore, replacement of Gly^{12} with D-Trp (Table 2.4) results in an antagonist that is approximately 10-fold more potent in the binding assay and 12-fold more potent at inhibiting agonist-induced (3 n*M* [Nle^{8,18},Tyr^{34}]bPTH-(1–34)NH$_2$) cAMP activity (Table 2.4). In contrast, the L-Trp analog (110) failed to alter either binding or cAMP activity, suggesting that introduction of hydrophobic side-chain groups in the D-configuration at position 12 provides auxiliary hydrophobic interactions with the receptor, which promotes binding. A combination of bulky hydrophobic residues at position 12 together with the substitution of Met^{8} and Met^{18} by Nle gave analogs having binding activity 10- to 15-fold more potent than that of [Nle^{8,18},Tyr^{34}]bPTH-(7–34)NH$_2$. These results suggest that introducing amino acid residues with aliphatic and/or bulky hydrophobic side chains in either the D- or L-conformer can improve antagonism in the 7–34 truncated series (Table 2.4).

4.1.6 Truncated PTH Analogs: Identification of the Minimum Sequence Requirements. Truncation of peptide hormones having more than 15 amino acid residues to smaller yet biologically active and potent analogs is very often a challenging task. Most often truncation not only allows researchers the flexibility of synthesizing large numbers of analogs with considerable ease, speed, and at less cost, but the truncated analogs also can be studied more systematically to elucidate the regions within the native peptide sequence that are important for binding and bioactivity. SAR studies of PTH have shown that the minimum and critical region necessary for full biological and binding activity resides in the first 34 amino acids (1–34) of the polypeptide (109, 110). To further determine the residues within this polypeptide fragment that play an important role in molecular recognition, several truncated synthetic fragments of bovine parathyroid hormone (bPTH) were made and tested for their ability to competitively inhibit the binding of ^{125}I-labeled [Nle^{8},Nle^{18},Tyr^{34}]bPTH-(1–34)amide, as well as serve as antagonists of PTH-stimulated cAMP in vitro (111).

Table 2.5 *In Vitro* Biological Potencies of Truncated Analogs of Bovine Parathyroid Hormone

Peptide	K_b (Renal), M^a	K_i (cAMP), M	[Analog]/[PTH]^b
[Nle^8,Nle^{18},Tyr^{34}]bPTH-(3–34)amide	1.2×10^{-8}	1.2×10^{-8}	n.d.
[Nle^8,Nle^{18},Tyr^{34}]bPTH-(7–34)amide	1.1×10^{-6}	3.0×10^{-6}	30
[Nle^8,Nle^{18},Tyr^{34}]bPTH-(10–34)amide	2.7×10^{-6}	7.0×10^{-6}	70
[Nle^8,Nle^{18},Tyr^{34}]bPTH-(15–34)amide	3.2×10^{-5}	3.7×10^{-5}	370
[Nle^8,Nle^{18},Tyr^{34}]bPTH-(20–34)amide	7.3×10^{-5}	7.0×10^{-5}	700
[Nle^8,Nle^{18},Tyr^{34}]bPTH-(25–34)amide	3.4×10^{-4}	No inhibition[f]	>4000
[Nle^8,Nle^{18},Tyr^{34}]bPTH-(28–34)amide	No binding	Not tested	n.d.

"The concentration of each peptide that inhibited specific binding of radioligand to a half maximal level was taken as the apparent binding constant (K_b) of that peptide.

[b]Molar ratio of inhibitory peptide to PTH required for 50% inhibition of cAMP in vitro (112). n.d., not determined.

'Testing at a concentration of peptide as high as that used in the binding assay was precluded. Nonspecific in bition of cAMP activity at peptide concentrations greater than 4×10^{-4} M.

Table 2.5 summarizes the results of this study. The inhibition of specific binding of radioligand was assessed for each peptide fragment over a concentration range from 1×10^{-10} to 1×10^{-2} M.

The binding studies indicate that deleting the first two residues from the amino-terminal completely eliminates any detectable biological activity, while maintaining potent affinity for the receptor (112). Further stepwise amino-terminal deletions from position 3 to 27 caused a progressive decrease in binding avidity. However, it was observed that all of the truncated analogs, except the [Nle^8,Nle^{18}, Tyr^{34}]bPTH-(28–34) amide fragment, could completely and specifically inhibit the binding of radioligand when added at sufficiently high concentrations (1×10^{-2} M). This suggests that a small chain of 10 residues (25–34), but not as small as a seven amino acid fragment, is able to satisfy all the requirements for binding to the receptor pocket (113). The 25–34 sequence was found to be conserved in hormones isolated from all species studied to date, indicating that this fragment contains important structural information for binding. The observation that the relative order and magnitude of the apparent binding constants observed correlates closely with the K_i values for each peptide fragment in the *in vitro* adenylate cyclase assay suggests that the mechanism of hormone antagonism caused by these peptide fragments is through direct competition between PTH and analog fragment for receptor occupancy. Competition for receptor-binding sites by the analog fragments is not attributed to nonspecific peptide effects, given that hPTH-(44–68) and hPTH-(53–84) fragments do not inhibit binding of radioligand at a maximal concentration of 1×10^{-2} M.

A second major direction toward the development of potent and biologically active agonists of PTH relies on the incorporation of D-amino acids in place of the naturally occurring L-enantiomer at selected positions along the native peptide sequence. This technique has been successfully used to generate stable, more potent and bioavailable analogs of various peptide hormones and other biologically active peptides (e.g., Refs. 114–117). Four analogs of PTH having D-amino acids at selected positions along the native peptide sequence were synthesized and tested for their ability to bind and activate the PTH receptor. Because earlier studies (118–120)had shown that even subtle changes near the amino- and carboxyl-termini conferred large changes in biopotency, the D-isomers were introduced in these regions. Table 2.6 summarizes the results of key analogs. substitutions at the carboxyl-terminus are very well tolerated, given that replacement of Tyr^{34} by its D-isomer gave a peptide that is biologically equipotent. In addition, this peptide may be more active *in vivo* than *in vitro* because of its greater resistance to enzymatic degradation. Structural modifications in the amino-terminal activation region are poorly tolerated in terms of biopotency. Substituting Val^2 by its D-isomer in [D-Tyr^{34}] bPTH-(1–34) gave analog [D-Val^2,D-Tyr^{34}] bPTH-(1–34) amide, which lacked nearly all of the *in vitro* biological activity compared to

Table 2.6 Biological Activity of Bovine PTH(1-34) and bPTH(2-34) in Rat Renal Adenylate Cyclase Assay

Substitution	Fragment	Potency"	Relative Potency (%)[b]
None	1–34	5400 (3,900–8,000)	100
[Tyr^{34}]	1–34 amide	16000 (11,000–23,000)	300
[D-Tyr^{34}]	1–34 amide	14500 (11,000–17,000)	270
[D-Tyr^{34}]	2–34 amide	130 (120–150)	3
[D-Val^{2},D-Tyr^{34}]	2–34 amide	90 (60–100)	2
[D-Val^{2},D-Tyr^{34}]	1–34 amide	80 (60–100)	1

"Potency estimates are based on three independent assays except for [D-Tyr^{34}]bPTH-(1–34)amide. Limits in parentheses represent SEM for each peptide except [D-Tyr^{34}]bPTH-(1–34)amide, for which 95% confidence limits are provided.

[b]Relative potency calculated on the basis of the mean potency with the activity of the reference peptide, unsubstituted bPTH-(1–34) taken as 100%.

that of the unsubstituted analog. A similar effect is seen when the first amino-terminus residue is deleted, as in [D-Tyr^{34}]bPTH-(2–34) amide. However, no additivity of effects (in terms of a further reduction in biopotency) was seen on combining D-Val^{2} and [Tyr^{34}] bPTH-(2–34)-amide in the same peptide fragment (Table 2.6).

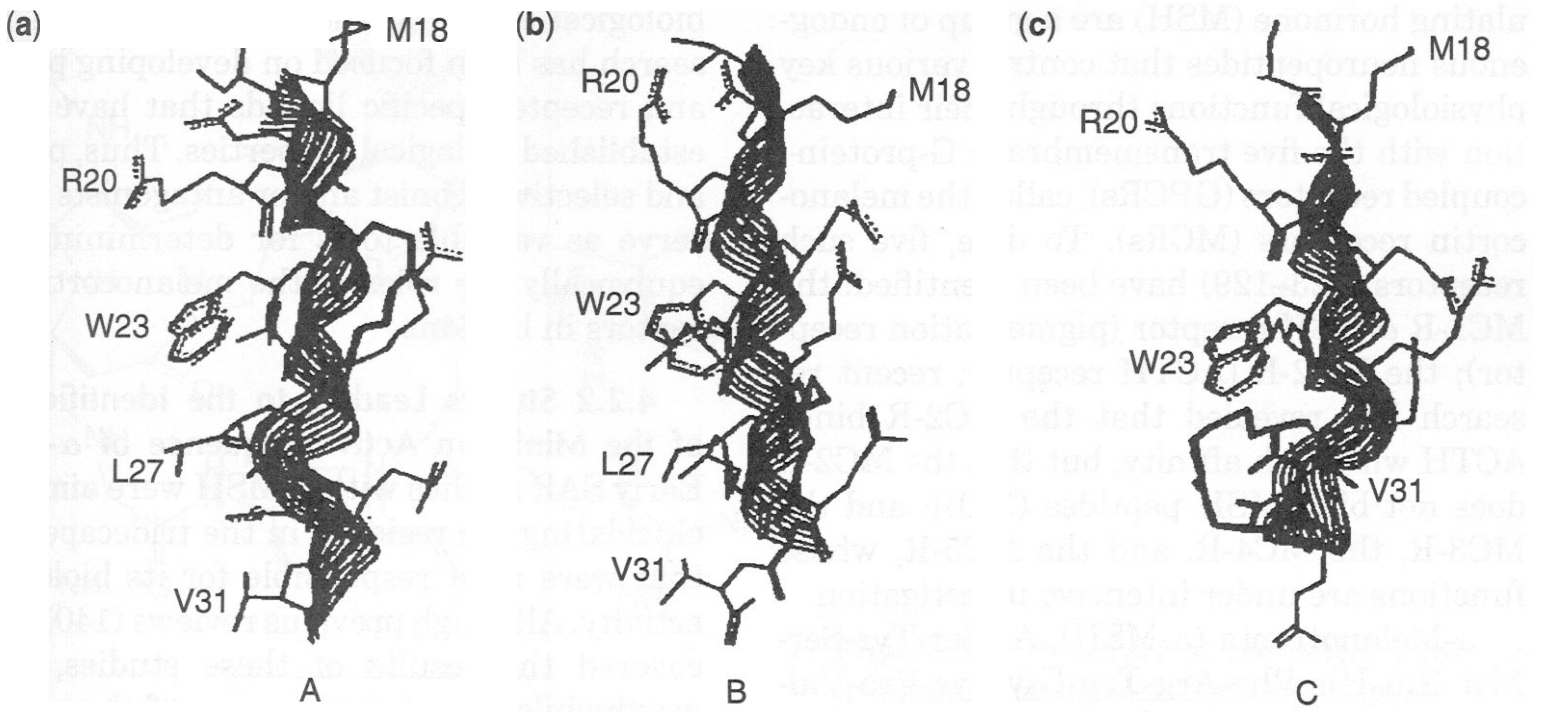
4.1.7 Conformationally Constrained Cyclic Analogs of hPTH(1–31)Amide

***4.1.7.1 In* Vitro Agonist Studies.** Cyclization at specific sites within a peptide is often used as tool for enhancing the selectivity, binding, and biopotency of the peptide ligand (3–5, 8–11). Cyclization introduces constraints that can stabilize the bioactive conformation of the ligand, thus improving the biological properties, and can also help to identify regions within the peptide sequence that may be important for molecular recognition (3–5). Given that the biologically relevant three-dimensional structure of PTH is at best speculative, cyclic lactam analogs of hPTH, designed to stabilize the amphipathic helical region between residues 17 and 30, and known to be important in binding to the receptor (120), were synthesized and tested for their ability to activate rat osteosarcoma 17/2 (ROS) cells in culture. Cyclization was achieved through the formation of side chain to side chain lactam bridges at three distinct sites within the 17–29 residue sequence of hPTH-(1–31)amide, previously identified by high resolution NMR to be involved in ion-pairing interactions in solution (121). There are two distinct ion pairs in the C-terminal a-helical region that could potentially contribute toward the stability of the helix. These are between Glu^{22}-Lys^{26} and Lys^{26}-Asp^{30} and have (i, i + 4) spacing, whereas the third ion pair between Lys^{27} and Asp$^{3'}$ having (i, i + 3) spacing is helix destabilizing. Lactam bridges between these residues would restrict their conformational flexibility, thus reducing the number of possible conformations the peptide can assume in solution.

Table 2.7 lists the results of in vitro cAMP-stimulation studies in ROS cells for the cyclic lactams as well as for the linear [Leu^{27}]hPTH-(1–31)amide analog. Cyclic peptide (5), having (i, i + 3) spacing, shows half the in vitro potency of its linear counterpart 1. In (5)a twist is introduced into the helix on lactam formation and may be responsible for destabilizing the helix and thus lowering the potency of this analog (Fig. 2.5). The increased bioactivity of analog 2 versus 1 is not attributed to an in-

Table 2.7 Biopotencies of Cyclic Constrained Analogs of hPTH(1-31)Amide in ROS Cells

Peptide	Adenylate Cyclase Activity, EC_{50} (n*M*)
hPTH-(1–31)-NH_2 (**1**)	19.9
[Leu^{27}]hPTH-(1–31)-NH_2 (**2**)	11.5
[Leu^{27}]*cyclo*(Glu^{22}-Lys^{26})hPTH-(1–31)-NH_2 (**3**)	3.3
[Leu^{27}]*cyclo*(Lys^{26}-Asp^{30})hPTH-(1–31)-NH_2 (**4**)	16.9
cyclo(Lys^{27}-Asp^{30})hPTH-(1–31)-NH_2 (**5**)	40.3



A: Peptide 3; B: Peptide 4; C: Peptide 5.

Figure 2.5. Models showing lactam positions in receptor-binding region of human PTH.

crease in the a-helical content of (2) (based on CD data), but rather is attributed to the replacement of a polar lysine residue (Lys^{27}) on the hydrophobic face of the helix by a hydrophobic leucine residue (Leu^{27}). Constraining analog (2) through a lactam bridge between Glu^{22} and Lys^{26} (analog 3) further improves bioactivity, presumably through stabilization of the a-helix. Although peptides (3) and (4) both have lactam bridges between the (i, i + 4) residues, and thus have the same helical content, the CD study indicates differences in the conformation populations. Peptide (3) presumably has a more nearly perfect helix than that of peptide (4), probably because of the fact that in (4) the constraint is toward the terminus to the helix region. This difference may account for the loss in bioactivity of (4) versus (3).

4.1.7.2 *In Vivo Studies.* The bioavailabilities of the cyclic analogs were tested by measuring their abilities to lower the blood pressure in rats when injected either intravenously or subcutaneously at a dose of 0.8 μmol/100 g of the analog (122). In addition, the time required by these analogs to lower the blood pressure to a maximum extent was also measured as a factor to evaluate their in *vivo* efficacies. Intravenous injections of the cyclic analogs elicited relative hypotensive effects not significantly different from those observed from the cAMP study. In addition, the time required to attain minimum blood pressure was not significantly different for these analogs.

However, subcutaneous injections produced quite different results. By this procedure, peptides (1), (2), and (5) produced significantly lower drops in blood pressure than did hPTH-(1–34)-NH_2 and peptides (3) and (4). However, the times required for attaining maximal drop in blood pressure after subcutaneous injections are quite different, in that analog (3) reached the minimum blood pressure much faster than either (4) or (5), a result consistent with data from cAMP studies. Finally, it was noticed that analogs truncated to the first 30 amino-terminal residues had poor biological properties such as hypotensive effects in rats when administered subcutaneously. However, these peptides showed similar hypotensive properties when administered intravenously, suggesting that Val^{31} might be playing an important role in the transport of these analogs from the site of injection into the vascular system.

4.2 Melanotropins

4.2.1 Structure-Activity Relationships. The melanotropins, a-, β-, and y-melanocyte stim-

ulating hormone (MSH) are a group of endogenous neuropeptides that control various key physiological functions through their interaction with the five transmembrane G-protein–coupled receptors (GPCRs), called the melanocortin receptors (MCRs). To date, five such receptors (123–129) have been identified: the MC1-R a-MSH receptor (pigmentation receptor); the MC2-R [ACTH receptor; recent research has revealed that the MC2-R binds ACTH with high affinity, but that the MC2-R does not bind MSH peptides (130)]; and the MC3-R, the MC4-R, and the MC5-R, whose functions are under intensive investigation.

a-Melanotropin (a-MSH), Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2, was one of the first peptide hormones isolated from the pituitary gland (131–135). This hormone plays an important role in skin pigmentation. Other effects ascribed to this hormone include: (1) regulation of the release of pituitary and peripheral hormones, such as somatostatin and corticotropin; (2) sebotrophic effects, adrenal steroidogenesis, immune response, cardiovascular and metabolic effects; and (3) important roles in the CNS such as *(a)* control over learning, memory, motivation, sleep, sexuality, and food intake (associated with obesity); (*b*) effects on neurotransmission such as cholinergic and dopaminergic systems; (*c*) neurochemical effects; (*d*) hypothermic and antipyretic effects; (*e*) effects on nerve regeneration; and (*f*) interactions with other neuroactive compounds such as opiates.

It is now well established that the MC1 receptor mainly expressed in melanocytes and leukocytes plays a key role in skin pigmentation and inflammatory response (136, 137). The MC2 receptor is expressed only in the adrenal gland and mediates glucocorticoneogenesis (123). The MC3 and MC4 receptors are both found in the brain, the MC3 receptor is found in the arcuate nucleus and the nucleus of the solitary tract, whereas the MC4 receptor is mainly found in the hypothalamus (138). Finally, the MC5 receptor is involved in exocrine functions and is localized predominantly in the sebaceous glands (139). The complex role of the melanotropins and the MC receptors, in controlling various physiological functions, has made it difficult to draw simple correlations between these receptors and the biological effects of their ligands. Hence, research has been focused on developing potent and receptor-specific ligands that have well-established biological properties. Thus, potent and selective agonist and/or antagonists could serve as valuable tools for determining unequivocally the roles of the melanocortin receptors in humans.

4.2.2 Studies Leading to the Identification of the Minimum Active Sequence of α-MSH. Early SAR studies with a-MSH were aimed at elucidating the residues in the tridecapeptide that were most responsible for its biological activity. Although previous reviews (140) have covered the results of these studies, it is worthwhile to point out some of the salient features.

1. Replacing Met^4 by norleucine (Nle^4) increases the potency of the resulting melanotropin, probably because of the chemically inert and hydrophobic side chain of Nle. Early studies had shown that Met is easily oxidized to its sulfone, which increased the hydrophilicity dramatically and thus decreased the bioactivity of the corresponding derivative in the amphibian skin assays.
2. The substitution of Phe^7 by D-Phe^7 increased the biopotency of the resulting analog, suggesting that a reverse β-turn conformation (141), formed in the active core sequence (His-Phe-Arg-Trp) of a-MSH, may be important in binding and activity. Consequently, Hruby et al. developed the first highly potent and stable analog of a-MSH, [Nle^4,D-Phe^7]-α-MSH [NDP-MSH, MT-I, (Ac-Ser-Tyr-Ser-Nle^4-Glu-His-D-Phe^7-Arg-Trp-Gly-Lys-Pro-Val-NH_2)] (141). This compound was found to be a potent agonist of the MC1 receptor in amphibian and lizard skin assays.

To further improve the biological efficacy of a-MSH analogs, and to test the p-turn hypothesis, a series of cyclic compounds with greater conformational rigidity compared to that of the linear analog were synthesized. The first-generation cyclic analogs replaced the Met^4 and Gly^{10} residues with a pseudoisosteric Cys + Cys residues, to give *c*[Cys^4,Cys^{10}]-α-MSH

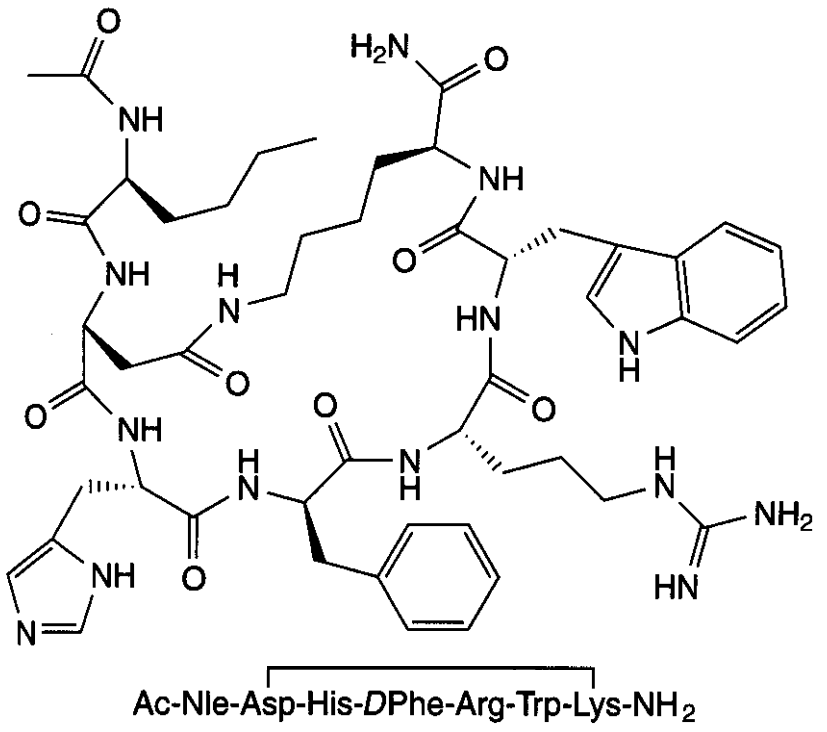


Figure 2.6. Structure of the potent nonselective human melanocortin receptor agonist MT-II.

(142). This compound was found to be more potent than a-MSH, but lacked prolonged biological potency. In view of the importance of melanotropic peptides in treatment of pigmentory disorders and as a tool to detect melanoma cancer, quenched dynamic simulation studies (143) were undertaken to develop a-MSH analogs with superpotent and prolonged biological activity in the lizard skin and tyrosinase assay. The key observations (143) from these studies were the following: (1) both a-MSH and NDP-a-MSH assumed folded conformations with a probable β-turn-like structure at residues seven and eight; (2) the hydrophobic side-chains of His^6, D- or L-Phe^7, and Trp^8 occupied one face of the folded molecule with the charged (hydrophilic) side-chains of Glu^5, Arg^8, and Lys^{11} segregated toward the opposite face of the molecule; (3) although Glu^5 and Lys^{11} occupied the same face, their oppositely charged side-chains were not in proximity for strong coulombic interaction. The molecular mechanic studies also indicated that if Lys^{11} was moved to replace Gly^{10}, the side-chain of Lys^{10} could be involved in a strong electrostatic interaction with the side-chain of Glu^5, and such an interaction may further stabilize the folded conformation and thus improve binding. These observations led to the development of Ac[Nle^4,D-Phe^7,Lys^{10}]-α-MSH and Ac[Nle^4,Asp^5,D-Phe^7,Lys^{10}]-α-MSH analogs, which both assumed folded conformations with Asp^5 (Glu^5) and Lys^{10} side-chains in proximity to each other when tested by molecular dynamic calculations (143, 144). Analog Ac[Nle^4,Asp^5,D-Phe^7,Lys^{10}]-α-MSH is approximately fivefold more potent than a-MSH in the lizard skin assay (144). To further improve potency the cyclic analog with a lactam bridge connecting Asp^5 and Lys^{10} side-chains was synthesized and tested in frog skin, lizard skin, and tyrosinase assays (145, 146). Interestingly, the cyclic analog Ac-Nle^4-*c*[Asp^5,D-Phe^7,Lys^{10}]-NH_2-(4–10)α-MSH (MT-II, Fig. 2.6) was a superpotent agonist in both lizard skin and tyrosinase assays, but had approximately half the activity in the frog skin assay (Table 2.8). MT-11 also showed prolonged biological activity in the in *vitro* assay.

While these studies were in progress, a series of extensive studies on the minimum active sequences necessary for biological activity in melanotropin peptide at pigment cell receptor. Through use of a series of assays and animals it was determined that the His-Phe-Arg-Trp segment of a-MSH was the minimum sequence for full agonist activity (147, 148).

4.2.3 Importance of D-Phe^7 of MT-II in Binding and Biological Activity. The bioactive conformation of a-MSH involves a β-turn in the message sequence and it has been proposed that the side-chain residues of groups in this region (His^6, Phe^7, Arg^8, Trp^8) play an important role in binding receptor selectivity and agonist activity (144,146,149,150). Thus, it was proposed early that replacing the Phe^7 residue by its D-isomer could alter the confor-

Table 2.8 Relative Biological Potencies of α-MSH and Related Analogs[a]

Melanotropin Analog	Bioassay System		
	Frog Skin	Lizard Skin	Tyrosinase
α-MSH	1.0	1.0	1.0
Ac-[Nle^4,D-Phe^7]α-MSH	60.0	5.0	100.0
c[Cys^4,Cys^{10}]α-MSH	20.0	4.0	1.0
Ac-[Nle^4,Asp^5,D-Phe^7,Lys^{10}]-NH_2-(4–10)α-MSH	1.0	5.0	n.d.[b]
Ac-Nle^4-c-[Asp^5,D-Phe^7,Lys^{10}]-NH_2-(4–10)α-MSH	0.5	90.0	100.0

[a]All potencies reported are relative to a-MSH = 1.0 in assay systems.
[b]n.d., not determined.

mation of the peptide and thereby its bioactivity, and thus earlier studies led to substitutions in this position (e.g., see Refs. 151–153). To further test this hypothesis, analogs of MT-II having a halogen at the *para* position in the phenyl ring and the D-Nal(2′)^7 analogs were synthesized and tested for activity at mouse and human melanocortin receptors (frog skin, mMC1-R, hMC1-R, hMC3-R, hMC4-R, and mMC5-R; Tables 2.9 and 2.10) (154). The D-*para*-chloro and D-*para*-fluorophenyl analogs were agonists at both mouse melanocortin receptors (mMC1 and mMC5) and also showed full agonist activity in the frog skin assay. In the frog skin bioassay (FSB), [D-Phe(pF)^7]-MT-II (EC_{50} = 0.10 n*M*) is 20 times more potent than [D-Phe(pCl)^7]-MT-II (EC_{50} = 2.0 n*M*). However, at the mMC1-R and hMC1-R, [D-Phe(pF)^7]-MT-II has approximately one-third the potency of that of [D-Phe(pCl)^7]-MT-II at both receptors (Table 2.9). At the hMC3, hMC4, and hMC5 receptors a similar trend was observed, with the [D-Phe(pCl)^7]-MT-II being a more potent agonist than [D-Phe(pF)^7]-MT-II (154).

However, for the D-p-iodophenyl and the D-Nal(2′)^7 analogs the results in the FSB were different. It was found that both of these compounds were potent antagonists in the FSB. Surprisingly, both analogs were found to be potent agonists at the mouse MC1 and hMC1 receptors. At the hMC3, hMC4, and hMC5 receptors the [D-Phe(pI)^7]-MT-II and [D-Nal(2′)^7]-MT-II (SHU9119) were both potent antagonists, with pA_2 = 9.3 at the hMC4R and a pA_2 = 8.3 at the hMC3 receptor. On the other hand, the [D-Phe(pI)^7]-MT-II analog showed partial agonist activity at the hMC5 receptor, whereas SHU9119 is a full agonist at this mammalian receptor. It is interesting to note that the [D-Nal(1′)^7]-MT-II analog showed full agonist activity at all human receptors, indicating that subtle differences in the topography of the peptide ligand can significantly alter bioactivity. Other MT-II derivatives having larger lactam rings have been tested for their binding potencies and receptor selectivity. However, the larger, mare flexible ring improved neither the potencies nor the selectivities (at hMCRs) of these analogs (155), suggesting that ligand-receptor interactions favor a 23-membered ring.

4.2.4 Effects of β-Substituents in Biological Potency. β-Methyl-substituted amino acids recently have been used to study the topo-

Table 2.9 Activities of MT-II Derivatives at Various MC1-Rs[a]

Compound	EC_{50} Values (n*M*)		
	Frog Skin	mMC1-R	hMC1-R
a-MSH	0.10	1.3	0.091
[D-Phe(pF)^7]-MT-II[b]	0.10	0.026	0.016
[D-Phe(pCl)^7]-MT-II	2.0	0.0095	0.0050
[D-Phe(pI)^7]-MT-II	Antagonist pA_2 = 10.3	0.19	0.055
[D-Nal(2′)^7]-MT-II	Antagonist pA_2 = 10.5	0.039	0.036

[a]Data adapted from Ref. 154.
[b]MT-II: Ac-Nle-c[Asp-His-D-Phe-Arg-Trp-Lys]-NH_2.

Table 2.10 Activity of MT-II Analogs at Various Human Melanocortin Receptors[a]

Compound	EC_{50} Values (nM)		
	hMC3-R	hMC4-R	hMC5-R
a-MSH	669	210	807
[D-Phe(pF)^7]-MT-II	191	19	1360
[D-Phe(pCl)^7]-MT-II	63	18	117
[D-Phe(pI)^7]-MT-II	1134	573	684
	$pA_2 = 8.3$	$pA_2 = 9.7$	
[D-Nal(2′)^7]-MT-II	2813	Antagonist	434
	$pA_2 = 8.3$	$pA_2 = 9.3$	

[a]Data adapted from Ref. 154.

graphical requirements for ligand-receptor interactions. Hruby et al. (156) studied the change in bioactivity that occurs on incorporating all four isomers of β-methyl Phe and β-methyl Trp in the **MT-II** template (Table 2.11) (154). β-Methyl analogs constrain the χ space and thus restrict the rotational freedom of the side-chain group. As a result, the peptide will have certain restricted conformations, which in turn can increase receptor-binding preferences. Incorporation of all four isomers of β-methyl Phe^7 in **MT-II** generally led to decreased potency in the FSB by 1 to 2 orders of magnitude (Table 2.11). However, in the lizard skin assay [(2*S*,3*S*)-β-Me-Phe^7]-MT-II was approximately 17 times less potent than **MT-II**; the [(2*S*,3*R*)-β-Me-Phe^7]-MT-II and the [(2*R*,3*S*)-β-Me-Phe^7]-MT-II were equipotent; and the [(2*R*,3*R*)-β-Me-Phe^7]-MT-II is 2 orders of magnitude less potent than **MT-II** (Table 2.11). In the β-Me-Trp^9 series the [(2*R*,3*S*)-β-Me-Trp^9]-MT-II showed much higher potency than that of the other three stereoisomers in the FSB. In the lizard skin assay [(2*S*,3*R*)-β-Me-Trp^9]-MT-II analog is 33 times less potent than **MT-II**, whereas the other three isomers showed nearly equal potency. These results demonstrate the sensitivity of the lizard skin melanocortin receptor for proper stereochemistry at the β-carbon atom and ultimate topographical presentation of the Phe^7 residue, which contrasts with the requirements at the frog skin receptor. Furthermore, the results in Table 2.11 suggest that incorporation of constrained amino acids into **MT-II** or other melanotropins can further improve bioactivities, when these new analogs possess the right side-chain conformation for binding to their target receptors.

4.2.5 Ring-Expanded Analogs of MT-II. To study the "bioactive" backbone conformations required for tight binding and receptor selectivity at the human melanocortin receptors, a series of ring-expanded MT-II analogs having alanine at position 10 were synthesized (157).

Table 2.11 Comparative Biological Activities of Topographically Modified MT-II Analogs[a]

Analog	Frog Skin		Lizard Skin	
	EC_{50} (nM)	Rel. Potency	EC_{50} (nM)	Rel. Potency
MT-II	0.1	1.0	0.2	1.0
[(2*S*, 3*S*)-β-Me-Phe^7]-MT-II	6.25	0.016	3.44	0.06
[(2*S*, 3*R*)-β-Me-Phe^7]-MT-II	6.25	0.016	0.2	1.0
[(2*R*, 3*S*)-β-Me-Phe^7]-MT-II	2.0	0.05	0.3	0.67
[(2*R*, 3*R*)-β-Me-Phe^7]-MT-II	16.67	0.006	20.0	0.01
[(2*S*, 3*S*)-β-Me-Trp^9]-MT-II	0.44	0.23	1.0	0.2
[(2*S*, 3*R*)-β-Me-Trp^9]-MT-II	28.60	0.004	6.67	0.03
[(2*R*, 3*S*)-β-Me-Trp^9]-MT-II	0.06	1.6	1.43	0.14
[(2*R*, 3*R*)-β-Me-Trp^9]-MT-II	0.33	0.3	1.0	0.2

[a]Data adapted from Refs. 155 and 156.
[b]MT-II: Ac-Nle-c[Asp-His-D-Phe-Arg-Trp-Lys]-NH_2.

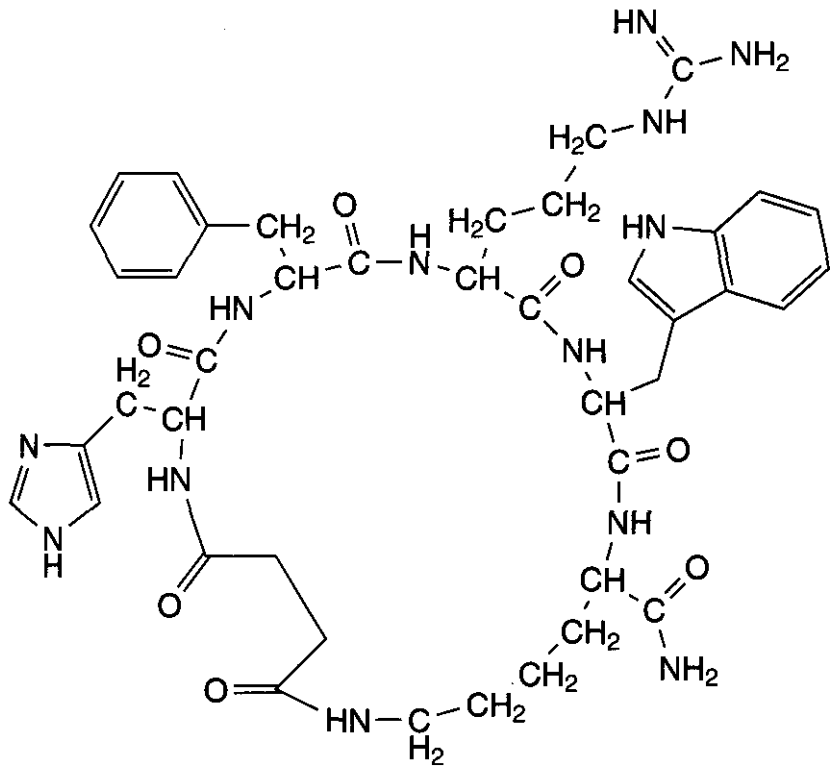
Table 2.12 Binding Affinities of the Ring-Expanded MT-II Analogs[a]

Analog	Structure	IC_{50} (n*M*) hMC1R	hMC3R	hMC4R	hMC5R
MT-II	Ac-Nle^4-*c*[Asp^5, Lys^{10}]-α-MSH	0.25	8.56	0.72	43.6
1	Ac-Nle^4-*c*[Asp^5-His^6-D-Phe^7-Arg^8-Trp^9-Ala^{10}-Lys^{11}]-NH_2	0.35	25.5	0.89	—
2	Ac-c[Asp^5-His^6-Phe^7-Arg^8-Trp^9-Ala^{10}-Lys^{11}]-NH_2	1.94	102	2.03	>1000
3	Ac-Nle^4-c[Asp^5-His^6-Phe^7-Arg^8-Trp^9-Ala^{10}-Lys^{11}]-NH_2	22.4	2400	77.8	>1000
4	Ac-Nle^4-c[Asp^5-His^6-D-Phe^7-Arg^8-D-Trp^9-Ala^{10}-Lys^{11}]-NH_2	0.91	137	71.5	1441
5	Ac-Nle^4-c[D-Asp^5-His^6-D-Phe^7-Arg^8-Trp^9-Ala^{10}-Lys^{11}]-NH_2	264	>1000	91.0	>1000

[a]Adapted from Ref. 157.

The introduction of Ala^{10} increases the cyclic lactam from a 23-membered (**MT-II**) to a 26-membered ring, thus allowing greater conformational flexibility, thought to be important for maintaining active peptide conformation. Furthermore, this 26-membered analog was modified through inversion of chirality at positions 5, 7, and 9 to test the stereochemical requirements of the side-chain groups at these positions in receptor selectivity. These studies showed that at the hMC1, hMC4, and hMC5 receptors, **MT-II** and [Ala^{10},Lys^{11}]-MT-II (analog **1**) had similar binding affinities, whereas at the hMC3R, [Ala^{10}]-MT-II had a fourfold decrease in binding potency (Table 2.12). Removal of Nle^4 from [Ala^{10}]-MT-II (analog 2) resulted in a 5.5-fold decrease in binding affinity at the hMC1R and a four- and twofold decrease at the hMC3 and hMC4 receptors, respectively. However, this analog was unable to competitively displace [^{125}I]-NDP-MSH from the hMC5R at ligand concentrations > 1000 n*M*. When D-Phe^7 in analog **1** was substituted by its L-isomer (analog **3**), the binding affinity at all melanocortin receptors was decreased substantially. However, it was interesting to note that this substitution resulted in a 100-fold ligand selectivity between the hMC1 and hMC3 receptors and a 30-fold selectivity between the hMC3 and hMC4 receptors. The introduction of a D-Trp^8 residue in place of Trp^8 in analog **1** gave a peptide with poor binding affinity at all receptors (analog **4**), and the maximum effect was seen at the hMC4R where an 80-fold decrease in binding was observed for analog (4) versus analog (**1**). Finally, the importance of the chirality of the side-chain of Asp^5 analog (**1**) was determined by substituting the D-isomer (analog 5). This compound was unable to competitively displace [^{125}I]-NDP-MSH at the hMC3 and hMC5 receptors, and was, respectively, 750 and 100-fold less tightly bound to the hMC1 and hMC4 receptors versus analog (**1**). All peptides were agonists at the human melanocortin receptors.

4.2.6 Novel Constrained Cyclic Lactam Analogs of a-MSH. Agonists. The important role of a-MSH in the control of various key physiological functions has prompted research in the development of novel tight-binding and receptor-selective linear or cyclic analogs. However, to date, many of these compounds that incorporate the core sequence show similar selectivity profiles. The recent observations that the hMC3 and hMC4 receptors might be involved in the control of feeding behavior and energy homeostasis in humans has heightened the interest to develop selective and potent agonists and/or antagonists for these receptors. The novel cyclic peptide [(O)C-CH$_2$-CH$_2$-C(O)-*c*-[His^6-D-Phe^7-Arg^8-Trp^9-Lys^{10}]-NH$_2$ (MK-1, Fig. 2.7) (158, 159) is a highly selective and potent agonist of the hMC4 receptor. This compound is a modified cyclic lactam analog of **MT-II**, the nonselective but potent agonist of the melanocortin receptors. In MK-1 cyclization is through the use of a linker arm involving a backbone to side-chain cy-



c[(O)C–CH_2–CH_2–C(O)–His^6–D–Phe^7–Arg^8–Trp^9–Lys^{10}]–NH

VJH-085

Figure 2.7. A novel selective cyclic lactam agonist of the human melanocortin 4 receptor.

clization strategy. In MT-II, however, cyclization is through a lactam bridge between the carboxyl side-chain of Asp^5 at the N-terminus and the ϵ-amino group of Lys^{10} at the C-terminus. Binding studies showed that MK-1 is 700-fold more selective at the hMC4R vs. hMC1R (IC_{50} hMC1R = 4200 n*M* and IC_{50} hMC4R = 6.0 n*M*). Additionally, this analog is 55- and 120-fold more selective at the hMC4R vs. the hMC3R and hMC5R, respectively (Table 2.13). In a related study Bednarek et al. (160) used a similar cyclization strategy to identify a selective and potent peptide agonist of the hMC4 receptor. They synthesized a series of cyclic peptides having the general structure c[X-His-D-Phe-Arg-Trp-Y]- NH_2, where X = succinyl or ω-amino-carboxylic acid (as linker arms) and Y = a-, ω-diamino-carboxylic acids. *In vitro* analysis identified two analogs that showed good agonist activity and a high degree of selectivity for the hMC4 receptor. The first analog involved the substitution of a 2,4-di-aminobutyric acid group in place of Lys^{10} and the use of a succinyl linker to cyclize the peptide (21-membered ring). This analog is a potent hMC4R agonist with 55-fold selectivity for the hMC4R versus the hMC3R receptor and is about 1000-fold more selective for the hMC4 versus the hMC5 receptor. Another analog having a Glu residue in place of Lys^{10} and using a 3-aminopropionic acid moiety as the linker arm was also a potent hMC4R agonist (EC_{50} = 0. 56 ? 0.04 n*M*) (BBRC). This compound showed a 90-fold greater affinity for the hMC4 versus the hMC3 receptor and was 2000-fold more selective for the hMC4 versus the hMC5 receptor. To test the importance of lactam ring size in selectivity and bioactivity analogs having smaller and larger rings were tested. The smaller lactam rings showed lower potency, whereas it was observed that rings having more than 21 atoms showed good potency and selectivity at the hMC4 receptor.

4.2.7 Antagonists. Although SAR studies of a-MSH have resulted in the development of potent agonist analogs in the past two decades, the search for modestly potent and selective competitive antagonists has not been as successful (161–164). Although a few early studies suggested weak or partial inhibition by a-MSH analogs or a-MSH-related fragment derivatives (132, 161, 163, 164), these results were difficult to improve on. Since then, the design and synthesis of a-MSH antagonists has been slow because of a lack of understanding of antagonist structure-activity relationships. Recently, SAR studies in the Hruby group, involving the use of novel cyclic

Table 2.13 Biological Activities of the Cyclic α-MSH Analogs at Various Human Melanocortin Receptors[a]

	hMC3R			hMC4R			hMC5R		
Peptide[b]	IC_{50} (nM)	EC_{50} (nM)	% Activity	IC_{50} (nM)	EC_{50} (nM)	% Activity	IC_{50} (nM)	EC_{50} (nM)	% Activity
VJH-085	332.0	70	95	5.96	1.55	100	710	61.52	78
MK-2	21.46	81.9	87.8	102.8	281.6	35	154.9	148.3	100
MK-3	6.67	3.75	36	50.68	>10 μM Antagonist	0.0	6118	117	100
MK-4	6.1	>10 μM Antagonist	0.0	231	1220	22.8	27.5	1	81
MT-II	1.52	1.85	100	6.86	2.87	100	7.47	2.45	100

[a] Adapted from Ref. 159.

[b] **VJH-085:** (O)C-$(CH_2)_2$-C(O)-*c*[His-D-Phe-Arg-Trp-Lys]-NH_2; **MK-2:** (O)C-$(CH_2)_2$-C(O)-*c*[His-D-(2′)Nal-Arg-Trp-Lys]-NH_2; **ME-3:** (O)C-(C_6H_5)-C(O)-*c*[His-D-(2′)Nal-Arg-Trp-Lys]-NH_2; **ME-4:** (O)C-$(CH_2)_3$-C(O)-*c*[His-D-(2′)Nal-Arg-Trp-Lys]-NH_2.

lactam analogs having side chain to backbone cyclization, have provided new insights into antagonist structure-activity relationships. It was found that the substituting hydrophobic residues at position seven of the message sequence (MK-2) resulted in small losses in binding potency at the hMC4R, accompanied by an increase in antagonist activity at this receptor (Table 2.9). More recently it was found that when a hydrophobic group such as o-phthalic acid was used as the cyclizing linker (MK-3), this analog was a potent antagonist of the hMC4 receptor ($pA_2 = 11$), even though MK-3 has a binding affinity of 13.5%, compared to that of the superagonist MT-II, used as a control in this study. Interestingly, both peptide MK-2 and MK-3 had strong binding affinity for the hMC3 receptor. Thus it appears that the combination of a bulky residue at position 7 and a hydrophobic phenyl ring in the linker arm (MK-1 to MK-3) significantly alters the conformational representation of the ligand presented to the hMC4 receptor, so as to convert the hMC4R-selective and potent agonist to a hMC4R-selective and potent antagonist (159). These SAR studies also showed that increasing the 23-membered lactam ring of MK-1 by one carbon atom (succinyl vs. glutaric linker) gives a highly selective and potent antagonist (MK-4) for the hMC3 receptor. At the hMC4 receptor, analog MK-4 is only a partial agonist, while concomitantly maintaining full agonist activity at the hMC5 receptor. Analogs MK-1, MK-3, and MK-4 therefore represent the first examples of a class of cyclic melanotropin ligands with high selectivity and defined biological activities at the physiologically important hMC3 and hMC4 receptors (159).

4.3 Oxytocin and Vasopressin

4.3.1 Structure-Activity Relationships. Oxytocin and vasopressin are neurohypophyseal hormones synthesized in the hypothalamus and then transported along with their carrier proteins to the posterior lobe of the pituitary gland. They were first discovered in the posterior pituitary gland, but they also are located in the CNS and numerous other organs. These peptides both consist of nine amino acids; they both contain a 20-membered disulfide and an acyclic tripeptide tail; and the two peptides differ only in positions 3 and 8.

Oxytocin causes contractions of uterine smooth muscle and is secreted during labor. Oxytocin also stimulates contraction of smooth muscle in mammary glands, to cause milk ejection in nursing mothers. A large percentage of deliveries in the United States are induced or augmented by i.v. oxytocin (165). Oxytocin also has some intrinsic antidiuretic activity. Less is known about the role of oxytocin in the central nervous system. It has been shown to be involved in memory and learning as well as grooming and sexual behavior. At low doses vasopressin controls the resorption of water by the distal tubules of the kidneys and regulates the osmotic content of blood. At high doses it causes contractions of blood vessels causing localized increases in blood pressure. It also has CNS activities.

4.3.2 Oxytocin. Oxytocin was the first peptide hormone to be used clinically. Extracts from the posterior pituitary were introduced in obstetric practice more than 90 years ago (166). Oxytocin also was the first peptide hormone to be sequenced (38) and synthesized (39). Oxytocin is, in itself, quite an effective therapeutic agent when used appropriately, and there has been relatively little interest in designing more potent oxytocin agonists. However, when administered in high doses in a glucose solution, oxytocin can cause water intoxication. Therefore, analogs of oxytocin having reduced antidiuretic activity would have some benefit.

4.3.3 Structure-Activity Relationships of Oxytocin. Hundreds of analogs of oxytocin have been synthesized since 1953. There have been several comprehensive reviews of this work (167–169).

Oxytocin (OT) H-Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2

Vasopressin (VP) H-Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH_2

Table 2.14 Biological Activities of Oxytocin (OT) and Selected Agonist Analogs

	Rat Uterus (IU/mg)	Reference(s)
Oxytocin	546	181
Oxytocinoic Acid	1.3	170
Deamino-OT	803	182
[1-carba]OT	734	172
Deamino-[1-carba]OT	1898	183
Deamino-[6-carba]OT	929	184
[D-Tyr^2]OT	Partial agonist	173,185
[Thr^4]OT	900	186
[Thr^4, deamino-1-carba]OT	272	180
[Thr^4,deamino-6-carba]OT	695	180

Fragment studies of oxytocin indicated that the 20-membered ring structure is necessary for full agonist activity at the uterus receptor. The acyclic tripeptide tail, although having no biological activity itself, is important for binding to the uterus receptor. The C-terminal amide group is important for activity. Replacement of the C-terminal $CONH_2$ with COOH to form oxytocinoic acid results in a 400-fold decrease in activity (170) (Table 2.14).

Deletion of the free alpha amino group from the Cys in position 1 to form 1-deamino-oxytocin enhanced both the oxytotic and antidiuretic activities (171). Replacement of the sulfur in Cys^1 with a methylene group to form [1-carba]OT resulted in an increase in potency (172) (Table 2.14).

The orientation of the aromatic ring of Tyr^2 is important for binding and transduction. Substituting a D-Tyr in this position results in a partial agonist (173,174). The Tyr hydroxyl group is also important for activity because [Phe^2]OT has only approximately 1/15 the potency of oxytocin (175).

Manning et al. (176) synthesized [4-threonine]oxytocin, which was the first oxytocin analog containing only naturally occurring amino acids that was more potent than oxytocin itself and had lessened antidiuretic activity (177). This indicates that an amino acid side chain that can form a hydrogen bond at position 4 is important for agonist activity. The substitution of Gly in position 7 resulted in an analog that retained its oxytotic activity, yet had very little antidiuretic activity (178). Combining these two substitutions to form [Thr^4,Gly^7]oxytocin resulted in an analog with high specificity (179).

Lebl et al. (180) combined the Thr^4 and the carba substitutions, synthesizing [Thr^4, deamino-1-carba]oxytocin and [Thr^4,deamino-6-carba]oxytocin. Although these analogs had slightly reduced potency at the uterus receptor, they were more selective, having much lower vasopressor activity.

4.3.4 Oxytocin Antagonists. Oxytocin antagonists would be of great therapeutic benefit in delaying labor. Some of the earliest oxytocin analogs demonstrated antagonistic properties (see Refs. 167, 168, 187 for reviews). Methylation of the tyrosine hydroxyl group to produce [MeO-Tyr^2]oxytocin resulted in an analog with antagonism of the vasopressor response but not the oxytocic response (188) (Table 2.15). Schulz and Du Vigneaud (189,190) found that substitution of penicillamine in position 1 of oxytocin and 1-deamino-oxytocin resulted in potent antagonists of the oxytotic response in *vitro* and partial agonist/antagonist properties *in vivo*. Substitution of leucine in position 2 to form [Pen^1,Leu^2]oxytocin increased antagonist activity (191). [D-Pen^1,Orn^8]oxytocin is about 10-fold more potent as an antagonist than either [D-Pen^1]oxytocin or [Pen^1]oxytocin (192). Substitution of threonine in position 4 of a series of [1-(β-mercapto-β,β-dialkylpropionic acid)]oxytocin doubled potency (193).

Sawyer and Manning (194) concluded that the C-terminal glycinamide is not necessary for oxytotic antagonists. Amino acids with bulky side chains can be substituted for the

Table 2.15 Oxytocin (OT) Analogs with Antagonistic Potencies at the Rat Uterus *In Vitro*

Analogs	Estimated pA_2[a] Values *In Vitro* (No Mg^{2+})	Reference(s)
[Pen[1]]OT	6.86	189,190
[Pen[1],Leu[2]]OT	7.14	191
[D-Pen[1]]OT	6.94	209
	7.14	193
[D-Pen[1],Orn[8]]OT	7.89	193
[D-Pen[1],Thr[4]]OT	7.52	179
[1-(β-Mercapto-β,β-diethylpropionic acid)]OT	7.55	179
[1-(β-Mercapto-β,β-diethylpropionic acid),Thr[4]]OT	7.72	179
[1-(β-Mercapto-β,β-cyclopentamethylenepropionic acid)]OT	7.61	179
[1-(β-Mercapto-β,β-cyclopentamethylenepropionic acid),Thr[4]]OT	7.91	179
[D-Tic[2]]OT	6.50	201
[Tyr(Me)[2]]OT	6.79	202
[D-Phe(4-Et)[2]]OT	8.15	203,204
[2-Br-Tyr[2]]OT	7.05	205
[3-I-Tyr[2]]OT	7.05	206
[Deamino-Pen[1],Tyr(Me)[2]]OT	7.76	193
[Deamino-Pen[1],Tyr(Me)[2],Orn[8]]vasotocin[b]	7.70	192
[1-(β-Mercapto-β,β-diethylpropionic acid),Tyr(Me)[2],Orn[8]]vasotocin	8.91	207
[1-(β-Mercapto-β,β-cyclopentamethylenepropionic acid), Tyr(Me)[2],Orn[8]]vasotocin	8.52	207
[1-(β-Mercapto-β,β-cyclopentamethylenepropionic acid), D-Tyr(Me)[2],Val[4],Cit[8]]vasopressin	8.61	194
des(Gly(NH_2)[1-(β-mercapto-β,β-cyclopentamethylenepropionic acid), D-Phe[2]]arginine-vasopressin	7.64	194
d-carba[6][D-Phe(Me)[2]]OT	8.73	203
des(Gly(NH_2)d-carba[6][D-Phe(Me)[2]]OT	8.80	203
[Pen[1],D-Phe[2],Thr[4],Orn[8]]OT	7.23	208
[Pen[1],D-Phe[2],Thr[4],Thr[5],Orn[8]]OT	7.16	208
[Pen[1],D-Phe[2],Thr[4],Leu[5],Orn[8]]OT	6.67	208
[Pen[1],D-Phe[2],Thr[4],Asp[5],Orn[8]]OT	7.21	208
[Pen[1],D-Phe[2],Thr[4],Tyr[5],Orn[8]]OT	6.76	208
[Mpa[1], Glu[4],Cys[6],Lys[8]10T(bicyclic)	8.2	195,196

[a] pA_2 is the negative algorithm of the molar concentration of antagonist that reduces the response to 2× units of oxytocin to equal the response to 1× unit in the absence of antagonist.

[b] Vasotocin is [8-Arg]OT.

Gly in position 9 without a loss of antagonistic efficacy (177). They also found several antivasopressin analogs that were also antioxytocics.

Hill et al. synthesized a bicyclic analog of the weak monocyclic agonist *c*[Mpa[1],Cys[6]]-c[Glu[4],Lys[8]10xytocin, which was found to have potent antagonist activity (195–197) as did many of its derivatives. Extensive NMR and computational studies of this led to the determination of the bioactive conformation of oxytocin antagonists (198, 199) and to the design of topographically constrained antagonist analogs (200) with unique biological properties and unique insight into the topographical requirements of OT receptors.

4.4 Delta Opioid Receptor Ligands

All of the current opioid drugs used for the treatment of pain are primarily ligands for the p-opioid receptor. Numerous studies since the discovery of enkephalin 27 years ago (210) have suggested that an opioid ligand that primarily interacted with the δ-opioid receptor would have far fewer of the toxicities generally associated with the μ-opioid ligand (respiratory depression, constipation, addiction, etc.).

Early efforts to convert enkephalin into a selective 6-opioid ligand were successful in the development of *c*[D-Pen^2,D-Pen^5]enkephalin (211) (DPDPE) and its analogs, which eventually led to analogs that were essentially specific for 8-opioid receptors as agonists, such as (2*S*,3*R*)β-methyl-2′,6′-dimethyl tyrosine-*c*[D-Pen^2,u-Pen^5]enkephalin (212, 213). The earlier aspects of this work have been thoroughly reviewed (214) and a selective update has recently appeared (215). A different kind of lead to δ-opioid ligands came from the discovery of the deltorphins (216, 217) such as H-Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2 that are found in amphibian skins and have intrinsically highly delta opioid receptor selectivity.

A quite comprehensive review of the structure-activity relationships of deltorphins has been published (218). SAR studies of modified deltorphin structures led to another class of linear delta opioid receptor ligands such as H-Tyr-Tic-Phe-Phe-OH (TIPP) (219) and H-Tyr-Ticψ[CH_2NH]Phe-Phe-OH (TIPP[ψ]) (220) and related analogs, which also have been recently reviewed (221). Our purpose in this section is not to repeat or summarize these reviews but rather to point to some aspects related to peptide design in conformational space for 6-receptors. Thus, we will be highly selective in our choice of ligands to discuss structure-activity relationships and the conformation and topographical properties that lead to delta agonist and antagonist activity. In this regard it has recently been reported that use of a new spectroscopic method, coupled plasmon waveguide resonance spectroscopy (CPWR) (222), allows one for the first time to examine changes in G-protein–coupled receptors protein structure parallel and perpendicular to the membrane normal to that accompanying binding of ligands. It was shown for the human 6-opioid receptor that agonist and antagonist binding leads to different structures for the δ-opioid receptor (210), and that inverse agonist binding leads to yet another conformation (223). These studies provide unequivocal evidence that agonists, antagonists, and inverse agonists lead to different conformations of G-protein–coupled receptors and suggest that the availability of multiple conformational states are of critical importance to the function of G-protein–coupled receptors. The precise conformational difference remains to be determined.

4.4.1 Analogs of Enkephalins that Lead to Receptor-Specific Ligands and Nonpeptide Ligands. The conversion of enkephalin to the cyclic enkephalin analog *c*-[D-Pen^2,D-Pen^5] enkephalin (DPDPE), to give a potent and high δ-opioid receptor ligand (211), demonstrated clearly the power of conformational constraint for both enhancement of potency and receptor selectivity (224). Subsequent NMR (225) and X-ray crystallographic studies (226,227) provided insights into the importance of a turn conformation to δ-opioid receptor selectivity and differences in conformational requirements for agonists and antagonists, but left unanswered the side-chain conformation of Tyr^1 and Phe^4 for potent and selective 6-opioid receptor bioactivity. To examine these requirements, we turned to topographical constraints, that is, to constraints in χ_1 and/or χ_2 space that can be made within the context of the same backbone conformation for agonist (and antagonist) biological activity (228). All four β-methylphenylalanine-4 analogs (229) and β-methyl-2′,6′-dimethyltyrosine-1 (TMT) (213) analogs of DPDPE were synthesized and evaluated for binding affinities and biological activities for their conformational and topographical properties. As seen in Table 2.16 for the [TMT^1]DPDPE analogs, only the [(2*S*,3*R*)-TMT^1]DPDPE analog was both highly potent and highly selective for the delta opioid receptor. Conformational analysis that used NMR and computation chemistry demonstrated that for Tyr^1, the trans χ_1 conformation, and for Phe^4, the gauche (−) conformation, were critical for biological agonist activity and potency.

In related studies, Mosberg et al. (230–232) carefully evaluated the cyclic truncated deltorphin analog H-Tyr-*c*-[D-Cys-Phe-D-Pen]-OH (JOM-13) in a series of structure-activity and conformational studies that used the β-MePhe^3 constraint (230) and alternative constraints for the Tyr^1 position (see ref. 231 for an excellent review). These studies led to the conclusion of the gauche (−) side-chain conformation for χ_1 in Phe^3 and the trans χ_1 side-chain conformation for the Tyr^1 χ_1 (232). As expected the backbone conformations of

Table 2.16 Binding Affinities for Delta Opioid Receptor Selective Ligands

Compound	IC_{50} or K_i (nM) δ	μ	μ/δ ratio	Ref.
c-[D-Pen^2,D-Pen^5]Enkephalin(DPDPE)	1.6	610	380	211
[(2*S*,3*S*)TMT^1]DPDPE	210	720	3.5	212
[(2*S*,3*R*)TMT^1]DPDPE	5.0	4,300	860	212
[(2*R*,3*S*)TMT^1]DPDPE	3,500	77,000	22	212
[(2*R*,3*R*)TMT^1]DPDPE	9% at 1000	0% at 1000	NA	212
H-Tyr-c[D-Cys-Phe-D-Pen]-OH JOM13	0.74	52	69	230
H-Tyr-(2*S*,3*R*)β-MeTic-Phe-Phe-OH	0.53	>10,000	>19,000	247
Tyr-Tic-(2*S*,3*R*)β-MePhe-Phe-OH	0.38	>10,000	>26,000	247
H-Dmt-Tic-OH	1.84	1,360	739	248
N,*N*-Me$_2$Dmt-Tic-OH	5.93	5,720	965	218
(2*S*,3*R*)TMT-Tic[a]	9.3	35,000	3,800	251
(2*S*,3*R*)TMT-Tic	120	>80,000	>500	251
H-Tyr-c[D-Pen-Gly-Phe-Pen]-Phe-OH	4.0	11,400	2,800	254
H-Tyr-c[D-Pen-Gly-Phe(pF)-Pen]-Phe-OH	0.43	1,600	3,800	254
H-Tyr-c[D-Pen-Gly-Phe(pBr)-Pen]-Phe-OH	0.20	4,200	21,000	254
H-Tyr-D-Met-Phe-His-Leu-nBuG-Asp-NH$_2$	0.045	820	18,000	258
H-Tyr-D-Met-Phe-His-Leu-(3*S*)secBuG-Asp-NH$_2$	0.082	800	9,700	258
H-Tyr-D-Met-(2*S*,3*R*)β-MePhe-His-Leu-Met-Asp-NH$_2$	2.4	>70,000	>29,000	259
H-Tyr-c[D-Pen-Phe-His-Pen]-Nle-Asp-NH$_2$	3.4	9,100	2,700	260

[a] A potent inverse agonist at the δ opioid receptor **(252).**

the DPDPE ring (14-membered) and the JOM-13 ring (11-membered) were found to be somewhat different. Mosberg et al. carried these studies forward by examining the binding of JOM-13 to a model of the δ-opioid receptor they developed (233, 234). By use of these models they developed a model of JOM-13 bound to the δ-receptor (Fig. 2.8), which nicely explains the structure-activity relationship they had found for JOM-13.

Hruby et al. used their bioactive conformation model for [(2*S*,3*R*)TMT^1]DPDPE for a different purpose (Fig. 2.9), that is, to design nonpeptide peptide mimetics. A major interest of medicinal chemists is the development of peptide mimetics (235). The concept of pep-

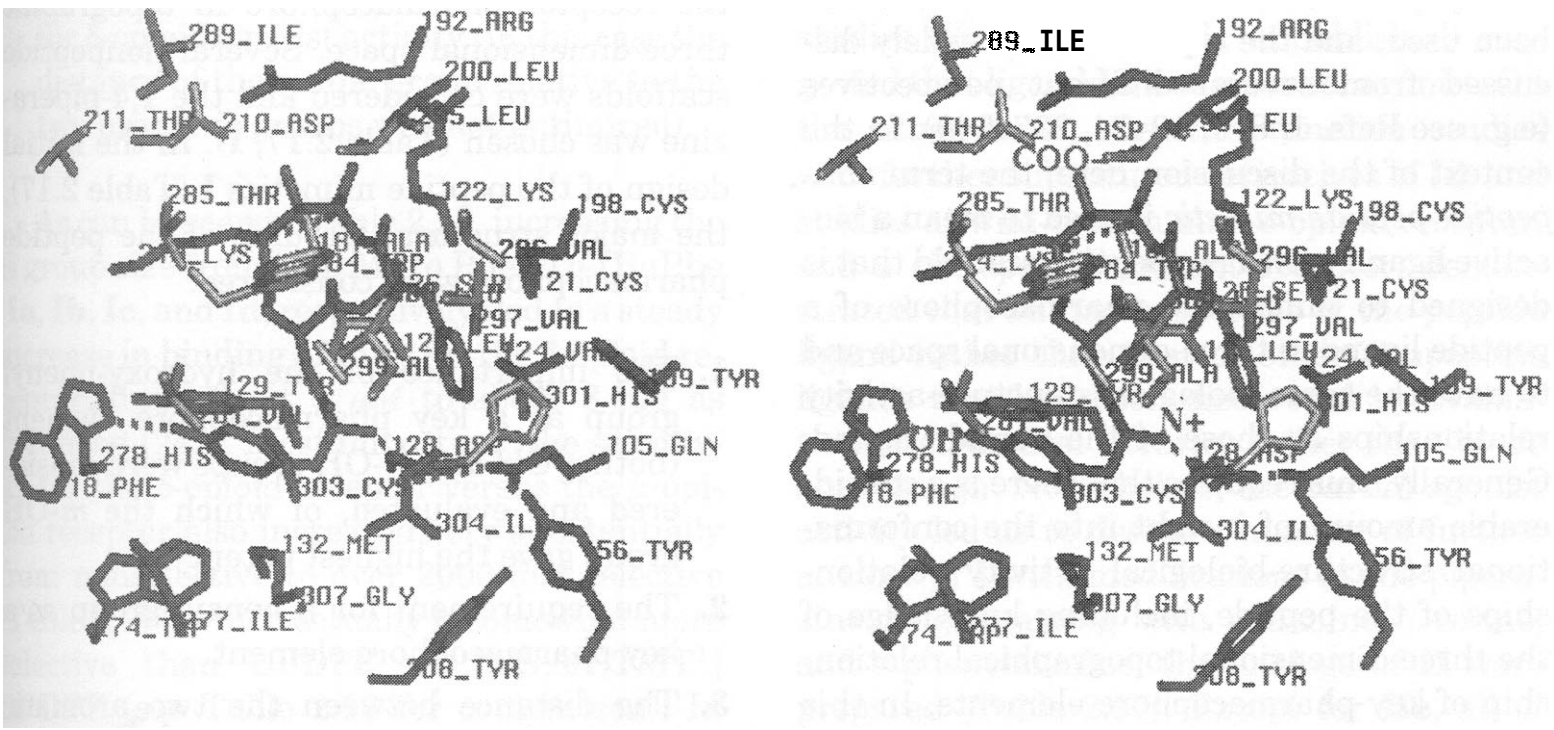


Figure 2.8. JOM-13 (blue) in the δ-opioid receptor binding pocket (stereoview). See color insert. [Taken from Fig. 2.9 in H. I. Mosberg, ***Biopolymers (Peptide Science), 51,*** 426 (1999). Reprinted by permission of John Wiley & Sons.]

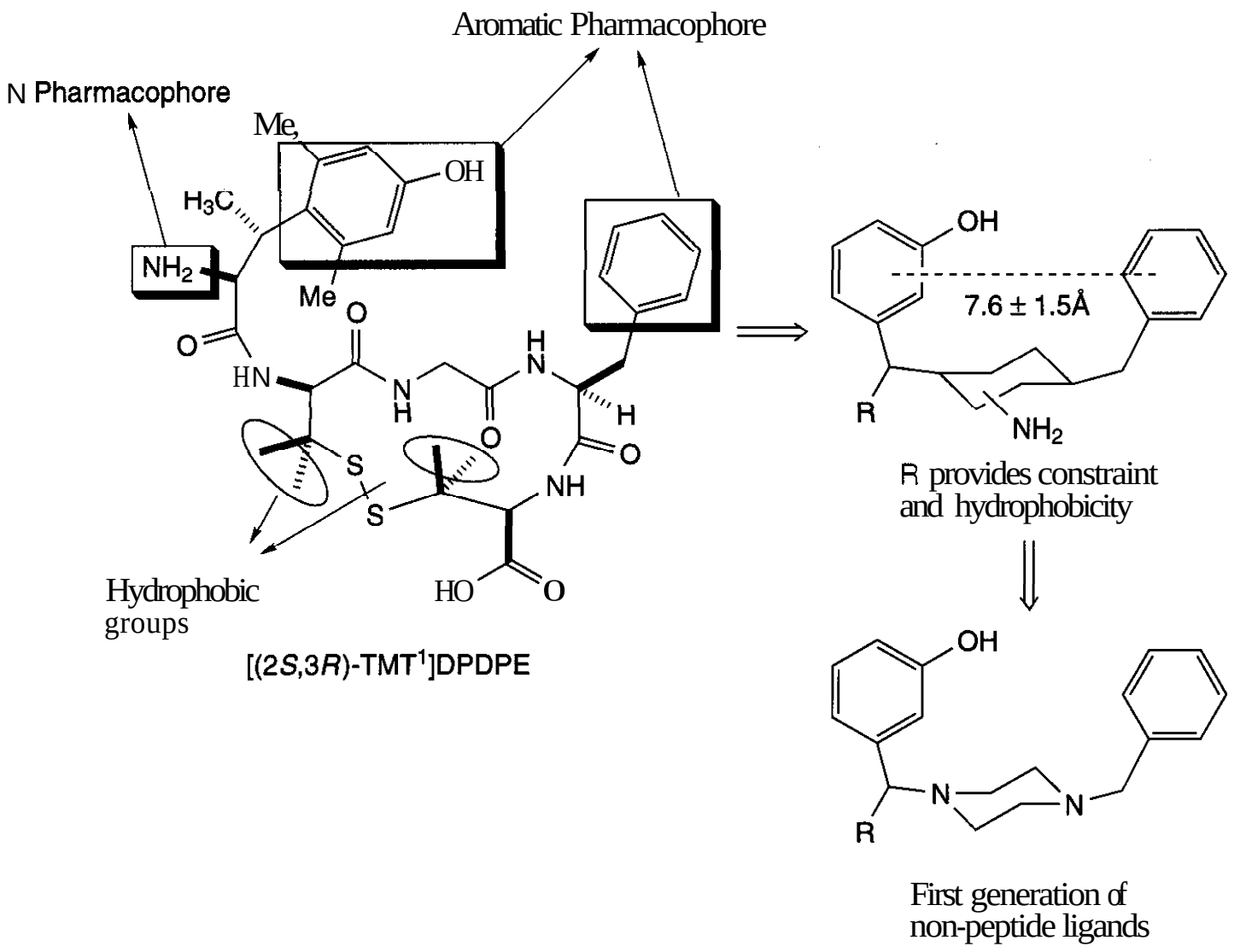
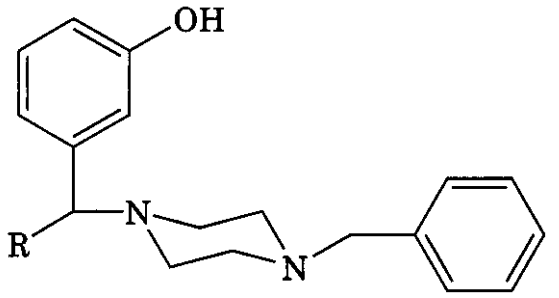


Figure 2.9. Conversion of (2*S*,3*R*)TMT[1]-c[DPDPE] into a nonpeptide peptide mimetic based on topographical considerations.

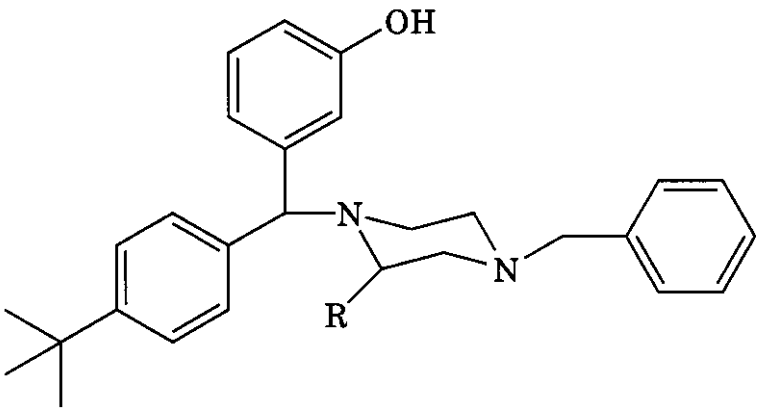
tidomimetics has been around for over 20 years, since the discussions of Farmer (236). There are many different ways in which the term *peptide* mimetic or peptidomimetic has been used, and the topic has been widely discussed from several different perspectives (e.g., see Refs. 1, 8, 9, 13–24,237–240). In the context of the discussion here. the term *non*-peptide peptide-mimetic is used to mean a bioactive ligand with a nonpeptide scaffold that is designed to mimic the pharmacophore of a peptide ligand in three-dimensional space and to have the same biological structure-activity relationships as those of the peptide ligand. Generally, this requires that there is a considerable amount of insight into the conformational structure-biological activity relationships of the peptide, including knowledge of the three-dimensional topographical relationship of key pharmacophore elements. In this case (Fig. 2.9) this involved comprehensive biophysical studies of the [TMT[1]]DPDPE analogs (Table 2.16), including extensive NMR studies, computational studies, molecular dynamic simulations, and molecular modeling (241, 242). These studies led to a proposal for the receptor pharmacophore in topographic three-dimensional space. Several nonpeptide scaffolds were considered and the 1,4-piperazine was chosen (Table 2.17, I). In the initial design of the peptide mimetics I (Table 2.17), the major structural features of the peptide pharmacophore were considered:

1. The importance of the hydroxy-phenyl group as a key pharmacophore element (bothp-OH and m-OH groups were considered and evaluated, of which the m-OH group gave the highest potency).
2. The requirement for a benzyl group as a key pharmacophore element.
3. The distance between the two aromatic group in three-dimensional space was a key to delta opioid receptor selectivity of peptide ligands.

Table 2.17 Nonpeptide Mimetics for Delta Opioid Receptors Based on Delta Opioid Peptides



I



II

Structure	Binding Affinity, IC_{50} (n*M*)		
	μ Receptor	δ Receptor	Selectivity
Ia[a] R = H	8,100	6,400	1.3
Ib[a] R = Me	780	610	1.3
Ic[a] R = Ph	500	34	15
Id[a] R = tBuPh	17,000	8.4	2,000
(+)**Id**[a] R = tBuPh	11,000	42	260
(−)**Id**[a] R = tBuPhe	7,700	4.1	1,900
IIa[b] R = Me	3,300	11	290
IIb[b] R = CH_2OH	8,000	38	210
IIc[b] R = $CH_2C_6H_5$	46,000	21,000	2.2
IId[b] R = CH_2-O-CH_2-C_6H_5	26,000	38	670

[a]See Ref. 226.
[b]See Ref. 227.

4. The need for a bulky R group for distinguishing peptide ligands for δ- versus p-opioid receptors.
5. The requirement for a basic amine group for 6-opioid agonist activity (in this case the distance of the amine group relative to the two aromatic groups was not optimized).

As can be seen in Table 2.17, increasing the R group size from H to Me to Phe to *p*-tBuPhe (Ia, Ib, Ic, and Id, respectively) led to a steady increase in binding affinity for the 6-opioid receptor; from about 6 μM to about 8 n*M* as predicted (242). Most important, the selectivity for the δ-opioid receptor versus the μ-opioid receptor also increased very substantially from nonselective to over 2000-fold selective (Table 2.17), which actually is somewhat more selective than DPDPE or [(2*S*,3*R*)TMT[1]] DPDPE (see Table 2.16 for comparison). In the functional assays that make use of the classical guinea pig ileum (GPI, for the μ-receptor) and mouse vas deference (MVD, for the &receptor) compound Id was still highly 6-opioid receptor selective but was found to be less potent in this assay than would be expected from its binding affinity. Subsequent studies (Yamamura et al., unpublished) suggested that ligand Id was a partial agonist. On the other hand, structure-function studies with further substituted derivatives of Id, and studies at wild-type human δ-opioid receptor, and a site-specific mutant receptor, demonstrated that Id had properties of the peptide ligand rather than that of other nonpeptide ligands that had been discovered by evaluation of structural libraries rather than by *de novo* design. Nonetheless, the partial agonist activity led us to design a number of further analogs of I with modifications in the piperazine ring. Starting with L-alanine, L-serine, and L-phenylalanine, the analogs of II were prepared (Table 2.17). Except for IIc, all of these compounds had nanomolar binding to δ-opioid receptors and were quite selective for the 6-opioid receptor, but again in functional

assays they had lower potencies than what would be anticipated from their binding affinities (243). The importance of the two nitrogens also was examined (243, data not shown). When the benzyl nitrogen was replaced by a CH, group, the potency at the δ-opioid receptor decreased by nearly 3 orders of magnitude, whereas when the other nitrogen was replaced good potency at the δ-opioid receptor was retained, with some loss in selectivity.

These studies suggest that, although *de novo* design of nonpeptide peptidomimetics with high binding affinity and receptor selectivity has an excellent chance for success, there still is much to learn about those structural factors that are key for distinguishing agonist from antagonist biological activity. Agonist and antagonists for G-protein–coupled receptors clearly have different structure-activity relationships, and in addition may have certain dynamic structural requirements that are needed to bind to the receptor to produce transduction for agonists and no transduction for antagonist. In this regard, we have recently shown (244), through the use of a new spectroscopic method, coupled plasmon waveguide resonance (CPWR or PWR) spectroscopy, which allows one for the first time to examine changes in the structure of GPCRs in membrane bilayers parallel and perpendicular to the membrane bilayer normal, that when delta opioid agonists and antagonists bind to the human delta opioid receptor, the receptors have different conformations, and that the changes in conformation are consistent with the differences in changes in structure for the receptor that would be expected for transduction to occur or not to occur. The implication of these findings suggests the need to be able to evaluate those structural features critical for agonist vs. antagonist activity at GPCRs in both peptide and nonpeptide scaffolds. This points again to the realization that there is still no general predictable strategy in going from agonist to antagonist ligands or vice versa for GPCRs (245, 246), although there are a number of approaches that have worked in specific cases.

Returning to peptides for the delta opioid receptor, a few other developments related to high 6-opioid receptor selectivity should be mentioned. The highly important TIPP and TIPP[ψ] analogs of Schiller et al. already have been discussed and a few of the most selective analogs are given in Table 2.16. Of considerable interest were related dipeptides based on the structure H-Tyr-Tic- OH, first reported by Lazarus et al. (for a review, see Ref. 248). Particularly interesting were the analogs H-Dmt-Tic-OH [DMT = (2*S*)-2′,6′-dimethyltyrosine] and the *N*,*N*′-dimethyl analog (*N*,*N*-Me_2Dmt-Tic-OH; Table 2.16). Originally, H-Dmt-Tic-OH was reported to be exceptionally potent and selective (250) but subsequent direct comparison with TIPP analogs (249) indicates that the most selective analogs in this series are the tetrapeptide analogs in Table 2.16. At the same time a more constrained series of dipeptide analogs were prepared by Hruby et al. (251), in which all four isomers of TMT were examined. As shown in Table 2.16, only the (2*S*,3*R*) analog was found to be highly potent and selective for the δ-opioid receptor (251). The (2*S*,3*S*) analog was much less potent but retained good δ-opioid receptor selectivity. Both of the 2R analogs, (2*R*,3*S*) and (2*R*,3*R*), were found to be essentially inactive at both δ- and μ-opioid receptors (251, data not shown). Subsequently, based on extensive second-messenger assays, it was shown that H(2*S*,3*R*)TMT-Tic-OH was a highly potent and selective (>6000-fold selective for the delta versus mu receptor) inverse agonist at the delta opioid receptor (252), providing an important tool for evaluating the effects of inverse agonists in δ-opioid receptor physiology and pharmacology.

There are a few other approaches that have led to highly potent and δ-opioid receptor-selective peptides. One of the most interesting involves modification of DPDPE at the carboxyl-terminal. Of particular interest was the discovery that modification of the DPen^5 residue with L-Cys or L-Pen (but not D-Cys or D-Pen) and then adding an aromatic residue led to analogs with unusual properties (Table 2.16) (253,254). As can be seen, the Phe^6 compounds are all as potent as or more potent (n*M* to sub-n*M*) in binding affinity than DPDPE, and have much higher selectivity, with the Phe(pBr),Phe^6 analog having an IC_{50} value of 0.20 n*M* and a 21,000-fold selectivity. Even more remarkable is the exceptional potency of these compounds in the MVD (δ-receptor) in

vitro bioassay (data not shown, 253,254) with the $Phe(pF)^4,Phe^6$ analog having an EC_{50} value of 16 pm and a selectivity vs. the GPI (preceptor) of 45,000 (254). The extraordinary potency and selectivity of these compounds can be attributed in part to their greatly enhanced efficacy (255, 256) at &receptors. The structural and biochemical origins of such large increases in the efficacy of signal transduction are still largely unknown, but insight into their origins could provide important clues for the design of more efficacious drugs (257).

Finally, various modifications of the deltorphins, which are naturally δ-opioid receptor-selective ligands (see above), can lead to even more potent and 6-opioid receptor-selective ligands. For example, Sasaki and Chiba (258) prepared a series of C-terminally modified peptide analogs related to the deltorphin, such as the $nBuG^6$- and (RS)$secBuG^6$-constrained analogs in Table 2.16, which are highly potent and highly selective 6-opioid receptor agonists. Misicka et al. (258) showed that use of topographically constrained amino acids in the Phe^3 position, such as the $(2S,3R)\beta$-$MePhe^3$-containing analog in Table 2.16, can provide a potent (IC_{50} = 2.4 nM) and highly selective (>29,000) delta opioid receptor ligand. It also is possible to obtain good binding affinity and δ-opioid receptor selectivity by modifying the deltorphin sequence through intermolecular cyclization such as the [D-Pen^2,L-Pen^5]-deltorphin analog in Table 2.16 (257).

5 THINGS TO COME IN PEPTIDE AND PEPTIDOMIMETIC DESIGN

The determination of the genome of humans and many other animals and living systems has opened up enormous opportunities for peptide and peptidomimetic design, and for the development of peptides and peptidomimetics as drugs, therapeutics, and diagnostic reagents. Most cellular processes and system activities, including those involving diseases, are controlled or modulated by peptide-protein and/or protein-protein interactions. In many protein-protein interactions fairly small structural regions of one or both of the partners are responsible for the biological effects. Thus the development of peptides and peptidomimetics that mimic these interactions with high specificity can be expected to have dramatic effects in their use as drugs of choice, especially given that many peptides have very low toxicities compared to those of current drugs.

Some of these approaches include:

1. Continued development of conformational constraints that can provide new peptide and peptidomimetic motifs for design.
2. Continued development of topographical constraints, especially in conjunction with conformational constraints, will provide new ways of evaluating peptide ligand-receptor/acceptor interactions.
3. Continued development of methods to stabilize peptides against proteolyte degradation and to improve biodistribution.
4. Further development of new and more robust methods for peptide and peptide mimetic delivery and biodistribution.
5. Further development of assay methods, especially methods for evaluating peptide ligands in disease and pathological states as a part of ligand design.
6. Further development of synthetic methods for synthesis of large peptides and proteins that will allow for more widespread use of novel amino acid residues to explore protein function.
7. Continued development of peptide, peptoid, and PNA analogs and derivatives that cross membrane barriers, target intercellular receptor/acceptors, and enhance bioavailability.
8. Development of a large variety of peptide and peptidomimetic-based conjugates for a variety of uses in diagnosis, drug delivery, and treatment of diseases.
9. Continued investigation of novel scaffolds that can mimic peptide secondary structures (ϕ and ψ space), such as a-helices, β-turns, β-sheets; peptide topographical structures (chi space); and, most challenging, that can mimic protein conformational changes such as α-helix to β-sheet transitions.

10. Continued development of computational methods for evaluation of the conformational and dynamic properties of peptides more quickly and more accurately.

It goes without saying that clearly there will be a need for continued development of biophysical methods that allow more rapid and complete analysis of three-dimensional structure and molecular dynamics. Methods for studying the structural, conformational, and dynamic properties in integral membrane proteins such as GPCRs are critical, given that more than 50% of current drugs use these receptors as a way of modulating biological functions including disease. Of course, at the same time biologists will develop better methods for making use of genes, cells, tissues, and organs for peptide and protein drug development. Better models for evaluating disease states are critically needed, and no doubt are high on the agenda of many biologists and medical clinicians.

The ability of chemists and other physical scientists to more effectively collaborate with biological scientists and medical doctors is essential for the future of mankind. Nearly all problems facing us in this area require multidisciplinary approaches and ideas. The ability to effectively communicate and collaborate to solve problems in disease and diagnosis with mutual respect and without any arrogance of any field or discipline is critically required. The very serious ethical and social issues that modern science raises requires all scientists and medical practitioners to take seriously their own responsibility for what they invent and how it will be used in society.

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CHAPTER THREE

Inhibitors of Gastric Acid Secretion

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1 INTRODUCTION

The inhibition of gastric acid secretion is a key therapeutic target for ulcer disease, gastro-esophageal reflux disease (GERD), Zollinger-Ellison syndrome (Z-E), and gastritis. Currently, this is achieved either by blocking the acid-secretory effect of histamine (HA) through the use of HA H_2-receptor antagonists or by irreversibly binding to the H^+/K^+-ATPase with proton-pump inhibitors (PPIs), to prevent the release of H^+ ions from the parietal cell. These pharmacological approaches are effective in alleviating ulcer disease, although disease recurrence rates are high. The discovery that ulcers are linked to *Helicobacterpylori* (H.pylori) infection has led to a new therapeutic approach, in which eradication of the bacteria is the principal target. In addition to ulcer disease, H_2-receptor antagonists and PPIs are still effectively used for alleviating the symptoms of gastritis, Z-E, and GERD. The symptoms associated with each of these conditions are similar; therefore correct diagnosis is essential. Recently, H_2-receptor antagonists have become available without prescription, leading frequently to omission of the critical diagnosis stage, and the self-administration of these medications may mask the symptoms of other diseases.

The aim of this review is to outline the drugs that are currently available and the medicinal chemistry that led to their discovery. The physiological processes associated with acid secretion and the mechanism of action of inhibitors of this process are described. Current research into inhibitors of gastric acid secretion is aimed at producing a reversible PPI or antagonist of receptor-mediated acid secretion. The medicinal chemistry and pharmacology of such approaches are considered.

2 THERAPEUTIC MARKET

The therapy for gastric acid-related gastrointestinal disorders has evolved from nonspecific gastro-protective agents to treatments directed at specific sites regulating the secretion of gastric acid. H_2-receptor antagonists and PPIs are currently the major therapies used to inhibit the production of gastric acid. The discovery that H. pylori infection was highly correlated with the presence of duodenal ulcer and hypersecretion of gastric acid has introduced an additional therapy that targets the eradication of H. pylori. This combination therapy of an antisecretory agent and an antibiotic has been shown to dramatically reduce the number of patients in which ulcer formation recurs (1).The incidence of upper gastrointestinal disorders such as ulcer and GERD shows an element of global variation. For example, in Western countries duodenal ulcers are more common, whereas in Eastern countries gastric ulcers predominate (2).These differences may be attributable to any of a number of factors, including diet and genetic make-up. Therapeutic strategy also differs from the East to West. In Western countries, the conventional therapy for duodenal and gastric ulcers is eradication of H. pylori through the use of PPIs combined with an antibiotic. In Japan, when eradication therapy is used, a cytoprotective agent is also included. Indeed, cytoprotective agents are used in abundance, accounting in 1998 for approximately 50% of all prescribed treatments for ulcer disease. In Japan, unlike the West, H_2-antagonists are commonly used for maintenance therapy, with PPIs consisting of only 5% of the antisecretory therapy market. This results from restrictions imposed by the Japanese government that limit the prescribing of

Table 3.1 Currently Prescribed Antisecretory Medicines in the UK

Generic Name	Trade Name	Originator	Chemical Class
Cimetidine	Dyspamet	Goldshield	H_2-receptor antagonist
	Zita	Eastern	H_2-receptor antagonist
	Tagamet	SmithKline Beecham	H_2-receptor antagonist
Dicyclomine hydrochloride	Merbentyl	Florizel	Anticholinergic
Esomeprazole	Nexium	AstraZeneca	Proton-pump inhibitor
Famotidine	Pepcid	M.S.D.	H_2-receptor antagonist
Hyoscine butylbromide	Buscopan	Boehringer Ingelheim	Anticholinergic
Lansoprazole	Zoton	Wyeth	Proton-pump inhibitor
Nizatidine	Axid	Lily	H_2-receptor antagonist
Omeprazole	Losec	AstraZeneca	Proton-pump inhibitor
Pantoprazole	Protium	Abbott	Proton-pump inhibitor
Ranitidine	Zantac	Glaxo Wellcome	H_2-receptor antagonist
	Pylorid	Glaxo Wellcome	H_2-receptor antagonist
Rabeprazole	Pariet	Eisai/Janssen	Proton-pump inhibitor

PPIs to a maximum of 8 weeks, after which patients are placed on H_2-R antagonists (2).

The incidence of ulcer disease appears to be reducing worldwide, although whether this is attributed to H. pylori eradication therapy is unclear. However, the incidence of GERD is increasing (3) and the control of this disorder is a high profile pharmaceutical target. The increased exposure of the esophageal epithelium to gastric acid has been linked in some cases to morphological changes of the normal squamous epithelium into specialized intestinal-like columnar epithelium. The formation of this precancerous disease state called Barrett's esophagus occurs in 15% of patients with chronic GERD (4). It has been suggested that reducing the exposure of this precancerous tissue to further acid may prevent its subsequent malignant transformation.

Gastric acid-related diseases are common and diverse, and their treatment should be designed to alleviate the symptoms, while keeping the risk of adverse events to a minimum. H_2-receptor antagonists, although not long-lasting inhibitors of gastric acid, may be adequate to treat gastritis, whereas PPIs may be required to totally inhibit gastric acid throughout the day to effectively treat GERD patients. Although PPIs have been available since the mid-1980s, GERD requires a much higher therapeutic dose than that used for ulcer disease. Currently, the long-term safety of high doses of PPIs is unknown.

A review of the leading pharmaceutical products in 1985 placed the H_2-receptor antagonists Tagamet and Zantac as numbers 1 and 2, accounting for $894.2 and $565.8 million, respectively. By 1990, Tagamet had been usurped by Glaxo's direct competitor product Zantac, which obtained the largest amount of sales for 1990 ($2457.9 million) and 1995 ($3523 million). The success of Zantac was ascribed to a major marketing effort that highlighted the antiandrogenic side effects of Tagamet. By 1990, Zantac's growth had slowed as H_2-receptor antagonist therapy was superseded by more potent therapies that inhibited the proton pump, such as Astra's Losec (omeprazole). In 1995 when the patent for Zantac expired, Losec became the third biggest selling pharmaceutical ($2305 million). By 1999 Losec had taken pole position as the largest selling pharmaceutical product of all ($5909 million), within an antiulcerant class of therapeutics, valued at $12.9 billion. The patent for omeprazole expired in 2001 but AstraZeneca has counteracted this by marketing the (*S*)-enantiomer of omeprazole (esomeprazole), which is reported to possess an improved pharmacokinetic profile and potency compared to that of the (*R*)-enantiomer (5). (See Table 3.1 for currently prescribed antisecretory medicines in the UK.)

The decisions regarding the potential for developing other novel inhibitors of gastric acid secretion are likely to be based on therapeutic efficacy, specificity, lack of adverse effects, long-term tolerability, and safety, as well as commercial issues such as market share, patent expiry, and generic competition (6). Self-medication has

Table 3.2 Time-Related Discoveries with Respect to Gastric Acid Secretion

Year	Author/Company	Discovery
1824	William Prout (7)	Identification of HCl in gastric secretion of animals
1826	William Beaumont (182)	Examination of acid secretion in humans
1832	William Brodie (183)	Performed first surgical vagotomy (dog)
1879	Jean Pean (8)	Performed first gastrectomy in humans
1902	Ivan Pavlov (184)	Examination of the neural regulation of gastric secretion
1916	Leon Popielski (185)	Found that HA stimulated gastric secretion in dogs
1921	Andre Rapheal Laterjet and Pierre Wertheimer (186)	Published on the physiological contribution of the vagus to acid secretion and motility
1925	E. McCrea (187)	Suggested that the vagus nerve might be implicated in ulcer disease
1942	Charles Code (188)	Found increased HA-stimulated acid secretion induced ulcer formation in animals
1943	Lester Dragstedt (189)	First truncal vagotomy in humans
1953	Andrew Kay (190)	Developed the HA test (acid secretory response produced by HA in the presence of H_1-receptor blockade)
1960	I. Marks, Wilfred Card (191)	Use of HA test to determine parietal cell mass
1972	James Black (69)	Discovery of H_Z-receptor and H_Z-receptor antagonists
1973	A. Ganser, J. Forte (110)	Discovery of H^+/K^+-ATPase in oxyntic cells of the bullfrog
1976–1982	SmithKline & French	Tagamet launched worldwide (H_Z-receptor antagonist)
1982	Glaxo	Zantac launched (H_2-receptor antagonist)
1988	AstraZeneca	Omeprazole launched (PPI)
1995	Glaxo Wellcome plc	Ranitidine (H_2-receptor antagonist) generic forms available
1995	Takeda-Abbott	Lansoprazole launched (PPI)
1997	Yamanouchi (licensed to Merck & Co.)	Famotidine launched (H_2-receptor antagonist)
1997	Eisai Co. (licensed to Janssen Pharmaceutica)	Rabeprazole launched (PPI)
2001	AstraZenceca	Esomeprazole launched (PPI)

been a major component of gastric acid inhibitory therapies and therefore safety is of prime importance. Both H_2-receptor antagonists and PPIs have been shown to be extremely safe and well-tolerated drugs and future novel inhibitors of gastric acid will have to show a comparable level of safety.

3 ACID SECRETION THROUGH THE AGES

The concept that the stomach secreted acid and digestive enzymes was not fully appreciated until the nineteenth century, although various hypotheses had been mooted since the time of the ancient Greeks. The discovery of gastric acid (7) was the first step to understanding the role of the stomach in digestion and diseases associated with hypersecretion of acid. Modlin (8) provided an excellent review of the discovery, hormones, mechanisms, and drug discovery process linked to gastric acid secretion. Table 3.2 summarizes these stages, while focusing on the changing drug regime used to treat disorders associated with the gastric acid secretion.

4 HOW AND FROM WHERE IS ACID PRODUCED?

4.1 The Stomach

The stomach functions as a reservoir for ingested food and as a primary site of digestion.

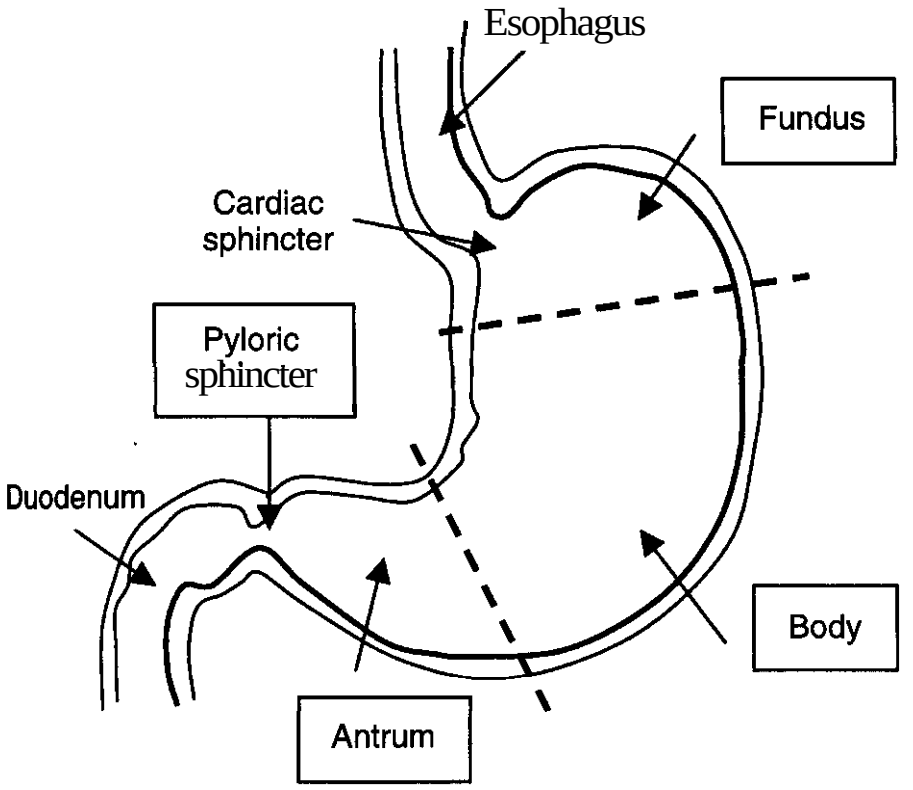


Figure 3.1. Anatomical regions of the stomach.

Once food has entered the stomach, gastric acid and enzymes are released and contraction of the circular and longitudinal muscle of the stomach wall produces thorough mixing of its contents, aiding the breakdown of food before it enters the duodenum. The stomach has both mechano- and chemosensitive receptors within its structure and the presence of food and its various components triggers specific responses within the stomach. The stomach is divided into four anatomical regions: the corpus or body, fundus, antrum, and pylorus (Fig. 3.1). Enzymes and gastric acid are secreted from glandular parts of the stomach located in the antrum and fundic regions. The oxyntic mucosa covers the fundic region and is the site of acid secretion. Mucus cells line the mucosa and invaginations of this epithelial layer form the gastric glands, which consist of specialized crypts that branch into specialized tubular gland structures. Columnar epithelial cells line the crypts, whereas numerous cell types are found in the tubular glands (9).

4.2 Hormones and Neurotransmitters Regulating the Secretion of Gastric Acid

The acid-secreting oxyntic cell, or parietal cell, is the main cell type in the gastric gland. Chief cells (pepsinogen-secreting cells), mucus cells, and D-cells (somatostatin-secreting cells) are also found within gastric glands. Somatostatin, released from fundic D-cells, inhibits the release of acid secretion from the parietal cell. These specialized cells are in close proximity to parasympathic nerve endings and neural inputs are able to regulate hormone release (10). The release of HA from enterochromaffin-like (ECL) cells can also be neurally mediated and subsequently stimulates parietal cells to secrete acid. Vagal nerve stimulation initiates the release of gastrin from antral G-cells, and acetylcholine (Ach) from postganglionic, cholinergic, and muscarinic nerves, which bind to their respective receptors on the ECL-cell to release HA and subsequently acid from parietal cells (Fig. **3.2).**

Ach, HA, and gastrin stimulate acid secretion by activating specific receptors on the basolateral membrane of the parietal cell. Once bound to the respective G-protein-coupled receptor, second-messenger systems are activated. Ach and gastrin activate phospholipase C to catalyze the conversion of membrane-bound phospholipids to diacylglycerol and inositol triphosphate. The release of Ca^{2+} from intracellular stores and the subsequent increase in cytoplasmic Ca^{2+} activates H^+/K^+-ATPase (proton pump). The binding of HA to the H_2-receptor activates adenylate cyclase, resulting in an increase in cAMP, which activates the proton pump (11).

Many peptides and neurotransmitters have been identified as having a direct or indirect effect on the parietal cell. Somatostatin, secre-

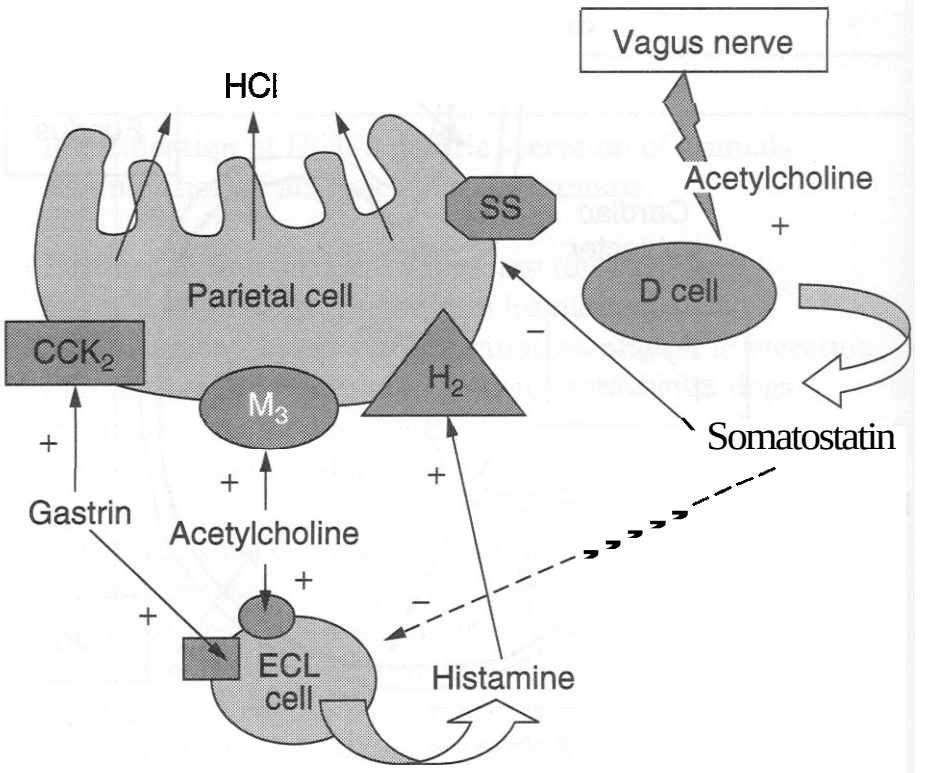


Figure 3.2. Stimulation of acid secretion from the parietal cell.

tin, and prostaglandin E reduce acid secretion either indirectly, by inhibiting the release of gastrin, or directly, by an action at the parietal cell (12). Interleukins, vasoactive intestinal peptide, cholecystokinin (CCK), calcitonin gene-related peptide, oxyntomodulin, neurotensin, adrenaline, and gastric inhibitory polypeptide may inhibit parietal cell secretion indirectly through the release of local somatostatin. Peptide YY, CCK, and 5-HT are thought to influence acid secretion by modulating neural tone to the stomach. The release of nitric oxide release may also inhibit gastric acid secretion.

In addition to mucus from goblet cells, bicarbonate ions are secreted from the stomach to protect the gastric mucosa from gastric acid. Bicarbonate secretion occurs when the luminal pH is less than 2 and its release is regulated both neuronally and hormonally.

5 PHYSIOLOGY OF GASTRIC ACID SECRETION

Gastric acid secretion is stimulated by food-related signals that stimulate the release of acid from specialized cells within the stomach. This secretory process has been divided into three phases: cephalic, gastric, and intestinal, with each phase leading to different amounts of acid being secreted (Table 3.3).

Cephalic Phase. The sight, smell, taste, and sensation of swallowing food causes central stimulation of the vagus nerve leading to Ach release from synapses within the fundic and antral regions of the stomach. Direct stimulation of the parietal cells within the fundus and, to a lesser degree, the indirect stimulation of HA release, through vagally stimulated gastrin release from G-cells within the antrum, causes the parietal cells to secrete acid into the stomach.

Gastric Phase. Gastrin is the main mediator of acid secretion. Acid secretion occurs in response to both the presence of nutrients and the physical distension produced by food entering the stomach. Distension-induced gastric acid secretion, relative to the total amount of acid released, is species dependent (human = 20%; dog = 50%; rat = 38%). The chemical constituents of a meal are the strongest stimulant of gastrin release and acid output during

Table 3.3 Relative Acid Secretory Response to Food

Phase of Acid Secretion	Percentage of Total Acid Secretion
Cephalic	50
Gastric	
Distension	20
Chemical	25
Intestinal	5

the gastric phase, with peptides and amino acids being greater stimulants of gastrin than proteins, carbohydrates, and fats.

Intestinal Phase. Only a relatively small amount of acid is secreted because of the numerous inhibitory mechanisms stimulated by the presence of nutrients within the intestinal lumen.

Inhibition of gastric acid secretion also occurs during the cephalic, gastric, and intestinal phases. In the cephalic phase, the release of various neuropeptides may contribute to the inhibition of gastric acid secretion. Central injection of neuropeptide Y (NPY), corticotrophin-releasing factor, bombesin, calcitonin, calcitonin gene-related peptide, neurotensin, interleukin 1, and prostaglandins have all been shown to inhibit gastric acid secretion. The exact mode of action of these peptides remains to be elucidated, although they inhibit gastric acid secretion through vagal and sympathetic nerves. The hypothalamus appears to be an important site of action of many peptide inhibitors of acid secretion. Of the peptides studied so far, only NPY exerts both stimulatory and inhibitory effects on acid secretion when injected into different hypothalamic sites. In the gastric phase, increasing gastric acidity initiates a mechanism that turns off gastric acid secretion. Once the acidity of the lumen has reached pH 2, gastrin release is inhibited and therefore acid secretion is reduced. Stimulation of somatostatin release from D-cells in the antrum of the stomach inhibits the release of gastrin from G-cells and thus reduces gastric acid secretion. In patients with a duodenal ulcer this inhibitory process is less efficient, especially when intraluminal pH falls below 3. Eysselein et al. (13) also demonstrated that patients with a duodenal ulcer exhibited higher rates of acid secretion yet a diminished capacity for inhibition of acid secretion when low concentrations of peptone were instilled intragastrically.

In the intestinal phase, inhibition of acid secretion is produced by the presence of fat, acid, or hyperosmolar solutions within the intestinal lumen. Fat, the most potent inhibitor, was proposed to cause the release of inhibitory substances from the intestine, and although no substances were formally identified, they were termed enterogastrones. Little is known about the neural pathways that participate in the fat-induced inhibitory reflex, although vagotomy does appear to reduce the inhibitory effect of intestinal fat. This response is attributed to the altered sensitivity of parietal cells to Ach in the absence of vagal tone. The release of CCK and somatostatin, in response to intestinal acid, inhibits gastric acid secretion.

6 DISORDERS ASSOCIATED WITH ELEVATED SECRETION OF GASTRIC ACID

6.1 Ulcer Disease

Peptic ulcers arise because of an imbalance of acid-secretory mechanisms and mucosal-protective factors, and the rationale for their treatment is aimed at restoring that balance. The loss of balance between acid secretion and mucosal-protective factors varies among peptic ulcer types. In type I ulcers, which occur high in the stomach, acid hypersecretion is not necessarily evident, suggesting the importance of impaired mucosal-protective factors in this clinical setting. Type II ulcers, in contrast, include gastric ulcers, distal antral (prepyloric) ulcers, and duodenal ulcers. They are associated with acid hypersecretion and the impaired negative feedback effects of acidification on gastrin release and on continued acid secretion. The causes of gastric ulcers include H. pylori infection, nonsteroidal anti-inflammatory drugs (NSAIDs), environmental factors, and malignancy. Duodenal ulcers can result from hypersecretion of gastrin, which is assessed by evaluating fasting gastrin levels in patients unresponsive to other therapies for duodenal ulcers.

6.1.1 NSAID-Associated Ulcer. NSAIDs inhibit the production of prostaglandins, prostacyclins, and thromboxanes from arachidonic acid by covalently modifying the enzyme cyclooxygenase (COX) and irreversibly inhibiting the ability of arachidonic acid to bind to the active site on the enzyme. Chronic administration of NSAIDs has been linked to ulcer disease, although there is no evidence that they are the direct cause of ulcer formation. In patients already diagnosed with ulcer disease, chronic administration of NSAIDs was associated with a fourfold increase in the risk of ul-

cer-associated complications (14). These complications, however, may remain undetected because of the reduction in pain produced by the inhibition of endogenous prostaglandins. Inhibition of prostaglandin-derived gastroprotective properties such as gastric blood flow and mucus production increases the exposure of the mucosa to toxic agents. In an attempt to restore the gastro-protective properties of the prostaglandins, the synthetic prostaglandin analog misoprostol may be coadministered. However, therapies targeted solely at the suppression of acid have been shown to be better tolerated (15). Two isoforms of COX have been identified: COX-1, a constitutively expressed enzyme is found in many tissues, whereas COX-2, an inducible enzyme, is predominantly expressed at sites of inflammation. Selective COX-2 inhibitors would therefore be anticipated to reduce prostaglandin-dependent inflammation, while leaving protective gastric mucosal prostaglandin synthesis intact. Ulcer healing involves the formation of granulation tissue, reepithelialization, scarring, and contraction of the ulcer base. Specific inhibitors of COX-2, however, have been reported to delay the healing of erosions in mice and rats (16). Although COX-1 appears to predominate in human gastric mucosa, both COX-1 and COX-2 are found at the rim of gastric ulcers. Myofibroblasts, which are considered to play an important part in ulcer healing, express COX-2 and synthesize prostaglandins when exposed to inflammatory stimuli. Inhibition of COX-2 may thus retard ulcer healing in human gastric mucosa (17).

Thromboxane, a major product of arachidonate metabolism, stimulates platelet aggregation and vasoconstriction. The chronic administration of low doses of aspirin, an NSAID, as a prophylactic antithrombotic agent, for patients with a history of heart disease, has been shown to double the incidence of acute biopsy-induced bleeding in patients (18). The antihemostatic properties of aspirin on platelets may be linked with the high number of ulcer hemorrhages occurring in this patient subgroup (19). Aspirin inhibits both COX-1 and COX-2 and as COX-2 selective inhibitors do not appear to alter platelet function they may act as a suitable antithrombotic agents for high risk cardiovascular disease patients with ulcer disease. Celecoxib, a selective COX-2 inhibitor, appears to produce much less gastroduodenal injury than standard NSAIDs. In several animal studies, however, selective COX-2 inhibitors have been shown to interfere with healing of ulcers and to exacerbate inflammation (20). Current clinical trials with COX-2 inhibitors have not directly examined hemorrhage and healing of ulcers in patients. However, given that a high percentage of patients with NSAID-related gastric ulcers do not experience disease-associated symptoms, the use of a COX-2 inhibitor, which might worsen or delay the healing of ulcers in a high risk patient population, might be dangerous.

6.2 Zollinger-Ellison Syndrome

In this disease, a non-beta-cell tumor of the pancreatic islets may produce gastrin in a quantity sufficient to stimulate secretion of gastric acid to life-threatening levels. The introduction of H_2-receptor antagonists has meant that total gastrectomy is no longer necessary in treating these patients. Acid secretion was initially controlled by high doses of cimetidine or ranitidine and in some cases additional surgery was carried out to remove resectable tumors and reduce gastric acid secretion by proximal gastric vagotomy. The development of H^+/K^+-ATPase inhibitors, such as omeprazole, has enabled adequate inhibition of gastrin-stimulated acid secretion to be achieved for longer periods. Once- or twice-daily dosing with omeprazole produces a profound inhibition of gastric acid secretion; therefore a vagotomy is no longer necessary.

Gastric ECL-cell carcinoids are rare events that have been described in association with pernicious anemia and Zollinger-Ellison syndrome. They usually relate to marked hypergastrinemia, atrophic gastritis, or a genetic defect, rather than the presence or absence of acid. Regression or disappearance of ECL-cell carcinoids may occur either spontaneously or after removal of gastrin. H_2-receptor therapy may result in up to a twofold increase in plasma gastrin, although no endocrine cell hyperplasia has been reported. Omeprazole causes a two- to fourfold increase in plasma gastrin and this results in hyperplasia in 7% of patients (21).

6.3 Helicobacter Pylori (H. *Pylori*)

H. pylori infection occurs in approximately 40% of the population over 40 years of age and most patients with peptic ulcer disease are infected with H. pylori. Because ulcers recur in patients who have undergone "successful" H. *pylori* eradication therapy, infection may not always be causative for the disease. Less than 5% of ulcer patients are H. pylori negative and in H. pylori positive patients only 10% of ulcers recur after eradicating the infection. Likely causes of ulcer recurrence are considered to be reinfection, the use of ulcerogenic drugs, and persistent gastric hypersecretion. However, true reinfection is rare and it is more likely that the most common cause of H. pylori recurrence is attributed to inadequate eradication. The significant reduction in H. pylori density produced by a combination of antibiotic and antisecretory therapy may reduce the levels of infection to below detectable levels (22). Rapid urease tests for H. pylori have a sensitivity of only 80–90%. Therefore histological examination is also used to confirm an initial noninvasive test result. Whole blood or serum antibody testing are rapid, accurate, and cost-effective tests for establishing *H.* pylori status in rapid urease test-negative patients. These less invasive techniques could be used in place of endoscopy when the patient has not previously been treated for H. pylori (23).

In duodenal ulcer (DU) patients, H. pylori infection causes inflammation of the antral gastric mucosa, which is associated with an elevation in gastric acid secretion. Gastritis increases the release of the acid-stimulating antral hormone gastrin and reduces the expression of the inhibitory peptide somatostatin. Bacterial products and inflammatory cytokines may produce these changes in endocrine function. Gastritis involving the corpus tends to decrease acid secretion, given that bacterial products and cytokines inhibit parietal cells. After eradication, gastrin levels are restored to normal levels.

Gastric metaplasia is found in 90% of H. pylori-infected DU patients and about 60% of non-DU patients with increased acid secretory levels. The induction of gastric acid causes severe inflammation and increases the areas of metaplasia within the gastric mucosa. Combined eradication and acid-suppression therapy produces greater reduction in gastric metaplasia than either treatment alone, indicating that both acid secretion and *H. pylori* infection contribute to ulcer formation. A number of putative virulence factors for *H. pylori* have been identified, including cagA, vacA, and iceA. Although disease-specific associations have been claimed, there are now sufficient data to state that none of these factors is specific for any disease. The presence of a functional cagA pathogenicity island, whose genes produce proinflammatory cytokines, increases mucosal IL-8 and inflammation, but is not predictive of the future development of a disease. The hypothesis that iceA has disease specificity has not been confirmed and vacA genotyping has also failed to predict the disease identification. Virulence appears to be a host-dependent factor. The pattern of gastritis is frequently used to indicate the different H. pylori-related diseases. The primary factors responsible for the different patterns of gastritis are suggested to be associated with environmental factors, with the H. pylori strain playing a minor role (24).

H. pylori infection is now proven to be a risk factor for gastric cancer and the organism was classified as a Group 1 carcinogen by the International Agency for Research on Cancer sponsored by the World Health Organization in 1994 (25). This has strengthened the case for H. pylori eradication to prevent gastric cancer. However, there are growing concerns that eradication may cause harm. In developed countries. an increase in the rate of cancers arising near the gastroesophageal junction may be linked to the disappearance of H. pylori. The conundrum is to either eradicate to avoid cancer of the distal stomach or leave it and hence avoid cancer of the proximal stomach or distal esophagus. More research is required to determine which patients should receive eradication therapy (26).

An increased incidence of GERD has been linked to the decrease in H. pylori infection produced by the current trend of eradication therapy. H. pylori may have a protective role by either reducing achlorhydria induced by PPIs or by increasing the activity of PPIs by increasing the formation of the active metab-

olite. The antisecretory effect of PPIs seems to depend on the presence of the infection because eradication of H. pylori has negative consequences on the efficacy of antisecretory drugs (27). Several hypotheses have been suggested to account for the reduction in efficacy of PPIs in H. pylori-negative patients. Hypersecretory disorders may be associated with increased expression of proton pumps on the membrane of the parietal cell; therefore PPIs would appear to be more effective in DU patients than in healthy controls. Other authors have suggested that H. pylori infection may inhibit proton-pump synthesis in the parietal cell because the amount of H^+/K^+-ATPase mRNA in the fundic gland mucosa was significantly increased in patients where H. pylori had been eradicated (28). If a decrease in the number of active proton pumps was the explanation for the higher effectiveness of PPIs in H. pylori-positive subjects, there should also be a lower acid output and consequently higher gastric pH in H. pylori-positive subjects during baseline recordings. However, under basal conditions, similar basal pH values are recorded both before and after the cure of infection. The most likely cause for the decreased effectiveness of PPIs is the production of ammonia by H. pylori in infected patients. H_2-receptor antagonists do not inhibit acid secretion to the same degree as do PPIs; thus the small amount of ammonia produced by H. *pylori* would be unlikely to significantly affect the pH. However, the high gastric pH produced by PPI therapy would be influenced by the reduction in ammonia produced by eradication of H. pylori. The reduction in H. pylori-related gastritis produced during omeprazole therapy has also been suggested to contribute to the reduced activity of omeprazole after eradication. However, histological improvement of gastritis after a cure of H. pylori is a slow process and the effect of reducing the effectiveness of omeprazole is rapid.

Although the usefulness of H. pylori eradication is still controversial, a randomized controlled study has shown that patients, in whom the organism has been eradicated, benefit with regard to quality of life and there is also a reduction in financial costs to the health system (22).

6.4 Reflux Esophagitis

Reflux esophagitis is a disorder of the defense mechanisms at the esophageal junction, which is caused by regurgitation of the gastric contents, especially of gastric acid. GERD is associated with decreased gastric emptying and/or increased incidence of transient lower esophageal relaxation (T-LESR). Smoking and obesity are factors that increase the incidence of GERD-like symptoms such as heartburn, belching, and bloating. Reflux has been observed in humans and dogs but not in rodents. Reflux can be subdivided into T-LESR. free reflux, and stress reflux. H. pylori infection does not necessarily correlate with GERD, although a reduction in acid secretion reduces the chances of reflux. The effectiveness of PPIs is reduced in the absence of H. pylori infection; therefore the majority of patients with GERD require >20 mg/day to provide symptom relief and to heal the esophagitis produced by gastric acid reflux. This dose is much higher than that required to inhibit acid secretion associated with DU disease. If PPI therapy is stopped, then GERD patients appear to produce greater amounts of gastric acid and their reflux is potentiated. It has been reported that if the prevalence of H. pylori continues to decline, then PPI consumption will continue to increase for GERD (29). Ideally, future therapy for GERD should be independent of H. pylori status and additionally be devoid of the acid-rebound effect produced with PPI therapy.

GERD causes significant discomfort but is not in itself life threatening. The incidence of adenocarcinoma of the esophagus is increasing in the United States (30), with almost 100% of cases occurring in patients with Barrett's esophagus, a condition in which mucin-secreting, metaplastic, columnar epithelium replaces the normal squamous epithelium of the esophagus (31). There is substantial evidence that GERD may increase the incidence of Barrett's esophagus. Fass reported that the length of Barrett's esophagus tissue correlated with the duration of esophageal acid exposure (32). The use of antireflux medication in patients with GERD leads to an improvement or alleviation of symptoms and healing of mucosal inflammation. Antisecre-

tory agents have been used to reduce the exposure of the esophagus to acid in Barrett's esophagus. However, even high doses of PPIs have not resulted in regression of Barrett's mucosa. Not all GERD patients develop Barrett's esophagus and the causative agents for this progression to a precancerous state have not been fully determined. The age of onset, duration of symptoms, and complications of GERD are markers of an increased risk of Barrett's esophagus. In additon, the longer the region of Barrett's mucosa, the higher the risk for the development of dysplasia (33).

7 TEST ASSAYS FOR STUDYING GASTRIC ACID INHIBITORS

Gastric acid secretion occurs through receptor-mediated or enzyme-mediated processes. Drug dissociation constants can be determined from *in vitro* bioassays, allowing affinity estimate comparisons to be made between compounds. Functional evaluation can be made using both *in vitro* and *in vivo* techniques, with the latter models being able to provide information on the pharmacokinetics of drug candidates. Technological progress made since the discovery of both H_2-receptor antagonists and PPIs means that some methods used in the original publications can now be replaced by cloned receptor systems.

7.1 *In Vitro*

Radioligand binding assays can be used to determine the affinity of H_2-receptor antagonists through the use of either cloned human H_2-receptors expressed in cultured cells or native H_2-receptors in guinea pig cortex membrane homogenates (34). Functional measurements (e.g., HA-stimulated cAMP production) can also be made in cell lines expressing either native or cloned receptors (35).

The effect of gastric PPIs on H^+/K^+-ATPase activity (ATP-induced phosphorylation) can be studied *in vitro* with partially purified H^+/K^+-ATPase preparations from pig gastric mucosa **(36).** Given that the enzyme assay needs to be performed at neutral pH, this system has been most effectively used to study the mechanism of action of PPIs rather than the structure-activity relationship (SAR) of inhibitors. PPIs are prodrugs, requiring an acid environment for conversion to the active molecule (37), and it is the most chemically labile molecules that appear particularly active in this assay. Thus, misleading results may be produced with regard to prediction of clinical activity.

Gastric membrane vesicle preparations enriched with H^+/K^+-ATPase have also been used to examine PPIs. The inhibition of hydrogen ion transport by PPIs is measured by use of the initial rate of acridine orange quenching as an index of acidification. However, steady-state acidification, as measured by aminopyrine accumulation, is inhibited with greater potency and this is consistent with the accumulation of PPIs in the intravesicular acidic space (38).

To study the effects of PPIs a more "physiological" situation is often used. Acid secretion *in vitro* has been studied by use of isolated parietal cells from guinea pigs (39), dogs (40), and rabbits. Rabbit parietal cells and chief cells, separated by density gradient centrifugation, copurify with H^+/K^+-ATPase. Stimulants of acid secretion cause the accumulation of radiolabeled aminopyrine, a weak base, into the acid compartment of the parietal cell. At pH 7 it can pass freely through biological membranes in its un-ionized form but becomes trapped within the secretory cannaliculi because of ionization. The [^{14}C]aminopyrine accumulation technique has been widely used to study structure-activity relationships of gastric acid inhibitors as well as to separate the inhibitory effect of PPIs from that of receptor antagonists. H_2-R antagonists inhibit HA-stimulated acid secretion and aminopyrine uptake, whereas omeprazole, a PPI, inhibits both HA-stimulated and db-cAMP-stimulated acid secretion (41). [^{14}C]Aminopyrine accumulation has also been used to examine acid secretion in intact gastric glands from rabbits (42) and humans (43).

A more recent cell-based *in vitro* assay involves the use of the microphysiometer, a sensitive extracellular pH sensor, which has been used to measure luminal (or apical) H^+ secretion and basolateral release of OH– as well as liberation of acidic metabolites in rabbit gastric glands. Adenosine 3′,5′-cyclic monophosphate stimulation produced a biphasic change

in the extracellular acidification rate (EAR) that is attributed to an initial excess of basolaterally released OH^- followed by delayed luminal efflux of simultaneously produced H^+. The elevated EAR at steady state reflected liberation of metabolic acid attributed to H^+/K^+-ATPase enzymatic activity. Both basolateral OH^- release and steady-state EAR were inhibited by the H^+/K^+-ATPase inactivators omeprazole (irreversible PPI) and SCH-28080 (reversible PPI) (44).

In vitro assays used to examine gastric acid secretion also include the use of isolated whole stomach preparations from the rat (45), mouse (46), and guinea pig (47). Whole, or part, stomach preparations are placed in an organ bath containing a buffered solution. The stomach is perfused with an unbuffered solution and changes in luminal pH are monitored in response to stimulation of acid secretion. Acid secretion is an oxygen-dependent process, so this technique is limited to young animals (45) with thin stomach walls or stomachs where the outer muscle layer has been removed. In older animals perfusion of the vascular bed with oxygenated buffer is used to maintain tissue viability (48). In these models, acid secretion can be produced through either direct or indirect stimulation of the parietal cells using HA, gastrin, and Ach. It is also possible to examine the effect of drugs on neurally mediated acid secretion by use of electrical-field stimulation (49).

7.2 *In Vivo*

Rats and dogs are generally used to examine gastric acid secretion *in vivo*. The anesthetized rat model most commonly used is the "Ghosh and Schild" rat (50). In this model, intragastric pH is monitored by means of the perfusion of an unbuffered solution through the stomach, passing over a pH electrode upon its exit. Drugs are generally administered through intravenous, subcutaneous, or intraperitoneal routes. Oral administration would require the flow of unbuffered solution to be temporarily stopped, preventing the continuous measurement of intragastric pH. The pylorus-ligated rat model, often termed the "Shay rat" (51), combines drug administration to the conscious animal with gastric acid collection under anesthesia (52). In this model, inhibitors of acid secretion are administered to the conscious rat through the oral, intraperitoneal (i.p.), or subcutaneous (s.c.) routes. After a specified time, during which gastric emptying should have occurred, the rat is anesthetized and the pyloric sphincter ligated to prevent further gastric emptying. Acid secretion is then stimulated by HA, pentagastrin, or Ach (usually s.c. or i.p.) for a fixed period of time, the animal euthanized, the stomach excised, and the pH of its contents determined. Both the Ghosh/Schild and Shay rat (53, 54) models have been used in the evaluation of HA H_2 and CCK_2/gastrin-receptor antagonists, and PPIs.

Conscious rat and dog models involve the use of surgically modified animals with a chronic gastric fistula (55), enabling gastric secretions to be collected and the pH determined by titration. The effect of drugs administered either orally or i.v. is examined on basal and stimulated acid secretion. For oral drug administration, the fistula must be closed for a period of time to allow gastric emptying. The surgical addition of an intraduodenal fistula allows drugs to be administered "orally," while being able to continuously monitor gastric pH. The brain is able to regulate the release of acid-secreting hormones, so a surgical modification of the gastric fistula was developed, the Heidenhain pouch model (56). In this model, part of the stomach is divided from the main body of the organ and within the pouch the nerves are cut, so that the pouch is isolated from the brain, but retains the local blood supply of the stomach. Hence, the pouch will secrete digestive juice in response to a circulating hormone (gastrin) produced by the main body of the stomach, but not in response to the extra stimulation from the brain. Because the pouch is separate from the body of the stomach, drugs and food can be provided, whereas gastric juice, free of food contamination, can be collected from the fistula (57).

Significant species differences have been demonstrated in gastric physiology and, whereas studies in primates are rare, their physiology has been shown to be similar to that of humans (58). Primates are trained to sit in a chair and, as in human studies, samples of gastric secretions can be taken through

a nasogastric tube for pH analysis. Unlike the dog, but in agreement with human studies, primates produce significant basal and stimulated acid secretion and the effect of inhibitors of gastric acid secretion can be examined (59).

The more recent use of microdialysis probes, implanted in the submucosa of the acid-producing part of the rat stomach, enables gastric acid secretion to be examined in conscious animals without surgical modification (60).

8 RECEPTOR-MEDIATED PROCESSES THAT REGULATE GASTRIC ACID SECRETION

Gastric acid secretion from oxyntic cells is produced in response to stimulation of second-messenger systems that trigger proton-pump activation. Vagal nerve endings in the stomach are stimulated by physicochemical factors causing the release of Ach. Neurotransmitter release from nerve endings that are in close proximity to ECL and G-cells stimulates the release of HA and gastrin, respectively. Each of these processes can be disrupted to reduce the amount gastric acid secretion. The ability to selectively and effectively inhibit gastric acid secretion determines the usefulness of such therapies.

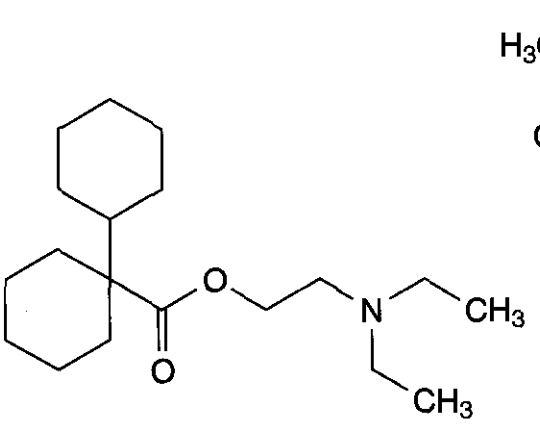
8.1 Ach-Muscarinic-Receptor-Mediated Acid Secretion

Ach, released after vagal stimulation, binds to muscarinic receptors present on both the acid-secreting parietal cell and the HA-secreting ECL-like cell (Fig. 3.3). Muscarinic receptors stimulate the secretion of acid, pepsinogen, and mucus in the gastric mucosa. Autoradiographic techniques have shown that the M_1 receptor is overexpressed in DU (61); therefore a selective M_3-receptor antagonist may provide a useful antisecretory therapy. Muscarinic receptors are currently subdivided into M_1, M_2, M_3, M_4, and M_5 (62). The receptors on rat and rabbit parietal cells and human and porcine gastric mucosa are of the M_1 subtype. Pfeiffer (63) and Kajimura (64) found that only the gene for the M_3 receptor subtype was expressed in rabbit parietal cells. However, the M_3-receptor is also associated with smooth muscle contraction and this may lead to unfavorable side effects (65). (See Fig. 3.2.)

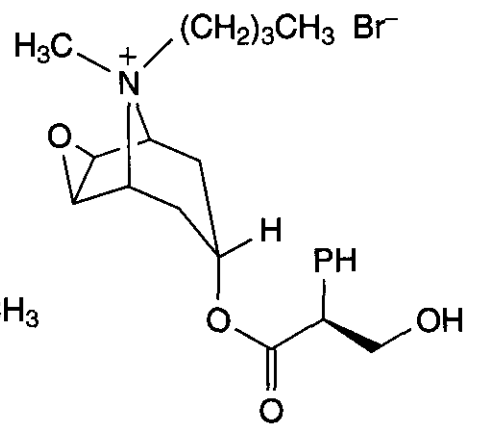
8.1.1 Structures and SAR of Anticholinergic Agents. HA H_2-receptor antagonists and PPIs have largely superseded the use of muscarinic anticholinergic agents in the control of acid secretion. Muscarinic receptor heterogeneity and wide tissue distribution, coupled with the low subtype specificity of the early anticholinergics such as propantheline, dicyclomine, and hyoscine bromide, led to their being associated with parasympathetic side effects such as dry mouth, dizziness, constipation, and blurred vision. Muscarinic antagonists, selective for receptors located on the parietal cells, are considered more suitable candidates for anticholinergic agents in the control of acid secretion. Moreover, the overexpression of the M_3 subtype in DU patients (61) has linked blockade of this receptor subtype to decreased pain through reduced duodenal motility. Pirenzepine, which in addition to helping distinguish muscarinic receptor subtypes, was developed as an antisecretory agent mainly on the basis of its preferential inhibition of receptors on the intramural ganglia of the stomach wall. Compared to the H_2 antagonist ranitidine, pirenzepine alone was less effective in the inhibition of acid secretion. The more potent derivative telenzepine improved healing rates (66); however, the increased incidence of side effects with the antimuscarinic agents, has failed to dislodge the H_2 antagonists as the preferred antisecretory therapy. The effectiveness of antimuscarinic therapy may be limited, given that pirenzepine, a muscarinic antagonist, inhibited peptone meal-stimulated acid secretion by 39%, whereas ranitidine, an H_2-receptor antagonist, produced 69% inhibition. In combination, however, the acid-secretory response was almost completely inhibited (99%) (67). Although this combination therapy is feasible, it is unlikely to be used because of the side effects associated with muscarinic antagonists.

8.2 Histamine H_2-Receptor–Mediated Acid Secretion

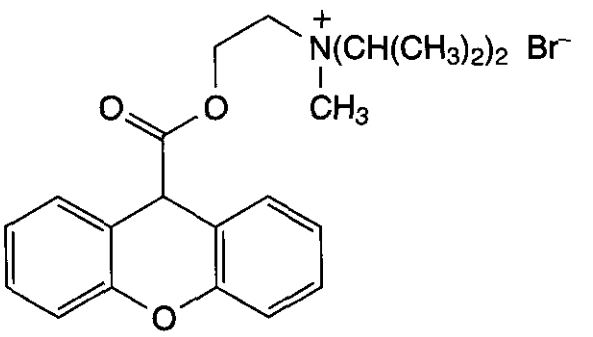
Leon Popielski first identified HA as a major stimulant of gastric acid secretion in 1916.



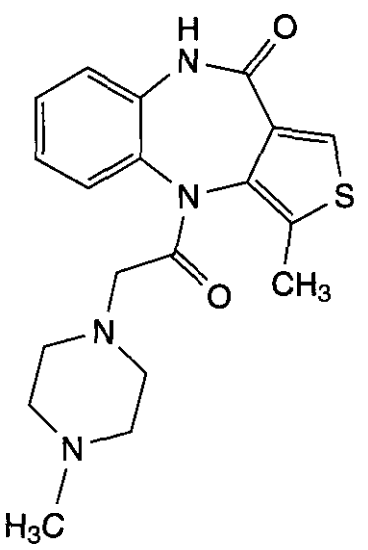
Dicyclomine



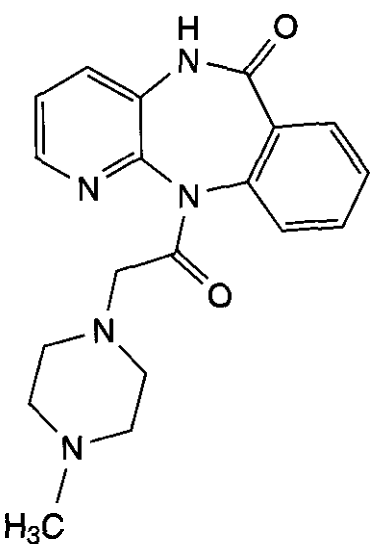
Hyoscine butyl bromide



Propantheline bromide



Telenzepine



Pirenzepine

Figure 3.3. Ach-receptor antagonists used in the control of acid secretion.

However, it was not until the synthesis of HA H_2-receptor antagonists that the role of HA in parietal cell stimulation was fully recognized (68, 69). An enlightening account of the H_2-receptor antagonist discovery program at SmithKline & French is provided in a review by Duncan and Parsons (70). Originally, HA was considered to be the final mediator of secretagogue-stimulated gastric acid secretion. However, although species dependent, the hormone gastrin is also able to stimulate gastric acid secretion, both directly (through the oxyntic cell) and indirectly (through the release of HA from the ECL cell). H_2-receptor antagonists inhibit both gastrin- and Ach-stimulated gastric acid secretion, indicating that the secretion of gastric acid can be mediated by the indirect release of HA (71).

HA is synthesized from dietary histidine by the enzyme histidine decarboxylase (HDC). High levels of HDC activity are found in the stomach, either in mucosal mast cells or in ECL cells. Gastrin, Ach, and adrenaline interact with their respective receptors on the ECL-cell to trigger the release of HA from storage granules located in ECL-like cells. HA, by activation of the H_2-receptor and subsequent elevation of cAMP levels, stimulates the parietal cells to secrete acid.

8.2.1 Histamine Receptor Classification. HA receptors are currently subdivided into H_1, H_2, H_3, and H,. Gastric acid secretion from the parietal cells is strongly stimulated by HA, an action that is exerted through H_2-receptors and'is not inhibited by the classic anti-HAS, which act at H_1-receptors. H_2-receptors are blocked by selective H_2-receptor antagonists, such as burimamide, cimetidine, ranitidine, and famotidine. H_3- and H_4-receptors are relatively newly discovered and their physiological relevance is still being delineated. H_3-receptors regulate neurotransmitter release from nerve terminals and, with regard to acid secretion, H_3-receptor stimulation has been shown to inhibit pentagastrin-stimulated gastric acid secretion in conscious Heidenhain pouch dogs (72). The recently

cloned H_4-receptors (73–76) have so far only been identified in bone marrow and eosinophils, where their role is believed to be in the regulation of the immune response.

8.2.2 H_2-Receptor Antagonists. H_2-receptors and the prototype H_Z-receptor antagonist burimamide were identified in a single paper by Black et al. (69). Burimamide was reported to inhibit both HA- and pentagastrin-stimulated gastric acid secretion in rats, dogs, cats, and humans. The potency of burimamide at inhibiting gastric acid secretion far exceeded that produced by anticholinergic drugs and was devoid of apparent side effects. Burimamide, however, had poor oral bioavailability and was subsequently replaced by metiamide, which was 10-fold more potent and its activity could be detected after oral administration (68).

Metiamide (400 mg) completely abolished gastric acid secretion, whereas the maximum tolerated dose of an anticholinergic, isopropamide, produced only 35% inhibition of food-stimulated acid secretion. In combination, however, submaximal doses of the two antagonists provided a potent antisecretory combination (77). Metiamide went into clinical trial in 1972 but was withdrawn because a small number of patients developed agranulocytosis during treatment. Initially, it was not certain whether this toxicological problem was caused by H_2-receptor blockade affecting maturation of the bone marrow, or whether the thiourea moiety in the metiamide side chain had a direct toxic effect on the cells (70). Cimetidine, a molecule not containing a thiourea group, was the third H_Z-receptor antagonist to be tested in humans. Cimetidine was similar to metiamide in its pharmacological profile, but did not cause the agranulocytosis observed with metiamide. Cimetidine was marketed in the UK in 1976 under the trade name of Tagamet.

Cimetidine therapy led to a revolution in the treatment of acid-peptic disorders, with oral therapy being able to reduce the necessity of surgical procedures. The discovery of this landmark molecule caused a number of pharmaceutical companies to be alerted to this therapeutic area and target the discovery of a "better" H_Z-receptor antagonist. Ranitidine was introduced by Glaxo in 1981 as a more potent drug with a superior side-effect profile and, backed by a skillful marketing campaign, superseded cimetidine as the world's most successful drug. Famotidine, synthesized by Yamanouchi, was the third antagonist to be registered and this remains the most potent H_2 antagonist available for clinical use. Two other H_Z-antagonists, nizatidine and roxatidine, are also commercially available, but they seem to offer no improvement over the other agents (6).

The clinically available H_2-receptor antagonists are extremely safe. Toxicity problems, however, were detected during the development of other H_Z-antagonists such as tiotidine, which induced glandular dilation and hyperplasia of the gastric mucosa. This observation highlighted the necessity to examine the effects of antisecretory drugs on the whole stomach, and later studies, either with H_Z-antagonists or proton-pump inhibitors, produced similar hyperplastic properties when administered for prolonged periods.

8.2.3 Structures and SAR of H_2-Receptor Antagonists. The success of cimetidine in the control of gastric acid secretion in humans was swiftly followed by a considerable effort to obtain other H_Z-receptor antagonists having a superior profile, either in terms of potency or duration of action. This process led to a wealth of medicinal chemistry, which has been the subject of a number of extensive and detailed reviews (78, 79) and to the evaluation of many new compounds in clinical studies. The SAR evolved from the imidazole-based antagonists metiamide and cimetidine, and is illustrated by representative compounds shown in Fig. 3.5. This led ultimately to the H_Z-receptor antagonists currently on'the market and shown in Fig. 3.4.

Although the H_Z-receptor antagonists span a diverse range of structures, they can for the most part be described by a general pharmacophore, that is, an aromatic or heterocyclic ring, attached by a flexible, preferably thioether chain, to a polar hydrogen-bonding group. However, the breadth of chemical functionality represented by each of these elements is testament to the rational approach successfully used in their design. Thus, it was largely by careful consideration of properties,

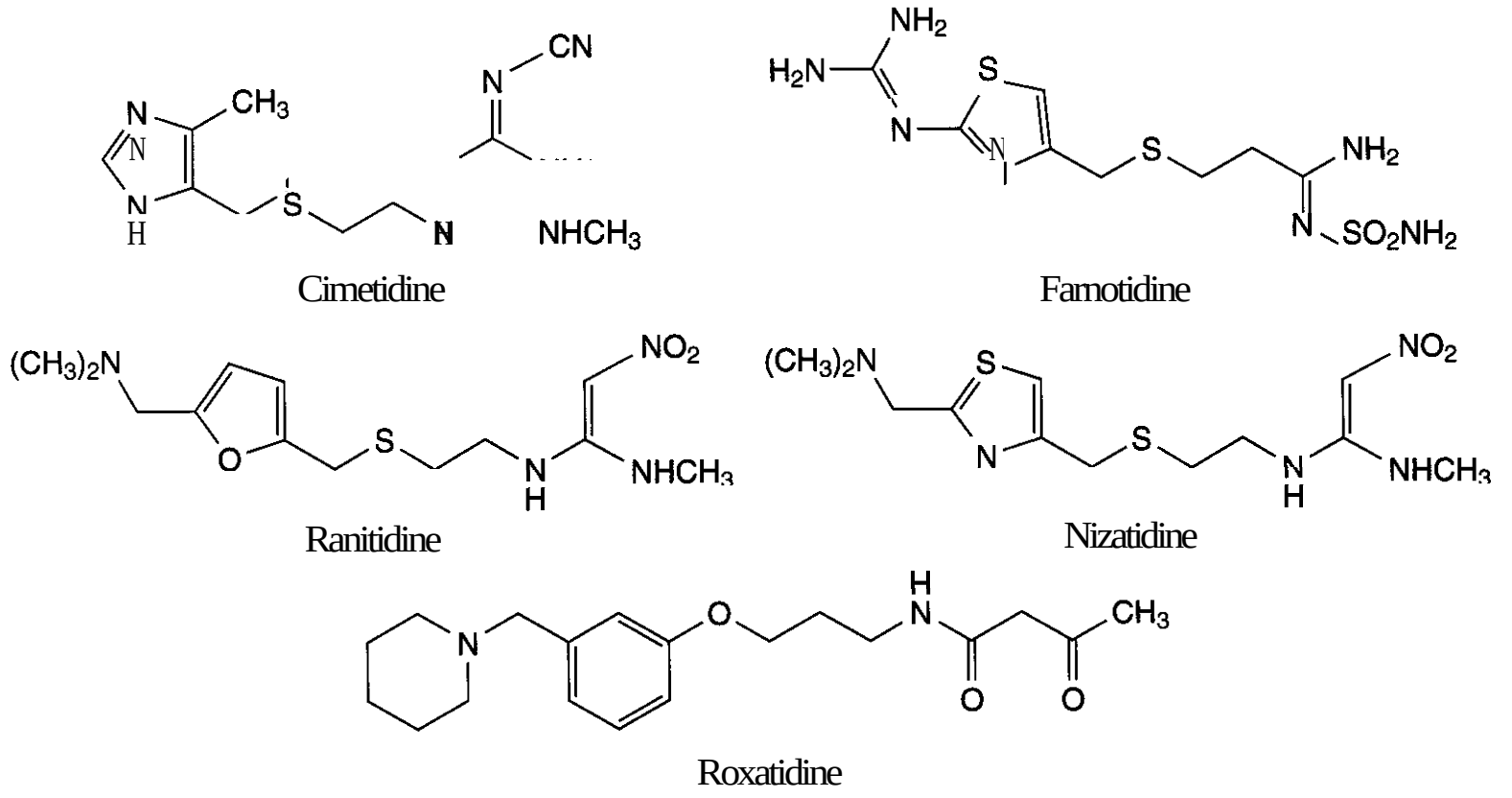


Figure 3.4. Marketed H_2-receptor antagonists.

such as acidity, hydrophilicity, dipole moment, conformation, and geometry of the hydrogen-bonding group (80), that it was possible to retain H_2-receptor antagonism in a variety of structures. For example, it was possible to replace the thiourea and cyanoguanidine groups of metiamide (81) and cimetidine (82) with nitroguanidine and diaminonitroethylene, respectively, which in this instance proved to be effective bioisosteres. Other groups such as guanidine (83), 2-amino pyrimidin-4-one (84), and 1,4-diamino butene-2,3-dione were less well tolerated, but they have been used successfully in H_2-receptor antagonists, where additional changes have also been made elsewhere in the molecule. Similarly, assessment of the potential ionic and tautomeric forms of the imidazole ring and the dynamics of its interaction with the receptor led to the suggestion that the neutral N^{τ}-H isomeric

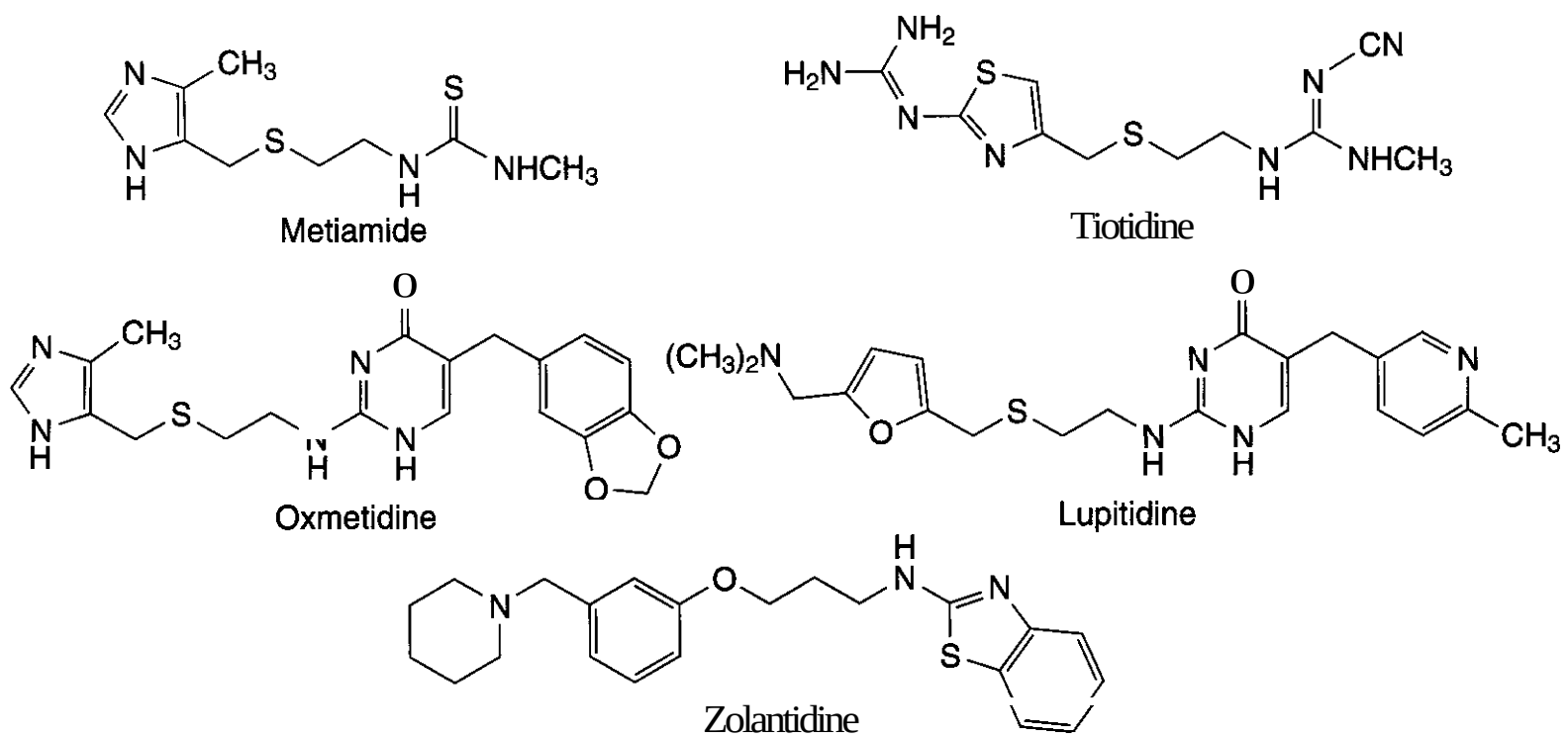


Figure 3.5. Representative H_2-receptor antagonists to illustrate SAR.

form is the preferred bioactive isomer (85,861. This prompted the preparation of many analogs in which either substituents such as chloro (87) and methylthio (88) were introduced or where the imidazole ring was replaced by other heterocycles.

Although 2-pyridine, 2-thiazole, and 3-isothiazole were all successfully used for this purpose, the most marked increase in potency with respect to cimetidine was achieved by replacing the imidazole ring by a guanidinethiazole group, to obtain tiotidine (89), as well as famotidine (90), in which the cyanoguanidine group had additionally been replaced by the more hydrophilic sulfamoylamidine substituent. Moreover, with greater basicity now residing in the exocyclic substituent than that in the heterocyclic ring itself, the importance of this characteristic in the latter substituent diminished. The presence of a furan ring as the heterocyclic component in ranitidine is consistent with this view (91), and a similar structural motif is also present in nizatidine (92). The dimethylaminomethyl-substituted furan of ranitidine evolved further to include piperidinylmethyl-substituted phenyl ethers, represented by roxatidine (93). An acetyl acetamide group was used as the hydrogen-bonding substituent in this case and higher affinity was achieved when this group was replaced by the bulkier and more lipophilic aminobenzothiazole ring, as in zolantidine (94). A similar trend toward higher affinity arising from such groups is observed in oxmetidine (95)and lupitidine (96), which can be considered to be more potent analogs of cimetidine and ranitidine, respectively.

In general, the introduction of branching to the linking chain afforded less potent compounds, whereas replacement by an aromatic linker was successful in the case of tiotidine analogs but only when the attached groups had a *meta* disposition (89). The thioether link has been retained in most H_2 antagonists because substitution of the sulfur atom by other heteroatoms or by methylene has been detrimental to affinity at the receptor. This has been attributed to a requirement to maintain an intramolecular hydrogen bond, between the polar termini, observed in crystal structures (97) and during infrared studies (98) of H_2 antagonists and which the thioether link uniquely helps to maintain. However, whether this is a necessary requirement for biological activity remains unclear.

8.2.4 Clinical Studies with H_2-Receptor Antagonists. HA H_Z-receptor antagonists are potent and selective inhibitors of gastric acid secretion, with numerous clinical studies to support their effectiveness in ulcer disease. Table 3.4 outlines the clinical effectiveness and pharmacokinetic profile of H_Z-receptor antagonists currently available in the UK.

Cimetidine, ranitidine, famotidine, and nizatidine exhibit similar profiles of absorption, distribution, and elimination. Each of the H_Z-receptor antagonists exhibits classical competitive drug–receptor interactions, with Schild slope parameters not significantly different from unity (99). The affinity of each of these drugs for the H_Z-receptor is reflected in their effectivenessin inhibiting gastric acid secretion (100). Famotidine is the most potent H_2-R antagonist, being 20–50 times more potent than cimetidine and 6–10 times more potent than ranitidine (101) and nizatidine. Each of these drugs is rapidly absorbed after oral administration, with peak plasma concentrations being achieved within 3 h of dosing. Oral bioavailability for these drugs ranges from 43% to 90%. With the exception of nizatidine, the bioavailability of H_Z-receptor antagonists is reduced because of extensive first-pass hepatic metabolism. Only minimum plasma protein binding occurs ($\leq$30%) and all of the H_Z-antagonists are eliminated quite rapidly, with a terminal half-life of 1 to 3 h and a total body clearance of 24–48 L/h. Elimination is mainly attributable to renal excretion, with renal clearances ranging from 13.8 to 30 L/h. Given that the values for renal clearance greatly exceed the glomerular filtration rate (6–7.2 L/h), it is apparent that renal tubular secretion plays an important role in this process (102).

8.2.5 Adverse Effects of H_2-Receptor Antagonists. The H_Z-receptor antagonists are generally extremely safe drugs, with few adverse effects being reported. Cimetidine was shown to possess antiandrogen properties in a small number of patients; however, this effect

Table 3.4 An Overview of the Clinically Marketed H_2-R Antagonists: Affinity, Clinical Pharmacology, and Pharmacokinetic Profile[a]

Drug	*In vitro* pK_B	Clinical Pharmacology: Effect on Gastric Acid Secretion			Oral Absorption	Plasma Half-life (h)	Volume of Distribution ($L\ kg^{-1}$)	Plasma Protein Binding	Excretion	Metabolism
		Normal		DU						
Cimetidine	6.1 (193)	i.v. IC_{50} = 2 μmol L^{-1} stimulant; pentagastrin, 40 $\mu g\ kg^{-1}\ h^{-1}$ (195)	p.o. 0.8–1.0 giday; 71% decrease in 24 h, mean H^+ (174)	p.o. 0.8 g/day; 55% 1.6 g/day; 67% decrease in 24 h, mean H^+ activity (174)	90% (194)	p.o. 2 (195)	0.8–1.2 (194)	13–25% (194)	Kidneys: i.v., –70% unchanged (195)	Oxidation to the sulfoxide (195)
Famotidine	7.8 (193)	IC_{50} = 4.3 $\mu g\ kg^{-1}h^{-1}$ stimulant pentagastrin, 0.1 $\mu g\ kg^{-1}\ h^{-1}$ (196)	5 mg; 40% inhibition 5 mg ≡ inhibition to cimetidine (300 mg) (197)	40–80 mg/day; improved healing rates; different dosing *regimes* examined. Effect on gastric acid secretion was not examined (198)	43% (199)	p.o. 3.8 (197)	1.1–1.4 (194)	20% (194)	Kidneys: i.v., 70% unchanged (199) p.o., ≈ 40% unchanged; remainder in bile or metabolized (194)	Oxidation to the sulfoxide (194)
Ranitidine	6.7 (193)	Ranitidine (150 mg) more potent than cimetidine (200 mg) (200)		PG-stimulated acid secretion, 40 mg; 42% 80 mg; 69% nocturnal acid secretion, 80 mg; 50% (201)	50% (202) 52% (203)	i.v. 2 (203)	1.82 (203)	15% (202)	Kidneys: i.v., 69.4 ± 6.1%; p.o., 26.7 ± 7.2% (203)	Hepatic, some enterohepatic recycling (202)
Nizatidine	≈7 (92)	25 mg 45%; 50 mg 58%; 100 mg 67% (204)	30 mg 57%; 100 mg 73%; 300 mg 90%; 75 mg ≡ . inhibition to cimetidine (300 mg) (205)	Nizatidine (300 mg) was significantly shorter-lasting than both ranitidine (300 mg) and famotidine (40 mg) (100); nizatidine (300 mg) produced similar healing rates to those of ranitidine (300 mg) (206)	70% (204)	i.v. 1.6 (204); p.o. 1.6 (207)	1.1 (194)	30% (194)	Kidneys (204)	–35% major metabolites: N-2 mono des methylnizatidine (–7% of dose), nizatidine N-2 oxide (–5%); nizatidine sulfoxide, minor metabolite (207)

[a]Abbreviations: i.v., intravenous administration; p.o., oral administration; i.d., intraduodenal administration; normal, healthy human volunteers; DU, duodenal ulcer patients.

was not detected with other H_2-receptor antagonists (103). Cimetidine also has high affinity for the cytochrome P450 system, whereas ranitidine has intermediate affinity and famotidine and nizatidine have little to no interaction with this pathway (104). Inhibition of this enzyme system can decrease the oxidative metabolism of other drugs, resulting in decreased clearance. H_2-receptor antagonists may therefore adversely affect the clearance of agents that have a limited therapeutic window (e.g., warfarin, theophylline, and phenytoin).

8.2.6 The Need to Develop More Potent Antisecretory Agents. The discovery and development of H_Z-receptor antagonists was the beginning of a novel therapeutic approach to diseases associated with the hypersecretion of gastric acid. The inhibition of acid secretion at the level of the parietal cell, by inhibiting the activation of HA released from ECL-cells, was the beginning of cellular-based antisecretory therapy. During the 1980s, H_Z-antagonists became first-line therapy in peptic ulcer disease and negated the necessity of surgery for a large number of patients.

It soon became apparent that H_2-receptor antagonist therapy had some drawbacks. In a small but significant number of patients, the disease was resistant or recurred after maximal H_2-antagonist therapy (105). In another small group of patients, H_2-receptor antagonists were particularly poor when it came to inhibiting the nocturnal secretion of gastric acid (106). The limitation of this therapy resulted from the actions of other mediators, such as Ach or gastrin, acting directly on the parietal cell, in stimulating the secretion of gastric acid. In some patients, tolerance to H_2-antagonist therapy also appeared to develop when long-term treatment was necessary (107, 108). A further problem was that acid rebound (elevated acid secretory response) occurred after cessation of H_2 therapy (109). The upregulation of other hormonal mechanisms, alterations in signal transduction, or receptor upregulation were hypotheses proposed to account for both the decreased effectiveness and acid rebound, which was produced by H_2-antagonist therapy.

The discovery of the proton pump (110) and the elucidation of its function (111) were the first steps toward the discovery of another novel antisecretory therapy. In 1977, a year after the launch of cimetidine, Astra reported on the first PPI, H83/88, a benzimidazole derivative that noncompetitively inhibited both receptor (HA)-mediated and nonreceptor (cAMP)-mediated acid secretion from isolated gastric mucosa (112).

9 H^+/K^+-ATPase (PROTON PUMP) INHIBITION

The enzyme H^+/K^+-ATPase, or proton pump, regulates the final stage in the cellular cascade that terminates in the secretion of gastric acid. Acid secretion is dependent on the cellular location of the enzyme and the close proximity of a potassium chloride efflux pathway. When the parietal cell transforms from a resting to a stimulated state, cytoskeletal rearrangement occurs and H^+/K^+-ATPase relocates to the apical plasma membrane. Potassium and chloride ions move across the apical cell membrane together with secreted protons. Potassium is recycled, whereas the hydrochloric acid of gastric juice is formed by chloride ions together with secreted protons (Fig. 3.6).

Neuronal, hormonal, and enzymic pathways influence the secretion of gastric acid. The diversity of therapies available for treating hypersecretory disorders appear to have developed with these targets in mind (i.e., vagotomy, H_2-, and muscarinic-receptor antagonists and, more recently, PPIs). The most potent inhibitor of gastric acid secretion is produced by inhibiting H^+/K^+-ATPase.

9.1 Structure of the Proton Pump

The H^+/K^+-ATPase enzyme, present in tubulovesicular and canalicular membranes of the gastric parietal cell, consists of two subunits, a 114-kDa a-subunit and a 34-kDa β-subunit. The a-subunit has been shown to contain 10 transmembrane helices, with the β-subunit possessing only a single transmembrane helix (113). The a-subunit carries out the catalytic and transport functions of the enzyme because it contains both ATP and cation binding sites; it also contains the sequences responsible for apical membrane localization. When

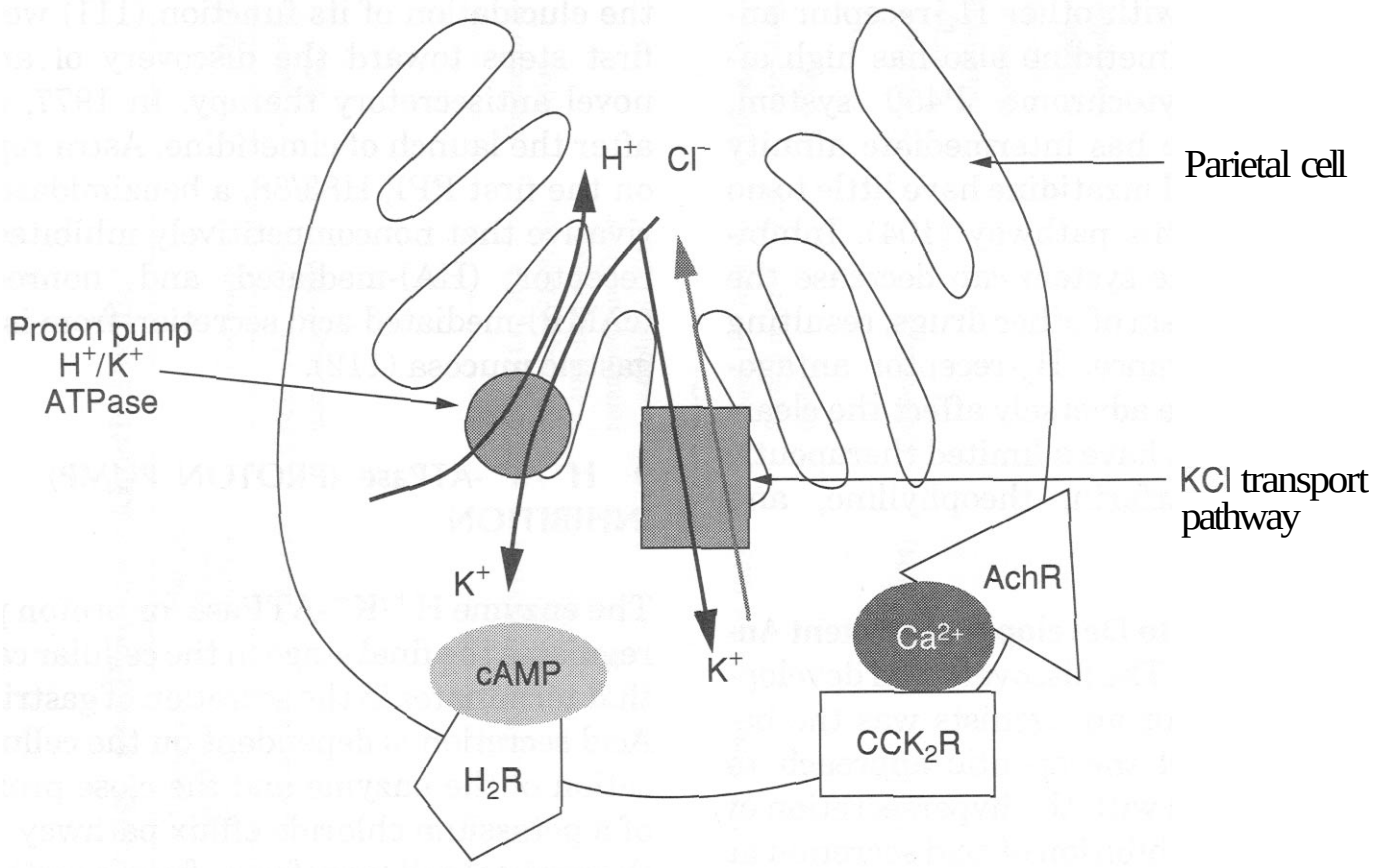


Figure 3.6. Activation of the proton pump in the parietal cell.

the parietal cell transforms from an active to resting state, the heavily glycosylated β-subunit is required for endocytic retrieval of the H^+/K^+-ATPase from the canalicular membranes. This subunit may also contribute to proper folding and membrane localization of the enzyme (114). H^+/K^+-ATPase belongs to the family of P_2-type ATPases and has homology with other members of this family such as Na^+/K^+-ATPase (65% homology) and Ca^{2+}-ATPases (23% homology).

9.2 Acid Secretion and the Proton Pump

When the resting parietal cell is stimulated by acid secretagogues, the tubulovesicles are transformed into the secretory canaliculus. The parietal cell has the largest mitochondria1 content of any mammalian cell (–34% of cell volume) and the ATP generated by this is mainly used for acid secretion. Hydrolysis of ATP results in a conformational change in the protein that mediates the electroneutral exchange of intracellular H^+ and extracellular K+. The pump is activated only when it is associated with a potassium chloride pathway in the canalicular membrane (Fig. 3.6). This allows potassium chloride efflux into the extracytoplasmic space and thus results in the secretion of HCl at the expense of ATP hydrolysis. The stimulation of hydrogen ion secretion by the ATPase leaves behind an equivalent number of hydroxide ions. These are converted to HCO_3 by the enzyme carbonic anhydrase, which is closely associated with the secretory membrane. The activity of the pump is determined by the access of K^+ to the extracytoplasmic surface of the pump. In the absence of K^+ on this surface of the pump, the pump cycle stops at the level of the phosphoenzyme.

In recent studies, mice lacking a functional H^+/K^+ATPase (achieved by ablation of the H^+/K^+ATPase a-subunit) were found to possess severe disturbances in the secretory membranes of the parietal cell, metaplasia of the gastric mucosa, achlorhydria, and hypergastrinemia (115). H^+/K^+-ATPase therefore forms a critical component of the ion-transport system mediating acid secretion in the stomach.

9.3 Mechanism of Action of Proton-Pump Inhibitors

PPIs are both more potent and of longer duration than H_2-receptor antagonists and therefore are frequently the drug of choice for the treatment of diseases associated with the secretion of gastric acid. PPIs inhibit gastric acid

secretion by inhibiting the enzyme H^+/K^+-ATPase, which is located on the luminal surface of gastric parietal cells. Currently prescribed PPIs are substituted benzimidazole-based structures. When administered at neutral pH, these weak bases are chemically stable, lipid-soluble, and have no effect on gastric acid secretion. The inactive PPI diffuses from the bloodstream into the parietal cells and subsequently into the acid environment of the secretory canaliculi, where it rearranges to form a sulfenic acid in equilibrium with a sulfenamide. Either chemical entity is then able to interact covalently with thiol groups at cysteine residues located on the luminal surface of the α-subunit of the H^+/K^+-ATPase. This covalent binding results in specific and essentially irreversible inactivation of the enzyme, leading to long-lasting inhibition of gastric acid secretion (Figs. 3.7 and 3.8). Such a profile is thus ideally suited to once-daily administration in the management of acid-peptic disorders.

Like omeprazole, the other commercially available PPIs, lansoprazole, rabeprazole, pantoprazole, and esomeprazole are inactive prodrugs that are activated in the acid environment of the gastric glands. This means that all these agents are best administered in a formulation that includes an enteric coating. Subtle differences in the inhibitory effects of these compounds on H^+ transport and acid secretion, however, have been reported. These have been attributed to binding of the drug to different cysteine residues within the proton pump. Unlike omeprazole and lansoprazole, which bind to two (cys813 and cys892) and three (cys813, cys892, and cys321) cysteine residues, respectively (116), rabeprazole is reported to bind to a single cysteine residue located at position 322 between transmembrane domain H3 and the lumen. This interaction does not appear to result in a conformational change of the enzyme, whereas omeprazole stabilizes the conformation of the enzyme in the dephosphorylated state, or so-called E2 form (117).

9.4 Structure and SAR of the Proton-Pump Inhibitors

In 1973 workers at AB Haessle in Sweden, identified timoprazole (118) as one of the first well-defined inhibitors of the newly discovered gastric proton pump. This compound stemmed from efforts to separate the toxicity and acid-inhibitory properties of 2-pyridylthioacteamide (CMN131). Removal of the thioamide group was considered to be the most likely solution to the toxicity of CMN131, which prompted the preparation of sulfur-containing heterocycles, as well as imidazoline- and benzimidazole-linked sulfides. However, it was the corresponding S-oxide analogs of the latter compounds that proved the more potent; it thus became clear that the mechanism of action of timoprazole was distinct from that of H_2-receptor antagonism. Timoprazole was subsequently followed by the more potent derivatives picoprazole in 1976 and omeprazole in 1979 (119). The three main structural features of omeprazole (i.e., the substituted pyridine ring; the substituted benzimidazole; and the methylsulfinyl linking group, by which these two ring systems are attached to one another) are essential, either in generating the active form from its inactive prodrug precursor or in binding irreversibly with the H^+/K^+-ATPase enzyme. For this reason, compounds inhibiting acid secretion by this mechanism and lacking one or more of these features are scarce. Moreover, for irreversible proton-pump inhibitors to achieve selective biological activity, their mechanism of action demands that they have relatively high chemical stability around neutral pH, but be readily activated at low pH. This chemical profile also influences attendant issues such as synthesis, formulation, and storage, and because biological activity of this class of compounds often correlates with chemical lability, not all compounds, which achieve potent inhibitory behavior in *vitro*, are viable drug candidates because they are inherently too chemically unstable.

The gastric proton-pump inhibitors currently available (Fig. 3.10) all retain the same key chemical features present in omeprazole, indicating that the structural requirements to achieve irreversible inhibition of the gastric ATPase enzyme are precisely defined. The clinical properties of this latter group of drugs is discussed more fully in section 9.6, whereas the remainder of this section focuses on other candidates currently or previously under de-

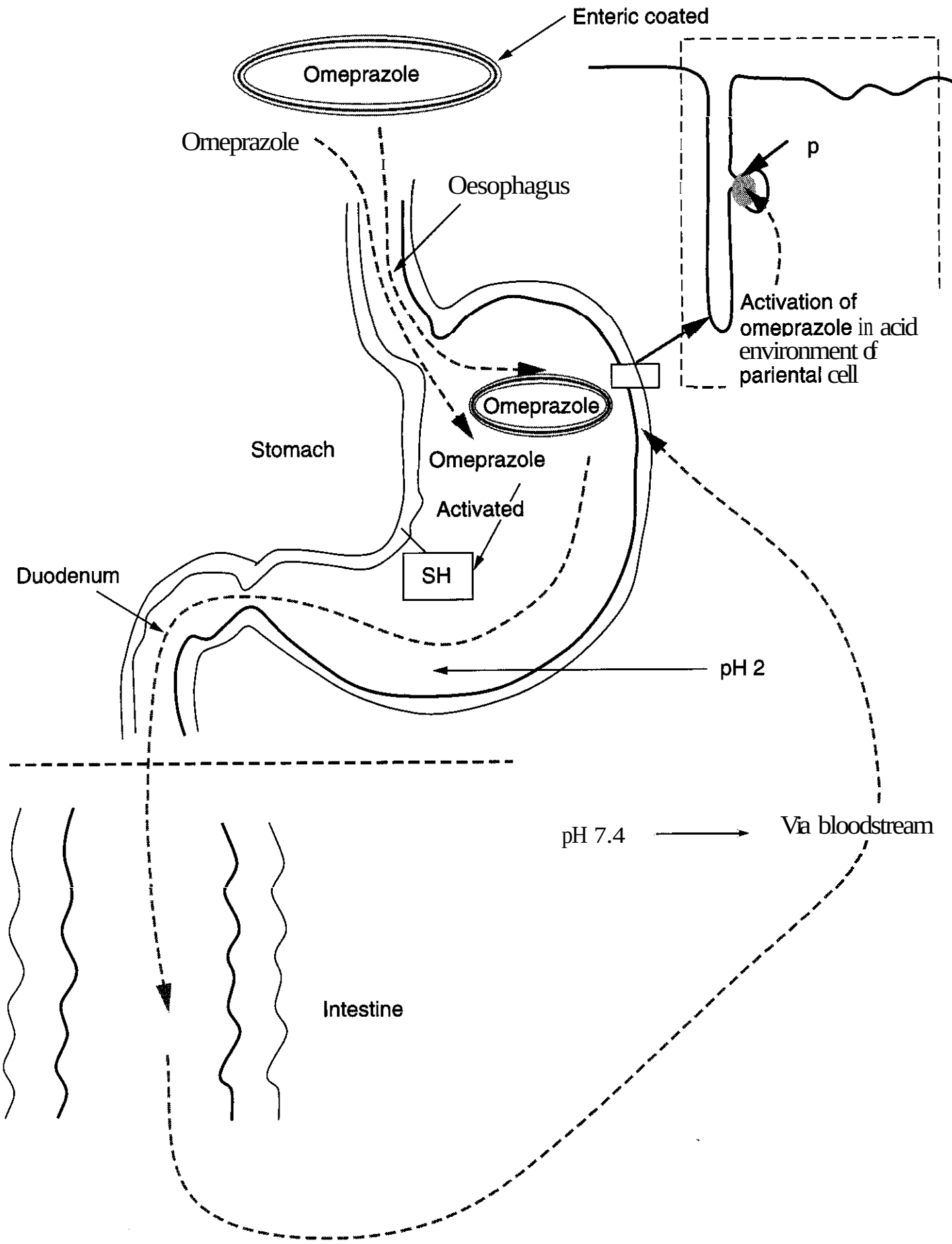


Figure 3.7. Conversion of omeprazole to its active state within an acidic environment. Oral administration of enterically coated omeprazole ensures maximal activation occurs within the parietal cell.

velopment. As discussed above, the potency of irreversible proton-pump inhibitors is a time- and pH-dependent property, making comparison of their in *vitro* potency difficult, given the wide range of assay conditions employed (Table 3.5 and Fig. 3.9). Where possible, their potency relative to omeprazole, measured under the same assay conditions, is indicated. However, notwithstanding differences in the stimulant used, comparison of their *in vivo*

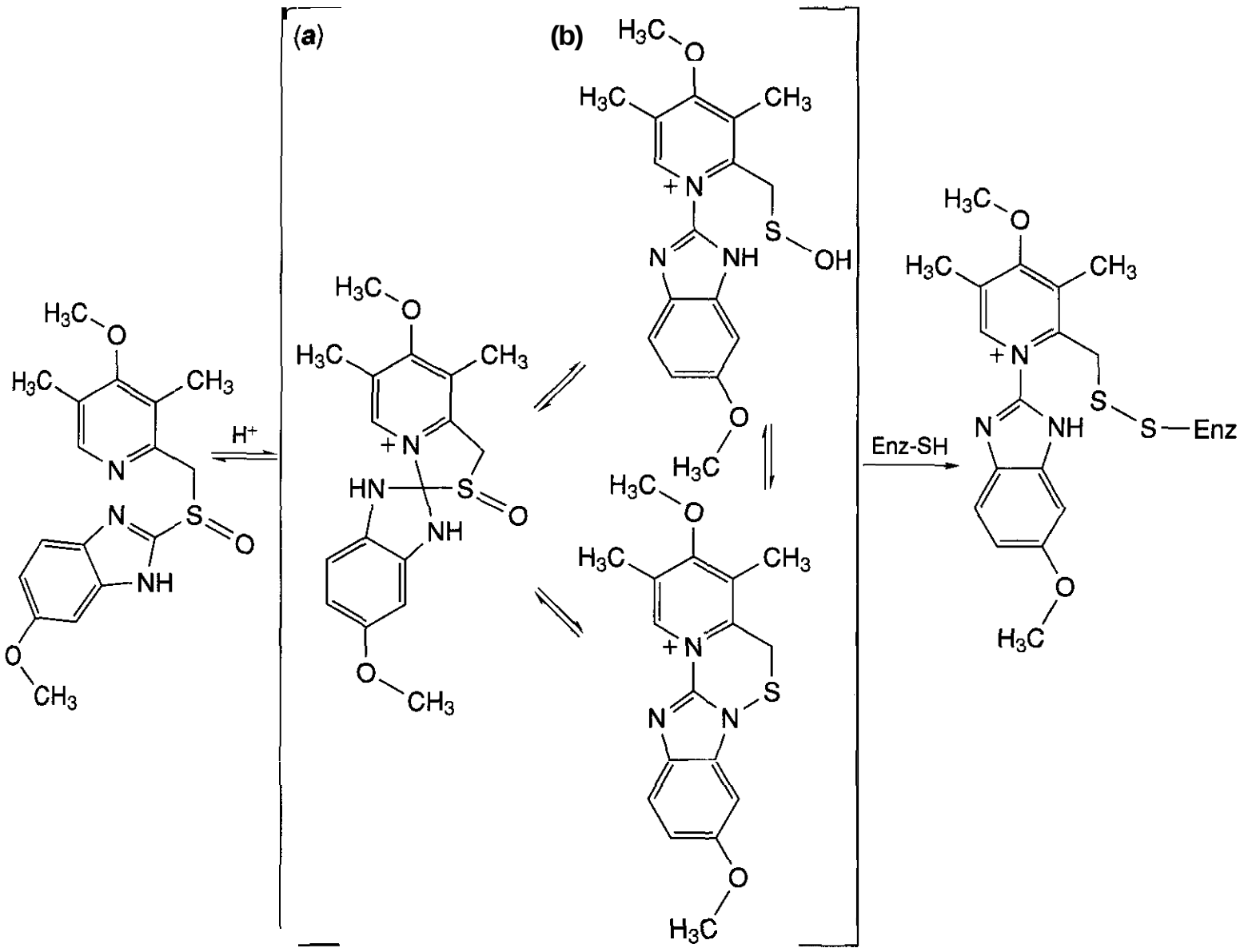


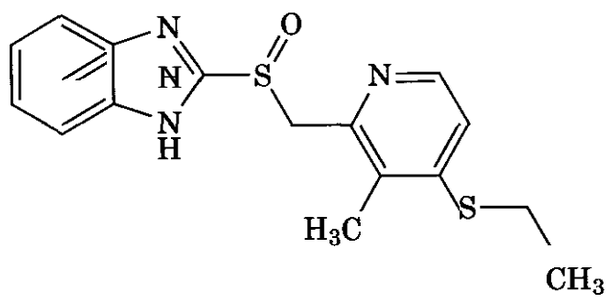
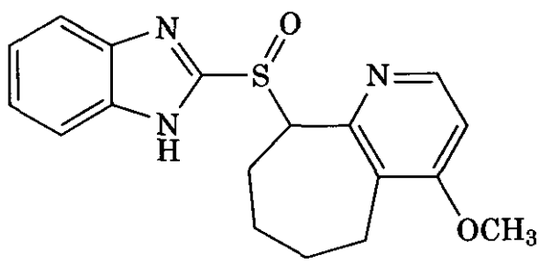
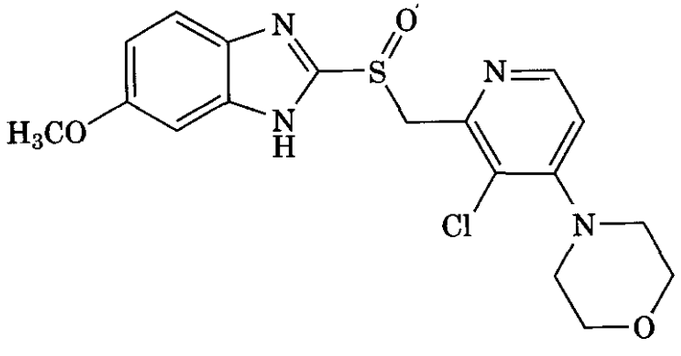
Figure 3.8. Acid-catalyzed activation of omeprazole to form the active pyridinium sulfenamide or sulfenic acid. Only one isomer of intermediates (a) and (b) is shown.

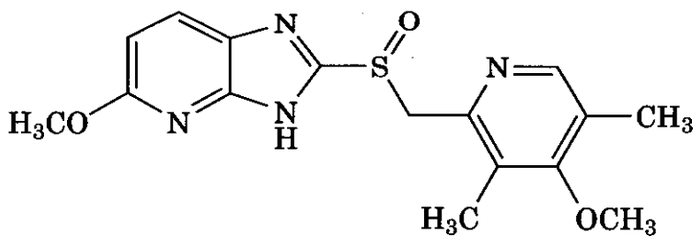
potency, as judged by inhibition of gastric acid secretion, is in many respects more reliable because interspecies variations are minimized and the data obtained are also unconfounded by differences in pH.

The same chemical features retained in the marketed compounds lansoprazole (120), pantoprazole (121), and rabeprazole (122) are retained in disuprazole (54) TY-11345 (123), Ro-18–5364 (124), and SKF 95601 (125). The benzimidazole group of omeprazole has been replaced by other heterocycles and its activity retained, with the methoxyimidazopyridine compound tenatoprazole **(126)** being the most advanced. Similarly, the fused benzene ring of the benzimidazole group has been substituted by a thiophene ring in the thieno[3,4-*d*]imidazole-based compounds saviprazole (127) and S-1924 (128); however, examples of the corresponding thieno[2,3-*d*]imidazole isomers (**1**), also described by Hoechst, are much less effective, most likely because of their greater chemical stability. Although many of the compounds lack substituents on the benzimidazole ring, the presence of electron-donating groups at the 5-position, such as methoxy (omeprazole), SKF 95601, OPC-22575 (129), S-337 (128), pyrrol-1-yl (IY-81149) (130), and difluoromethoxy (pantoprazole), also provided an optimum balance of chemical stability and reactivity. In fact, the presence of electron-withdrawing substituents in this position, such as nitro, methylsulfinyl, and trifluormethyl, increases the basicity of the benzimidazole ring to the point where the behavior of these compounds is dominated by activation at neutral pH, resulting in compounds having poor chemical stability and limited practical value (131).

In contrast, increasing the nucleophilic character of the pyridine ring, by the incorporation of electron-donating substituents, en-

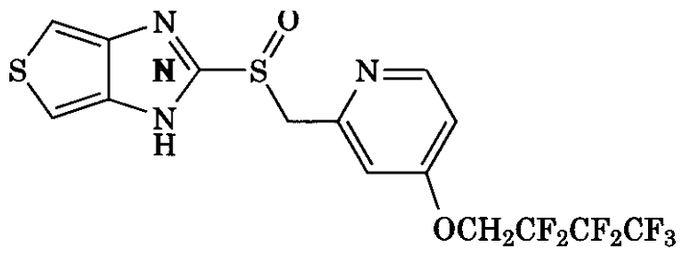
Table 3.5 Biological Activity of Representative Irreversible PPIs

Structure	*In Vitro* IC_{50}, μM (IC_{50}, μM, omeprazole)	*In Vivo* Inhibition of Gastric Acid Secretion in Rat by Enteral Administration, ED_{50}, mg kg^{-1} (ED_{50}, mg kg^{-1}, omeprazole)	Ref.
 Disuprazole (Upjohn)	20 (50)[a]	2–5 (2.5)[d]	54
 TY-11345 (Tao Eiyo KK)	3.3 (11)[b]	1.3 (10.2)[e]	123
 SKF 95601 (SmithKline & French)		1.14[d]	125



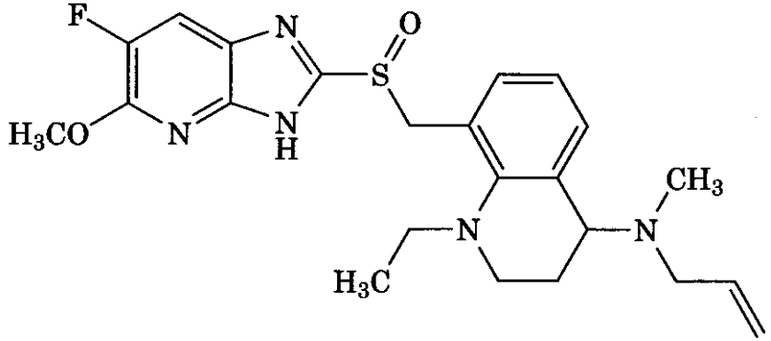
Tenatoprazole (Mitsubishi–Tokyo Pharm. Inc.)

6.2 (4.2)[c] | 4.2[d] | 192



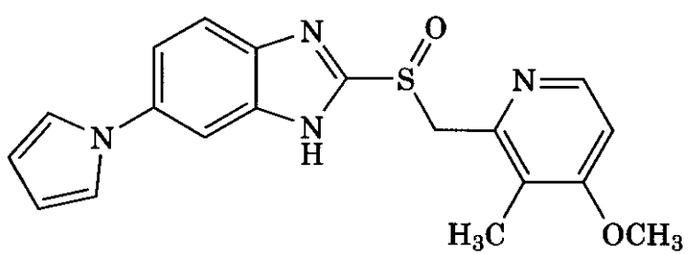
Saviprazole (Hoechst AG)

1.3[b] | 127



OPC-22575 (Otsuka Pharm. Co. Ltd.)

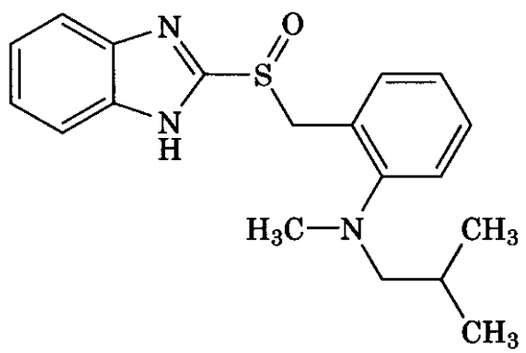
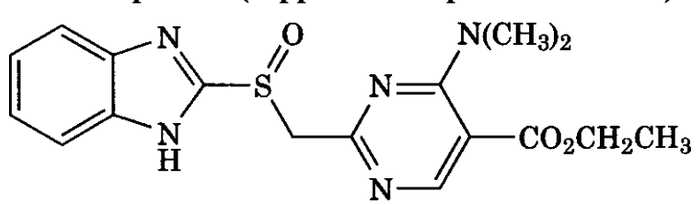
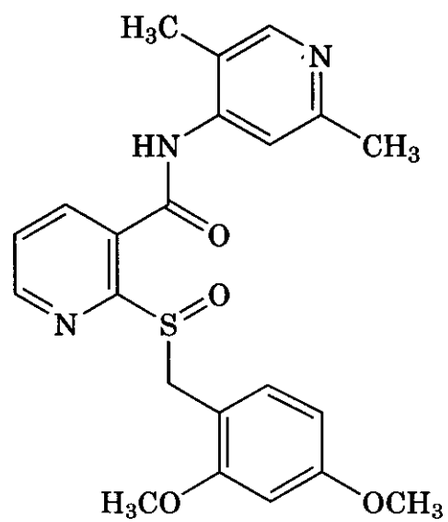
38 (2)[c] | =64.9 at 3 mg kg^{-1} (78.6% at 1 mg kg^{-1})[e,f] | 129

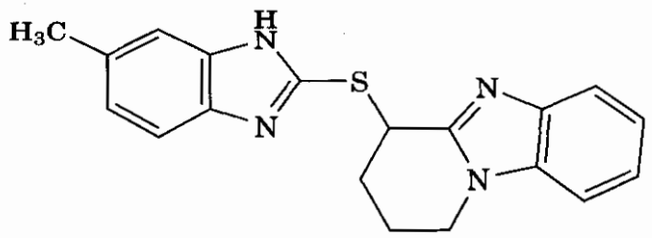


IY-81149 (11-Yang Pharm. Ind. Co. Ltd.)

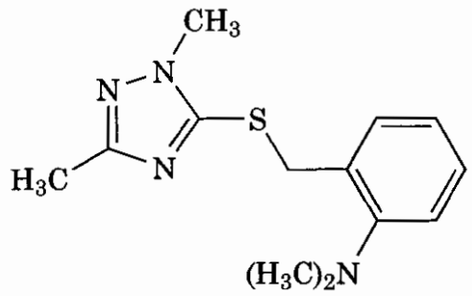
23 (160)[c] | 3.6[d] | 130

Table 3.5 (Continued)

Structure	In *Vitro* IC_{50}, μM (IC_{50}, μM, omeprazole)	In *Vivo* Inhibition of Gastric Acid Secretion in Rat by Enteral Administration, ED_{50}, mg kg^{-1} (ED_{50}, mg kg-', omeprazole)	Ref.
	0.28 (0.05)[b]	11.5	132
Leminoprazole (Nippon Chemipharm. Co. Ltd.)			
	7.5 (5.8)	2.2 (0.8)	133
(2) (Roussel Morishita Co. Ltd.)			
	0.24 (0.37)[b]	2.8 (4.1)[d]	134



YJA 20379-4 (Yungjin Pharm Co. Ltd.) | 32 (25)[b] | 23.6 (2.4)[d] | 136



GYKI-34655 (Egis Gyogyszergyar RT) | >100 (16.3) | 6.3[d] | 137

[a]Canine gastric (H^+/K^+) ATPase.
[b]Rabbit gastric (H^+/K^+) ATPase.
[c]Porcine gastric (H^+/K^+) ATPase.
[d]Shay rat.
[e]Ghosh and Schild rat.
[f]Intravenous administration.

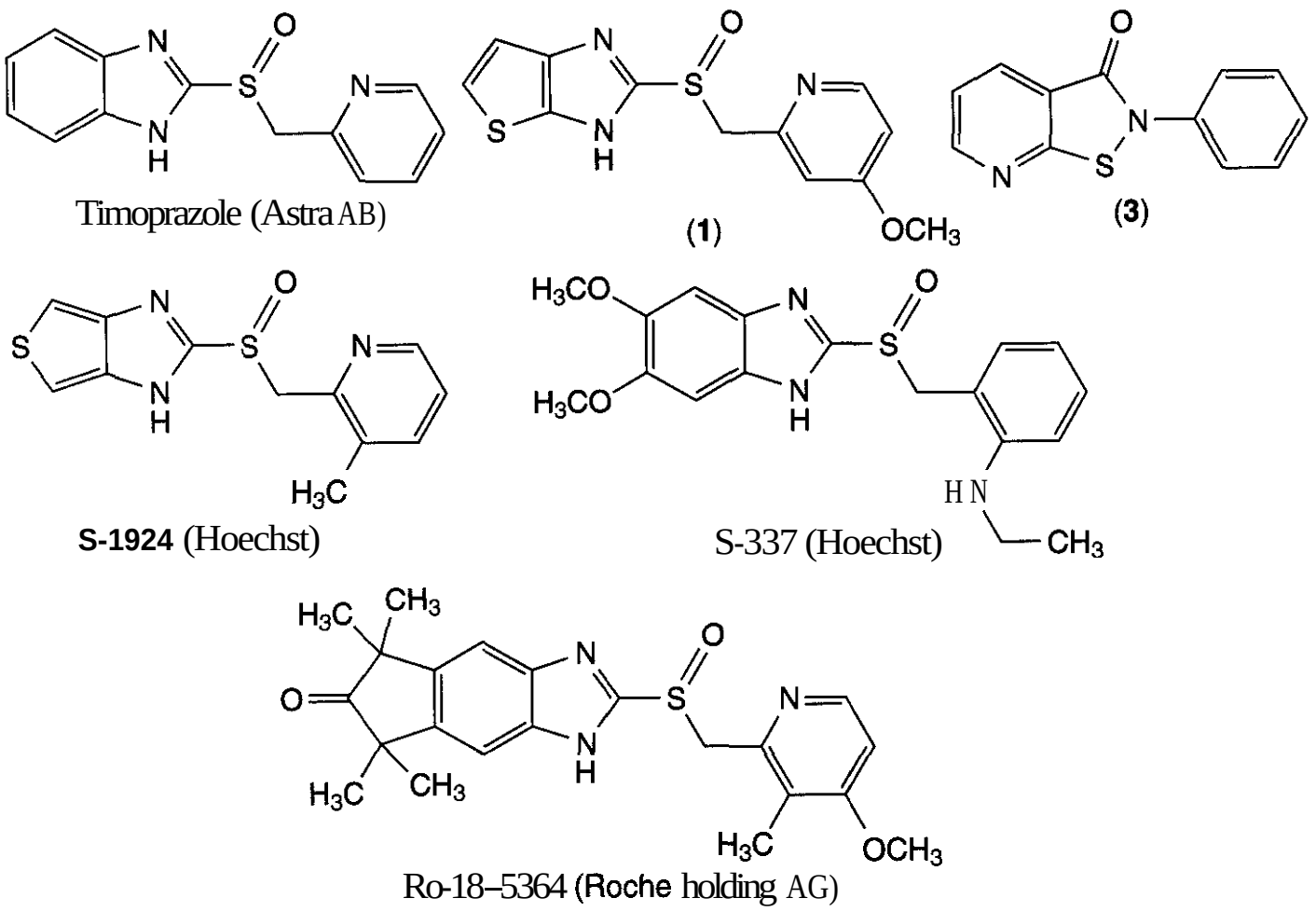


Figure 3.9. Structures of representative irreversible PPIs to illustrate SAR.

hances the rate of attack of the C-2 position of the benzimidazole group and thereby promotes the acid-catalyzed rearrangement to the active species. This electronic feature is present in omeprazole (3,5-dimethyl-4-methoxy) and pantoprazole (2,3-dimethoxy), whereas it is combined with increased lipophilic character in 4-fluoroalkyl benzimidazole-substituted compounds, such as lansoprazole (2,2,2-trifluoroethyloxy) and saviprazole (2,2,3,3,4,4-heptafluorobutyloxy). The 3-methoxypropoxy substituent present on the pyridyl group of rabeprazole has a particularly strong electron-donating character, but the resulting enhancement in reactivity in this case is mitigated by formulation as its sodium salt, so as to increase chemical stability. Similarly, the 3-chloro substituent is used to similar effect in the case of the 4-aminopyridyl-substituted compound SKF 95601 (131).

As an alternative to the 2-pyridyl group of omeprazole, compounds containing a substituted benzene ring in this position, made sufficiently electron rich by the presence of a 2-amino group, display potent inhibition. Although other examples of this type are known (S-3337, OPC-22575), leminoprazole (132) is the most advanced clinically and displays an enhanced duration of action relative to that of omeprazole. Similarly, the electron-rich-substituted pyrimidin-2-yl group (2)has also been used in place of the 2-pyridylmethyl moiety in compounds described by Roussel-Morishita (133).

AD-9161 represents the most potent of a novel but mechanistically similar class of irreversible H^+/K^+-ATPase inhibitors (134). Compounds of this type were designed based on the finding that the oxoisothiazolo[5,4-*b*]pyridine (3) displayed potent inhibition of ATPase in vitro but failed to achieve inhibition of gastric acid secretion in vivo (135). This disparity was ascribed to the rapid and preferential attachment to and inactivation of (3), by thiol groups located outside the region of the ATPase enzyme in the parietal cells. AD-9161 is considerably more stable than (3)and displays potency comparable to that of omeprazole both in vitro and in vivo.

Although all the proton-pump inhibitors described so far are prodrugs that rely on the participation of a sulfoxide group to effect their activation, related sulfide analogs are also known. Because compounds of this class,

such as YJA-20379–4 (136) and GYKI-34655 (137), would first need to be oxidized to the sulfoxide before activation, they are considerably less potent than omeprazole *in vitro*, but *in vivo* they are at least as effective in models of gastric acid secretion and ulceration.

In an effort to obtain a second-generation, irreversible proton-pump inhibitor having a superior profile, renewed interest has recently focused on the relative potency of the optical isomers of omeprazole and other benzimidazol-2-yl 2-pyridylthiomethyl S-oxides. The optical activity arises from chirality at the sulfur atom. One aim was to reduce liver clearance and thereby minimize variations in interpatient bioavailability and efficacy. The enantiomers of omeprazole were long expected to be of equivalent potency to one another and to omeprazole, given that acid-catalyzed conversion to the nonchiral active forms would be expected to occur at the same rate. Use of a single enantiomer was also an unattractive approach, given that, once separated, the enantiomers slowly racemized, although it was subsequently confirmed that they displayed similar behavior *in vitro* (138). It was only with the availability of larger quantities of the respective enantiomers (139) and their stabilization by formulation as alkaline salts, that their relative potency *in vivo* could be rigorously investigated. In rats, the enantiomers displayed distinct profiles in their potency and bioavailability because of selectivity in their rates of metabolism. Subsequently, in human studies, the relative potency of the enantiomers was found to be reversed, with the S-isomer now being the more potent, because of the presence of the CYP2C19 cytochrome P450 metabolizing enzyme for which the R-isomer is a substrate. This is an enzyme that is absent from rats (140).

Based on its superior bioavailability in human studies (141), esomeprazole, the *S*-enantiomer of omeprazole, has now been launched as a successor, with the aim of providing a higher level of management in acid-related disorders. Single-enantiomer versions of lansoprazole [(*S*)-lansoprazole] and pantoprazole [(−)-pantoprazole] (142) are currently in the early stages of clinical development.

9.5 Profile of Proton-Pump Inhibition

Omeprazole is a potent and irreversible inhibitor of H^+/K^+-ATPase. Subsequently developed compounds (lansoprazole, pantoprazole, esomperazole, and rabeprazole) have a similar mechanism of action, although small differences in bioavailability, potency, and metabolism have been reported.

Comparative studies with lansoprazole and pantoprazole suggest that they have a potency similar to that of omeprazole (143). Rabeprazole is readily converted to the active drug form and has a more rapid onset of effect than that of omeprazole in both *in vitro* and *in vivo* studies. In the rat, there is evidence to suggest that rabeprazole may have a slightly shorter duration of action than that of other PPIs. Compared with lansoprazole, the onset of action of rabeprazole was faster and its duration of action was shorter, as determined by measuring acid output and microsomal enzyme activity (144). However, in another study in dogs, the rate of recovery of acid output was similar when rabeprazole was compared with omeprazole (145). The rate of activation of PPIs is pH dependent, with rabeprazole being fully activated at pH 5.0, whereas omeprazole and lansoprazole are only partially activated. These different pH selectivities may have an impact on drug interactions and safety (146). Rabeprazole is claimed to possess properties different from those of the other PPIs, with regard to the number of cysteine binding sites; its effect on the conformation and partial reactivity of the enzyme; the rate of acid activation (122) and thus onset of action (147); antisecretory potency *in vivo* (148); and the kinetics of reversal by scavenger mechanisms (117). Unlike omeprazole and lansoprazole, rabeprazole is reported to bind to just one cysteine residue located at position 322 between transmembrane domain H3 and the lumen. This interaction appears not to result in a conformational change in the enzyme. Conversion of rabeprazole into a reactive intermediate also appears to occur more rapidly than for other PPIs, which results in a faster onset of action and greater potency in isolated cell systems. Compared with omeprazole, the rate of reversal by glutathione is more rapid and the compound has a shorter duration of action, as

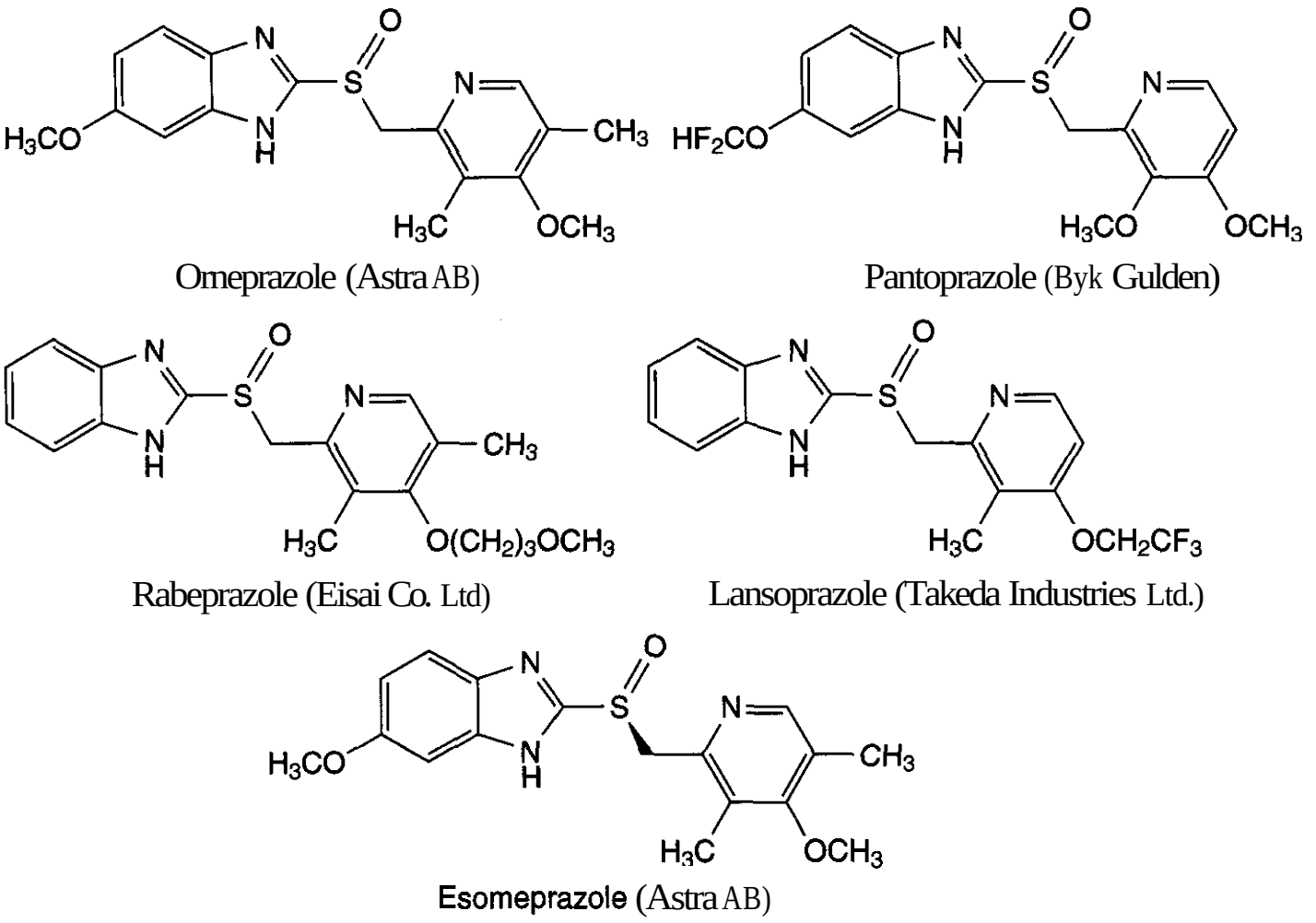


Figure 3.10. Marketed irreversible PPIs.

measured in isolated gastric glands and some animal models. Compared with lansoprazole, the onset of action of rabeprazole was faster and its duration of action was shorter, as determined by measuring acid output and microsomal enzyme activity (144).

9.6 Clinical Studies with Proton-Pump Inhibitors

PPIs are currently the most rapid, potent, and long-lasting treatment for hyperacidity disorders. Omeprazole was first marketed in 1988 and still remains the drug of choice for many patients. Like omeprazole, the majority of subsequently marketed PPIs, pantoprazole, lansoprazole, and esomperazole, bind irreversibly to the proton pump. Acid secretion can be restored only through endogenous synthesis of new H^+/K^+-ATPase, which has a half-life of production of approximately 50 h (116). Rabeprazole, however, is converted more rapidly into its activated forms and dissociates more readily from the H^+/K^+-ATPase, resulting in a faster rate of inhibition and a shorter duration of action. This property of rabeprazole is most likely linked to its activation within a more neutral pH range than that of any of the other PPIs, with pantoprazole being the most stable (143). (See Fig. 3.10.)

The plasma half-life of the PPIs is relatively short (~1 h) and accumulation is unlikely to occur, even when clearance is significantly reduced by renal impairment. The oral bioavailability of esomeprazole is lower than that of the other PPIs, with lansoprazole being the most orally bioavailable (149). All PPIs are highly protein bound and are metabolized in the liver.

Although PPIs are currently the most potent inhibitors of gastric acid secretion available, omeprazole has been reported to produce interindividual variations in its ability to inhibit gastric acid secretion. Recently, one of the isoenzymes of cytochrome P450, CYP2C19, which is integral to the degradation of PPIs in the liver, was shown to have two genetic phenotypes: extensive metabolizers and poor metabolizers. Variations in the phenotypes of CYP2C19 have been shown to affect the acid-suppressing effects of omeprazole, although the effectiveness of rabeprazole was not affected by CYP2C19 ge-

notype (150). The potency of omeprazole, lansoprazole, and pantaoprazole was dependent on metabolism by the enzyme CYP2C19 and, in subjects not expressing this genotype, the effectiveness of these PPIs was increased significantly. The genetically polymorphic CYP2C19 enzyme is absent from 3% of Caucasians and 20% of Asians.

It is difficult to make direct comparisons of the relative activity of each of the PPIs, given the different doses and dosing regimes used in clinical studies . However, for most PPIs, effectiveness has been compared to that of the prototype PPI, omeprazole. Table 3.6 outlines the results obtained in some of these clinical studies as well as the pharmacokinetic profile determined for each of the PPIs marketed in the UK. Clinical studies for H_2-receptor antagonists tended to measure effectiveness related to inhibition of acid secretion. In contrast, healing rates were generally the parameter of choice for PPIs. The PPIs are clearly more potent than the H_Z-receptor antagonists, with clinically used doses being at least 15 times lower than those of H_Z-receptor antagonists used in the treatment of DU (151). The pharmacokinetics and acid suppression produced by omeprazole, pantoprazole, lansoprazole, and rabeprazole from numerous clinical studies were recently reviewed (143). The authors concluded that these PPIs were of equivalent potency, although rabeprazole and lansoprazole displayed a more rapid onset of action. As part of the triple therapy used for eradication of H. *pylori,* each of the PPIs was equally effective.

Until recently, all PPIs were marketed as mixtures of enantiomers. However, the development of esomeprazole has prompted numerous studies to test its therapeutic benefit over that of existing PPIs. Within the last 2 years more than 40 publications have reported studies involving the use of esomeprazole. Esomeprazole has an improved pharmacokinetic profile relative to that of omeprazole: esomeprazole (20 mg per day for 5 days) had a 70% higher area under the plasma concentration-time curve than that of omeprazole (20 mg per day for 5 days). The S-isomer of omeprazole (esomeprazole) was found to undergo less metabolism by CYP2C19 than the R-isomer in human liver, this decreased metabolism accounting for esomeprazole's improved pharmacokinetic profile (152). The other advantages of esomeprazole over the existing PPIs are also related to its reduced metabolism by cytochrome P450 enzymes in the liver. With most other PPIs, drug interactions are of considerable importance, especially in situations where decreased metabolism is likely, for example, where the CYP2C19 enzyme is absent or there is hepatic or renal impairment. Furthermore, because esomeprazole undergoes less hepatic metabolism compared with that of omeprazole and other PPIs, there is less interpatient variability in the metabolic profile, the presence or absence of CYP2C19 being of less importance. This makes the drug easier to manage (153).

Although esomeprazole is a potent inhibitor of acid secretion, clinical trials comparing its effectiveness to that of other agents have tended to use disparate doses for each PPI examined (154). Kromer et al. considered that there was no pharmacodynamic argument in favor of single-enantiomeric formulations of any PPI (146). Moreover, potential pharmacokinetic differences between the enantiomers seem to be of little if any importance in the patient. A clinical study comparing the cost effectiveness of esomeprazole and omeprazole in the acute treatment of GERD found that esomeprazole 40 mg once daily was more cost effective than omeprazole 20 mg once daily (155). This cost-cutting marketing approach used by AstraZeneca may encourage the prescribing of esomeprazole over omeprazole or its imminent generic substitutes.

9.7 Adverse Effects of Proton-Pump Inhibitors

Headache is one of the most frequently reported adverse events in clinical trials where PPIs have been examined (frequency 1.3–8.8%). Patients with headache also had a significant incidence of diarrhea, nausea, and dizziness. A discontinuation of PPI therapy resulted in a cessation or reduction of the headache in 80.0% (20 of 25) (156).

Possible risks of prolonged treatment with PPIs had been thought to include hypergastrinemia (157), atrophic gastritis, and enteric infections. However, no data have established any true risk to patients, despite many years

Table 3.6 An Overview of the Clinically Marketed PPI Inhibitors; Affinity, Clinical Pharmacology, and Pharmacokinetic Profile

Drug	In *Vitro* pK_B	Clinical Pharmacology: Effect on Gastric Acid Secretion			Oral Absorption	Plasma Half-Life (h)	Volume of Distribution ($L\,kg^{-1}$)	Plasma Protein Binding	Excretion	Metabolism
Omeprazole	9 (208)	Normal		DU	54% (209)	0.6–1 (143)	0.31–0.34 (143)	95% (143)	Urine:feces 4:1 (210)	Hepatic hydmxylation through CYP2C19 (211,212)
		i.v. 40 mg, fewer pre-op patients with pH < 2.5 and a volume of 25 mL (213)	p.o. inhibition of: basal acid, 30 mg, 66%; 60 mg, 92%; PG stimulated acid, 30 mg, 71%; 60 mg, 95%; 7-day treatment, 30 mg and 60 mg, 100% inhibition (214)	p.o. 10, 20, and 30 mg/day for 1 week caused a 37, 90, and 97% decrease of 24-h intragastric acidity (215)						
Pantoprazole	8.6 (208)	80, 120 mg maximal inhibition for up to 21 h; onset of action < 1 h (216)	Maximal inhibition with 60 mg; 40 mg, pH < 3 for 8 h, equieffectivewith omeprazole (217)	Healing rate, 20 mg, 58%; 40 mg, 89%; 80 mg, 82% (217); 4-weeks treatment; healing rate: omeprazole, 20 mg, 77%; pantroprazole, 40 mg, 88%	77% (217)	0.9–1.9% (217)	0.13–0.17 (143)	98% (217)	Renal, ~80% (217)	Hepatic hydmxylation through CYP2C19 (212); subsequently metabolized by sulfotransferase (218)
Lansoprazole	9 (208)	—	Maximal inhibition with ≥30 mg (219)	Dose/healing rate 30 mg/86%; omeprazole 20 mg/82% (220)	85% (221)	0.9–1.6 (143)	0.39–0.46 (143)	97–99% (143)	Biliary, renal (222)	Hepatic hydroxylation through CYP2C19 (212)
Rabeprazole		—	10, 20, 30, and 40 mg, dose-dependent inhibition of acid secretion (223)	20 mg in conjunction with antibiotic eradication rates: omeprazole, 69%; rabeprazole, 84% (224)	52% (224)	1 (143)	—	95–98% (143)	Renal, ~90% (224)	Hepatic hydmxylation through CYP2C19 and CYP3A4 (143)
Esomeprazole	≈ 9	—	20 mg Day 1, 46%; Day 5, 90% (140)	20 mg in conjunction with antihiotic eradication/healing rate: omeprazole, 88192%; esomeprazole 86191% (225)	20 mg, 68%; 40 mg, 89% (226)	0.8–1.2 (211)	—	97% (210)	Urine:feces 4:1 (210)	Hepatic hydmxylation; mainly metabolized by CYP3A4, compared to *R*-omeprazole, which is almost completely metabolized through CYP2C19 (140)

of experience with these agents. PPIs are remarkably safe and well tolerated (158).

10 TARGETS UNDER INVESTIGATION

10.1 Reversible Proton-Pump Inhibitors

The effectiveness of clinically available PPIs relies on the number of active pumps at any one time and the recovery of pumps after biosynthesis. The prolonged suppression of gastric acid secretion produced by both H_2-receptor antagonists and PPIs produces extended periods of hypergastrinemia, which has been associated with the formation of precancerous changes in human gastric mucosa and gastric carcinoids in long-term animal studies. In fact, the development of omeprazole, a proton-pump inhibitor, was delayed because high doses were shown to induce ECL-cell hyperplasia in rodents. However, Astra disputed scientifically that this would occur in humans and omeprazole was eventually marketed in 1988.

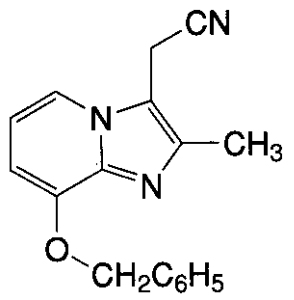
Nonetheless, some research efforts are currently targeted at obtaining reversible PPIs, so-called acid pump antagonists (APAs). These are proposed to offer distinct advantages over both the irreversible proton-pump inhibitors and HA H_2-receptor antagonists (159). Acting at the final stage of gastric acid secretion, molecules of this type have the potential to combine profound inhibition of gastric acid secretion elicited by all stimuli and the dose flexibility available with H_2-receptor antagonists. Several companies have progressed APAs into development, although currently none has progressed beyond phase III clinical trials.

From the medicinal chemistry point of view, the imidazopyridine-based compound SCH 28080 was the prototype of the reversible proton-pump inhibitor (160). As early as 1983, it had been suggested that the antisecretory effect of this compound was directly mediated by the gastric proton pump, and this has been further demonstrated by its ability to antagonize the binding of the irreversible proton-pump inhibitor omeprazole (161). The marked liver toxicity of this compound necessitated follow-up compounds, of which SCH 32651 (162) is an example. Yamanouchi and Shin-Nippon have also described the properties of structurally similar compounds, respectively YM-020 (163) and SPI-447 (161). Development of SPI-447 is ongoing and it has been shown that the compound has no effect on Na^+/K^+-ATPase activity. The mechanism of SPI-447 is thought to be SH-group independent, K^+-competitive, and highly specific against gastric H^+/K^+-ATPase (161) (Fig. 3.11).

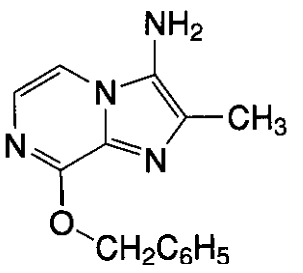
Another program to obtain a reversible proton-pump inhibitor came from workers at SmithKline & French, who selected the substituted quinoline compound SK&F 96067 as an early clinical candidate (164). SKF 96067 is a reversible inhibitor of the H^+/K^+-ATPase protein of the parietal cell (164). In clinical trials SKF 96067 was found to be a more potent inhibitor of gastric acid secretion than the H_2-receptor antagonist ranitidine. This compound reached Phase III clinical trials but has now been discontinued (165). The compound was followed by SKF 97574 (159) that, although of similar potency to that of SKF 96067, displayed a significantly longer duration of action in *vivo*.

As an alternative strategy to escape the drawbacks of the initial leads, the quinoline ring was retained but constrained in a pyrroloquinoline ring system as in SKF 96356 (166). This ring system has also formed the basis of compounds described by the Korea Research Institute of Chemical Technology [AU-006 (167), AU-461 (168), and DBM-819 (169)]. DBM-819 displays potency comparable to that of omeprazole in models of gastric acid secretion and ulceration (169).

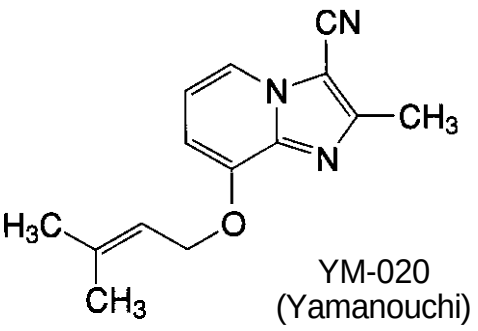
A tetrahydroisoqunoline-based compound, YH-1885, discovered by Yuhan, is currently one of the most clinically advanced reversible proton-pump inhibitors (170). It is now being codeveloped with GlaxoSmithKline for stomach ulcers and gastroesophageal reflux disease. Clinical data on YH-1885 have demonstrated that it is safe and well tolerated when administered as a single dose (60 to 300 mg) or multiple doses (150 to 300 mg) to healthy volunteers. The compound significantly increased gastric pH and increased the fraction of time above pH 3 at doses above 150 mg. During multiple dosing, YH-1885 exhibited a reversible mode of action with no significant



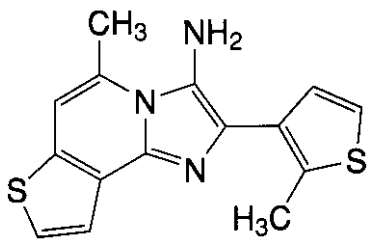
SCH 28080
(Schering-Plough)



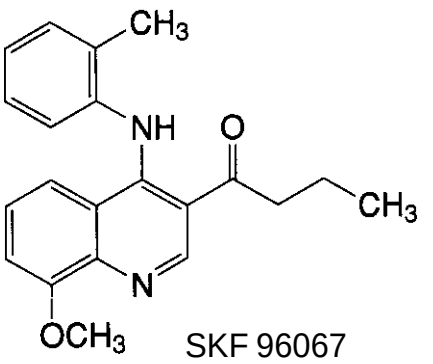
SCH 32651
(Schering-Plough)



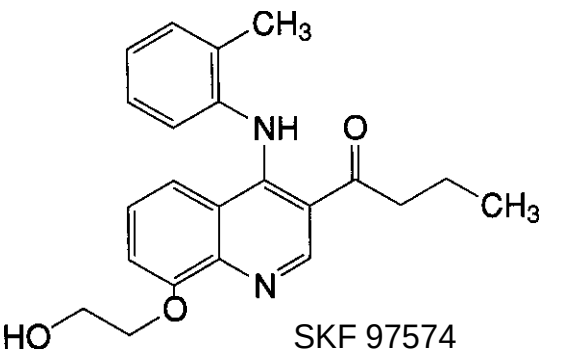
YM-020
(Yamanouchi)



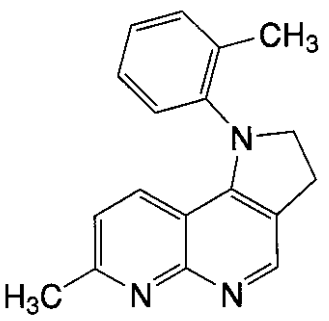
SPI-447
(Shin Nippon)



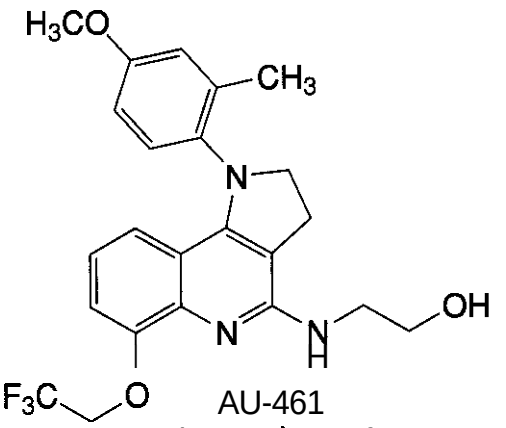
SKF 96067
(SmithKline and French)



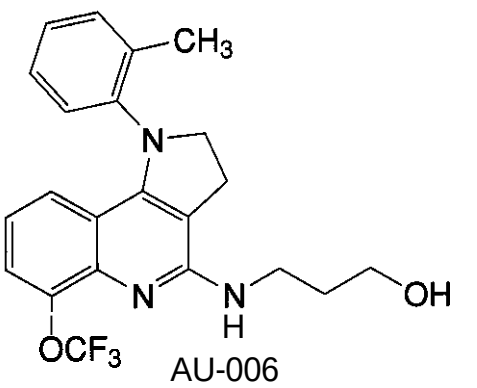
SKF 97574
(SmithKline and French)



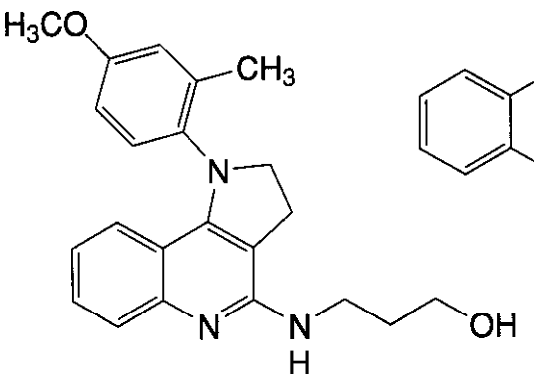
SKF 96356
(SmithKline and French)



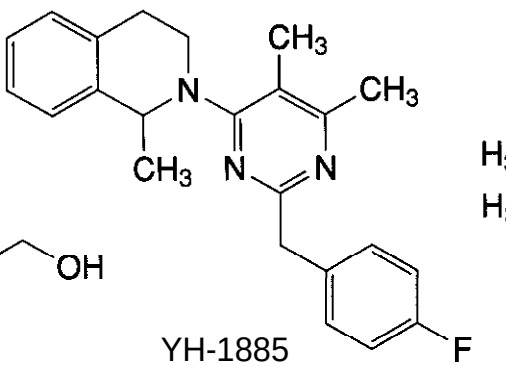
AU-461
(Korea Inst. of Chem. Technology)



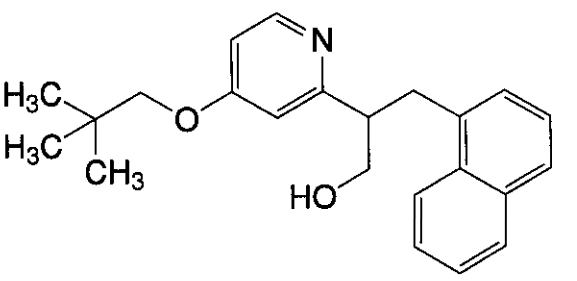
AU-006
(Korea Inst. of Chem. Technology)



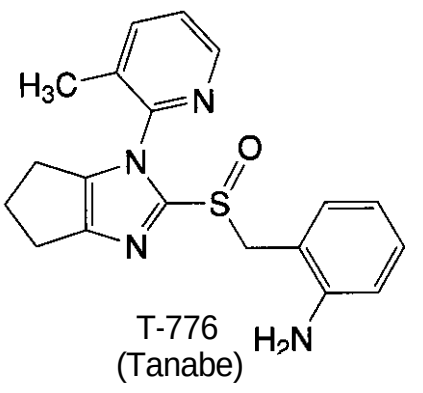
DBM-819
(Korea Inst. of Chem. Technology)



YH-1885
(Yukan)



(4) Banyu Pharm Co.



T-776
(Tanabe)

Figure **3.11.** Representative reversible **PPIs** to illustrate SAR.

accumulation (171). YH-1885 recently entered phase I clinical trials for the treatment of GERD.

Banyu Pharmaceutical Co. has disclosed the properties of proton-pump inhibitors such as (4), which were obtained by chemical modification of the structure of omeprazole, but which are reversible in their interaction with the ATPase enzyme (172). Tanabe, also, has described a proton-pump inhibitor (T-776), which contains many of the structural elements found in the irreversible inhibitors, but which has been shown to possess a reversible mechanism of action (173).

10.2 CCK_2/Gastrin-Receptor Antagonists

Gastrin is the only gut peptide hormone released from the stomach that mediates gastric acid secretion. Gastrin-stimulated release of gastric acid is produced either by direct stimulation of CCK_2/gastrin-receptors on the parietal cell or, in some species, indirectly after CCK_2/gastrin-receptor-mediated HA release from ECL cells. HA activates H_2-receptors on the parietal cell to stimulate gastric acid secretion. The regulation of gastrin and HA-stimulatedgastric acid secretion are key therapeutic targets in controlling hyperacidity disorders. Acid secretion is regulated by several hormones and the inhibition of acid secretion through H_2 and PPIs has a positive feedback effect on the release of gastrin (174, 175).

A number of chemically diverse CCK_2/gastrin-receptor antagonists have been examined in clinical trials as antisecretory agents or inhibitors of panic/anxiety attacks. To date, however, none has been marketed (Table 3.7). CCK_2/gastin-receptor antagonists, including those compounds in preclinical development as well as those that have progressed to humans, were the subject of a recent review (176).

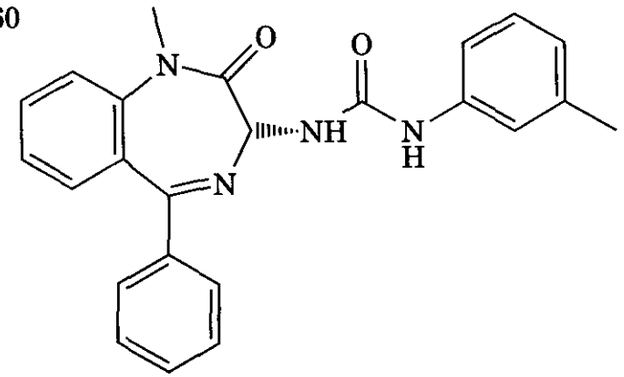
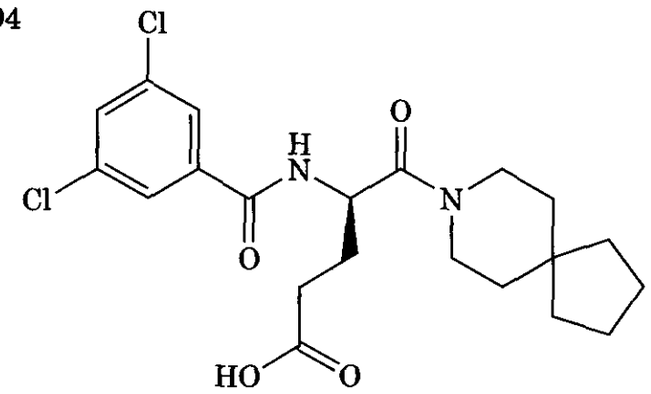
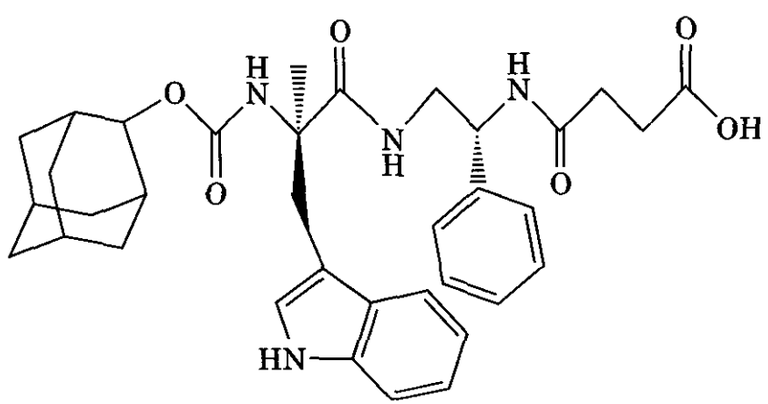
The importance of gastrin in development was investigated through the use of hormone and receptor knockout mice. Gastrin and gastrin-receptor knockout mice are viable, fertile, and develop without any gross abnormalities. Although gastrin expresses trophic properties in conditions of hypergastrinemia (e.g., pernicious anemia), no general atrophy of the gastric mucosa was observed in knockout mice. There was, however, a decrease in the acid-secretory capacity of both mutants. Basal acid secretion was abolished and parietal cells were unresponsive to stimulation by HA, gastrin, or Ach. In gastrin knockout mice the ECL cells contained fewer secretory granules and were located closer to the base of the gastric glands compared to that in normal, litter-mate control mice, an indicator of poor ECL cell activity. In both the gastrin and gastrin-receptor knockout mice, the number of parietal cells was reduced (177).

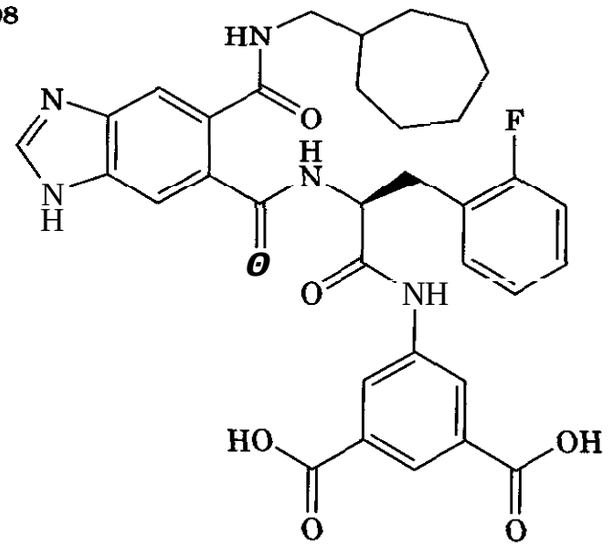
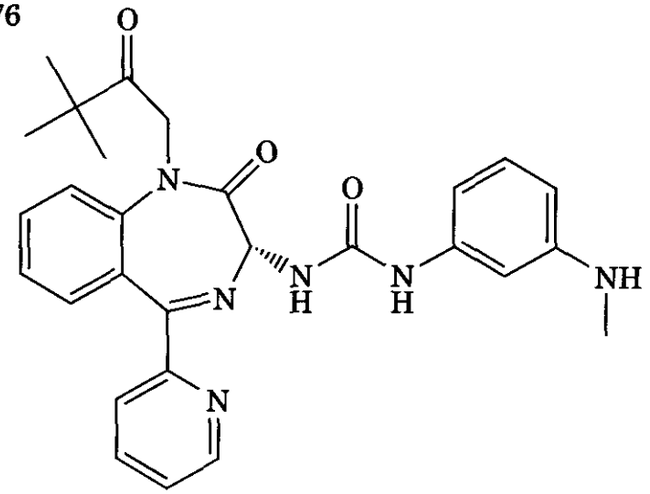
Current gastrin-receptor antagonists (CCK_2-receptor antagonists) inhibit the action of amidated gastrin, although they do not interact with the site at which glycine-extended gastrin binds (178). The gly-gastrin receptor has not been cloned, although there is strong functional evidence that its stimulation by glycine-extended gastrin is a link to proliferative behavior (179). The Aphton Corporation has developed an immunogen, gastrimmune, which is composed of the amino-terminal portion of G17 linked to a diphtheria toxoid. This immunogen stimulates the production of antibodies to both the amidated and glycine-extended forms of gastrin-17 and has been demonstrated to reduce the growth of implanted colon tumors in rats and mice (180). After encouraging results in phase II clinical studies (1811, gastrimmune, in combination with chemotherapy, entered phase III clinical trials in October 2000 for the treatment of stage IV gastric cancer, colorectal cancer, and late-stage pancreatic cancer. These trials have now been extended to include gastric/esophageal cancer, colorectal cancer, pancreatic cancer, and primary liver cancer.

11 SUMMARY

The involvement of more than one endocrine hormone and neurotransmitter in the control of gastric acid secretion has resulted in a number of therapeutic approaches directed toward achieving its inhibition. Of these strategies both muscarinic and histamine H_2-receptor antagonists have been used, with the latter being the method of choice for the control of acid-related disorders, particularly peptic ulcer disease, until the late 1980s. Along with the successful treatment of many patients

Table 3.7 CCK_2/Gastrin Receptor Antagonists That Have Been Evaluated in the Clinic

Compound Identifier	Company	Radioligand Binding (n*M*)	Clinical Evaluation
L-365,260 	**Merck**	8.5	Inhibition of gastric acid secretion: 50 mg, no **effect** on basal acid secretion; **2.5, 10, 50** mg **p.o.** inhibited pentagastrin-stimulated acid secretion (0.05–2 μg kg^{-1} h^{-1}) (227). Inhibition of CCK-4 induced panic attacks: 50 mg **p.o.** reduced frequency and intensity of attacks **(228);** 30 mg **p.o. o.d.** for 7 days failed to inhibit panic in patients (229).
CR2194 	Rotta	**600**	Inhibition of gastric acid secretion: **CR2194 (1, 2.5,** or 7.5 mg kg^{-1} h^{-1}) dose-dependently inhibited gastrin (6.4–800 pmol kg^{-1} h^{-1})-**stimulated** acid secretion. **CR2194** (7.5 mg kg^{-1} h^{-1}) inhibited basal and sham fed acid secretion (230).
PD134,308 	Parke Davis	**1.7**	Inhibition of panic attacks: In a randomized, double-blind study CI-988 (100 mg **TID** for 6 **weeks)** was compared to placebo in patients with panic disorder. *All* patients improved during treatment and no difference in the weekly rate of panic attacks **was** seen between the treatment groups (231).

JB95008		James Black Foundation	10	Inhibition of gastric acid secretion: JB95008 (0.02 and 0.4 mg kg^{-1} h^{-1}) produced 27 and 63% inhibition, respectively, of pentagastrin (1 μg kg^{-1} h^{-1}).
YF476		Yamanouchi Ferring	0.1	Inhibition of gastric acid secretion: YF476 (5, 10, and 25 mg p.o. o.d. for 14 days) acutely (day 1) inhibited inhibited meal-stimulated acid secretion but tachyphylaxis was produced after 14 days' administration (232).

with H_2-receptor antagonists, certain limitations on efficacy became apparent, the most significant of which were a lack of effectiveness in a subset of patients during nocturnal periods and that acid-rebound occurred upon cessation of treatment. Both these limitations were ascribed to the upregulation of other hormonal mechanisms during periods of elevated pH. The realization that histamine and acetylcholine ultimately exerted their effect through the K^+/H^+-ATPase enzyme or proton pump on the parietal cell, provided a potentially more complete means of controlling acid secretion and a new focus for medicinal chemistry. The currently available compounds that control acid-related disorders by inhibiting this enzyme do so in an irreversible manner, achieving sustained acid inhibition.

PPIs and H_Z-receptor antagonists between them have successfully treated disorders caused by increased gastric acid secretion in a vast number of patients worldwide. Their use has continued to be applicable even with the identification of H. *pylori*, a bacterium that is highly correlated with the incidence of ulcer disease. H_Z-receptor antagonists and PPIs have proven to be highly effective, are well tolerated, and are considered safe. Although significant progress in obtaining reversible K^+/H^+-ATPase inhibitors as well as CCK_2/gastrin-receptor antagonists has been made in recent years, compounds from either of these groups seem unlikely to dislodge the irreversible PPIs as the preferred treatment in acid-related disorders at present. Rather, single-enantiomer versions of compounds such as omeprazole, because of their associated pharmacokinetic advantage over the racemic compounds, seem set to retain their position among the highest earning prescription drugs for some time to come.

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CHAPTER FOUR

Chemokine and Cytokine Modulators

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Contents

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1 INTRODUCTION

Cell/cell communication is an important event in an organism's ability to respond to insult. Cytokines and chemokines play an important role in this process **(1).** Disease processes in which one or more of these factors are overexpressed, or deficient, may benefit from modulation of these cell-signaling events.

Cytokines can exist as both extracellular soluble proteins (8–40 kDa) and cell surface molecules. In response to insult, they are able to regulate host defense, differentiation, cell division, apoptosis, repair, and inflammation in a variety of cell types. Originally termed lymphokines and monokines, based on their cellular source, it was eventually recognized that these soluble mediators are more generally products of leukocytes. Because many of them communicate signals between leukocytes, many of the cytokines are now designated as interleukins. Cytokines are both pleiotropic and redundant in their ability to communicate and initiate biological responses. They can regulate cell function in an autocrine, paracrine, or intercrine fashion through cell surface receptors that are classified into families on the basis of structure and function (2). Cytokine receptors are composed of single-spanning membrane domains that form high affinity complexes upon binding with their ligands. Signaling does not require high receptor occupancy and can occur with very low concentrations of ligand, proceeding through tyrosine phosphorylation using nonreceptor tyrosine kinases such as Janus kinase (JAK). This cytoplasmic signal then triggers a distinct cellular response. Recombinant proteins have been evaluated therapeutically in cytokine-deficient patients in an effort to enhance a desired physiologic function. For example, erythropoietin has been used in cancer patients on chemotherapy or in chronic renal failure patients, as a method to stimulate red blood cell production. In contrast, overproduction of cytokines may result in an untoward response such as inflammation, cancer, or autoimmune disease, and blocking the production or activity of these cytokines has also been shown to have therapeutic benefit.

Chemokines and chemokine receptors play an important role in chemotaxis (cell trafficking). The term chemokine is derived from *che*motactic cytokine. Chemokines are protein

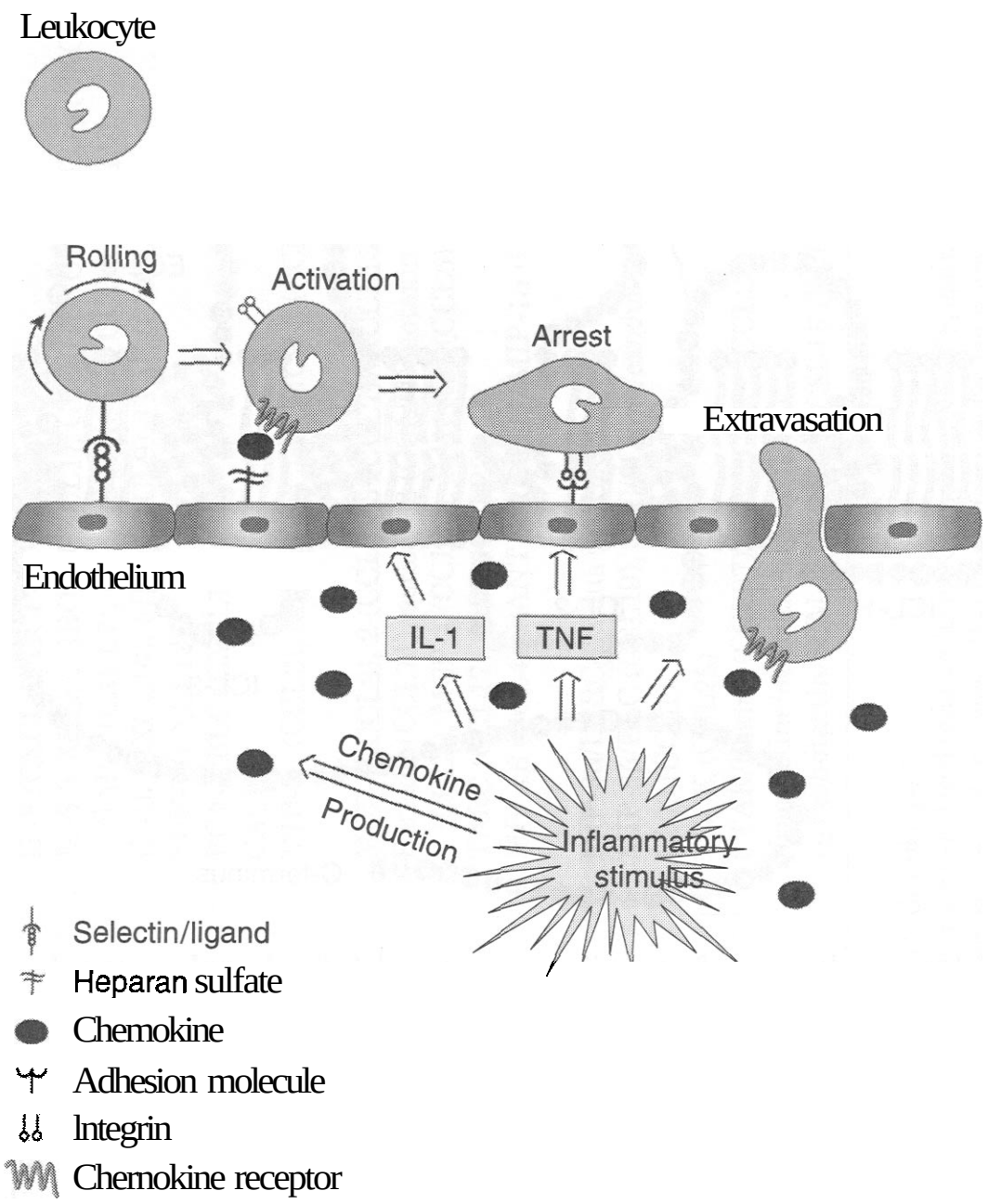

Figure 4.1. Schematic representation of leukocyte transmigration induced by chemokines. The first step involves rolling attributed to interaction between the leukocyte and the endothelial cells. This process is mediated by selectins and selectin ligands expressed on the surface of both cell types. In the next step a chemokine interacts with its receptor, inducing leukocyte activation and conformational changes in the adhesion molecules (integrins) and resulting in firm adhesion to the endothelial surface. It is believed that the chemokine is immobilized on the endothelial surface by interactions with glycosaminoglycans. The leukocytes then transmigrate into the tissues.

ligands (8–10 kDa) that signal through interaction with seven-transmembrane G-protein-coupled receptors (GPCRs). Two primary functions for chemokines are recruitment of leukocytes from circulation to a site of injury or inflammation, and regulation of homeostatic leukocyte trafficking. In addition to being able to initiate a specific cell migration, chemokines are also implicated in leukocyte activation, proliferation, angiogenesis, and lymphopoiesis, and can act as coreceptors for HIV. Although the chemokine field has a young history, significant advances have been made and modulators of chemokine/chemokine receptor interaction are currently being evaluated in clinical trials.

2 CHEMOKINES AND CHEMOKINE RECEPTOR BIOLOGY

Leukocyte migration to tissues is essential for development of an inflammatory response. The process involves two steps: arrest and firm adhesion of circulating cells on endothelial surfaces and migration through the endothelium into the interstitium (Fig. 4.1) (3). It has become clear in recent years that both pro-

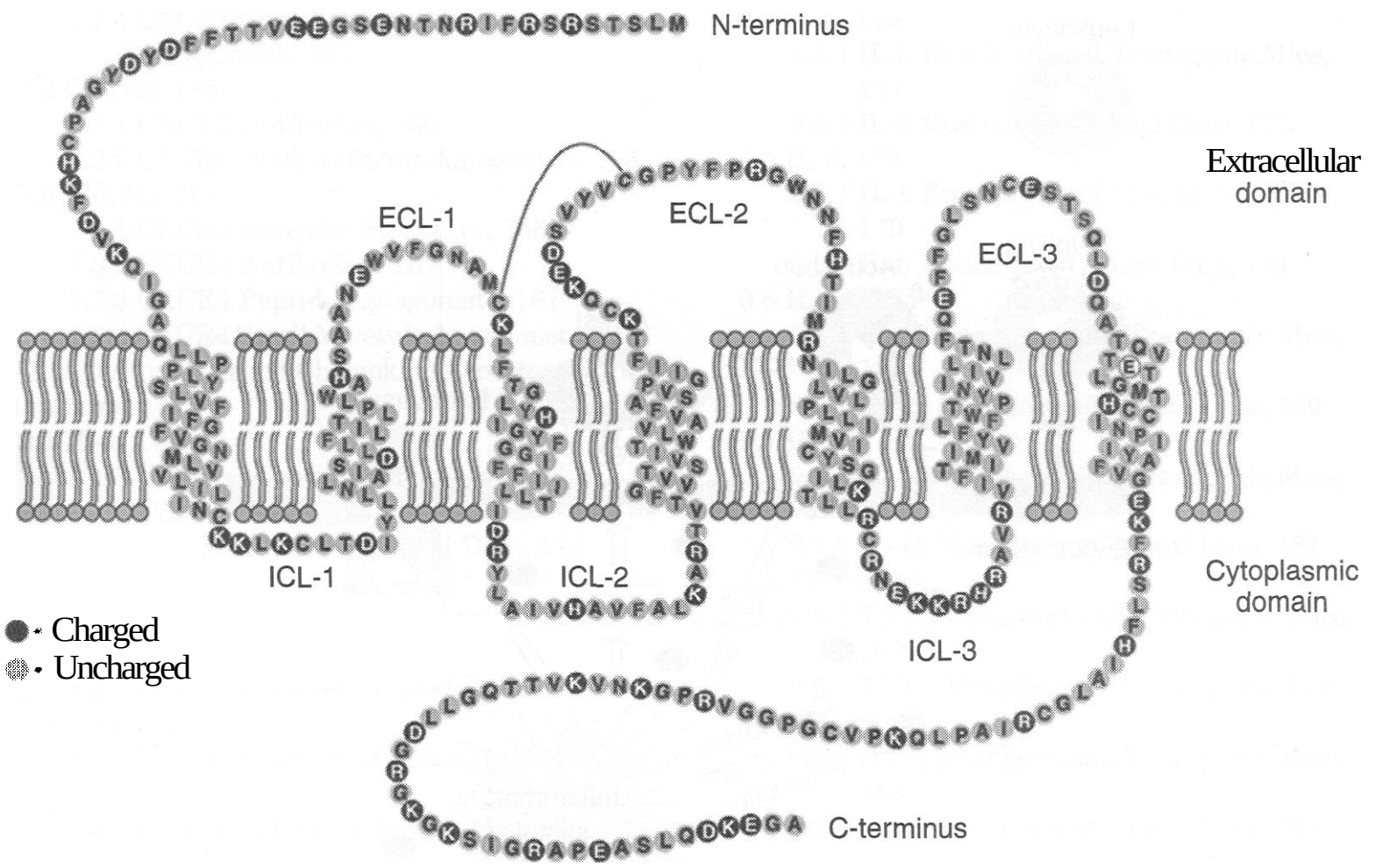

Figure 4.2. Schematic diagram of a two-dimensional model of a chemokine receptor. The extracellular loops (ECL), the putative helical regions that traverse the cell membrane, and the intracellular loops (ICL) are shown. The GPCR (family A) signature disulfide bridge between Cys residues of ECL1 and ECL2 is also shown.

cesses are regulated largely by a group of low molecular weight cytokines, the chemokine family. Over 40 chemokines have been identified to date (for reviews, see Refs. 4–7). Based on the spacing of two amino terminal cysteines, they are classified into two major and two minor families. In the CXC family (α family) one amino acid separates the first two cysteines, whereas in the CC family (β family) the two first cysteines are adjacent to each other. The two minor families of chemokines include only one member each, the C family represented by lymphotactin and the CX_3C family represented by fractalkine.

Chemokines exert their action through seven-transmembrane receptors, which are GPCRs (Fig. 4.2). Six CXC receptors, 10 CC receptors, and one receptor each for lymphotactin and fractalkine have been identified. Table 4.1 summarizes the human chemokine receptor family, their ligand specificity, and the cell types that predominantly express these receptors. The new nomenclature for chemokines is also included in the table (8).

One important feature of the chemokine and chemokine receptor family is the high degree of promiscuity with regard to ligand binding. Only a few receptors have been identified that bind with high affinity to only one ligand, and most ligands interact with more than one receptor. This apparent redundancy could be a potential problem in the development of receptor antagonists as therapeutics. However, it is not clear whether the overlapping activities of chemokines are relevant in the *in vivo* situation. It may be that each chemokine plays a specific function in a specific setting at a specific time, orchestrating a coordinated chemotactic response. In addition, CXC chemokines bind with high affinity only to CXC receptors and CC chemokines bind to CC receptors, so there is some degree of selectivity in the family.

From the functional point of view, chemokines from the CC family in general do not act on neutrophils but attract monocytes, eosinophils, basophils, lymphocytes, and dendritic cells (9, 10). (One notable exception is the abil-

Table 4.1 Chemokine Receptor Families[a]

Name	Cell type[b]	Ligands[c]
CXCR1	Neutrophils	IL-8 (CXCL8), GCP-2 (CXCL6)
CXCR2	Neutrophils	IL-8 (CXCL8), GROα (CXCL1), GROβ (CXCL2), GROγ (CXCL3), NAP-2 (CXCL7), ENA-78 (CXCL5), GCP-2 (CXCL6)
CXCR3	T-cells (Thl and Tcl)	IP-10 (CXCL10), MIG (CXCL9), ITAC (CXCL11)
CXCRA	Naïve T-cells, B-cells	SDF-1 (CXCL12)
CXCR5	B-cells, subset of T-cells	BCA-1/BLC (CXCL13)
CXCR6	Thl and Tcl cells	CXCL16
CCR1	Activated T-cells, monocytes, eosinophils, immature dendritic cells, basophils	MIP-la (CCL3), MIP-1β (CCL4), MCP-3 (CCL7), RANTES (CCL5)
CCR2	Monocytes, macrophages, activated T-cells, NK cells	MCP-1 (CCL2), 2 (CCL8), 3 (CCL7), 4(CCL13)
CCR3	Eosinophils, basophils, activated T-cells (Th2)	Eotaxin (CCL11), eotaxin 2 (CCL24), eotaxin 3 (CCL26), MCP-3 (CCL7), MCP-4 (CCL13), RANTES (CCL5)
CCRA	Activated T-cells, basophils, platelets	TARC (CCL17), MDC (CCL22)
CCR5	Activated T-cells, monocyte/macrophages, immature dendritic cells	MIP-1β (CCL4), RANTES (CCL5), MIP-la (CCL3)
CCR6	T-cells, immature dendritic cells, B-cells	MIP-3α/LARC/exodus (CCL20)
CCR7	Mature dendritic cells, subset T- and B-cells	MIP-3β/ELC (CCL19), SLC/6Ckine/exodus 2 (CCL21)
CCR8	Monocyte/macrophages, activated T-cells (Th2)	I-309 (CCL1)
CCR9	Thymocytes, T-cells	TECK (CCL25)
CCR10	T-cells, Langerhans cells	CTAK/Eskine (CCL27), MEC/CCK1 (CCL28)
CX_3CR1	T-cells, natural killer cells	Fractalkine/neurotactin (CX_3CL1)
XCR1	T-cells	Lymphotactin/SCM-1α (XCL1), SCM-1β (XCL2)

[a]Data from Refs. 9, 11, and 12.

[b]Th1, T helper cell type 1; Th2, T helper cell type2; Tcl, T cytotoxic cell type 1; Tc2, T cytotoxic cell type 2.

[c]BCA, B-cell–activating chemokine; BLC, B-lymphocyte chemoattractant; ELC, Epstein–Barr virus-induced receptor ligand chemokine; ENA-78, epithelial cell–derived neutrophil-activating factor, 78 amino acids; GCP-2, granulocyte chemoattractant protein; GRO, growth-related oncogene; IP-10, interferon-inducible protein 10; I-TAC, interferon-inducible T-cell a chemoattractant; LARC, liver- and activation-related chemokine; MDC, macrophage-derived chemokine; MIG, monokine induced by γ interferon; MIP, monocyte inflammatory protein; MCP, monocyte chemoattractant protein; NAP, neutrophil-activating protein; RANTES, regulated on activation, normal T expressed and secreted; SDF-1, stromal cell–derived factor 1; SLC, secondary lymphoid tissue chemokine; TARC, thymus- and activation-related chemokine; TECK, thymus-expressed chemokine.

ity of CCR1/MIP-1α to generate a chemotactic response for neutrophils in rodents.) On the other hand, chemokines from the CXC family can be further subdivided into two classes: those that contain an ELR sequence, which attract neutrophils, and the subset that lacks the ELR motif, which attracts lymphocytes (13, 14).

Based on their expression patterns, chemokines can be classified as inducible or constitutive. Inducible chemokines participate primarily in inflammatory responses and are the majority members of the family. The constitutive chemokines are expressed primarily, but not exclusively, in secondary lymphoid organs, and recent evidence suggests they play a major role in lymphocyte and dendritic cell homing (15). It has been shown that these chemokines can also be responsible for the organization of specialized structures inside lymphoid organs, such as formation of germinal centers (16). Chemokine receptor expression is crucial for determining the migration pattern of leukocytes. Some receptors are restricted to certain cell types. For example, CXCRl is expressed predominantly in neutrophils, whereas others like CCR2 are expressed on a variety of leukocytes, including monocytes, T-cells, basophils, dendritic cells, and natural killer cells. Most leukocytes express more than one receptor at any given time. Regulation of chemokine receptor expression has recently been shown to occur upon activation or deactivation of monocytes, dendritic cells, and T-cells (5, 15, 17).

3 CHEMOKINE RECEPTOR SIGNALING

The intracellular signals involved in chemotaxis are not yet fully understood, and much of the information available today has been deduced from signaling information for other GPCRs. However, significant new data regarding the chemotactic process have accumulated in recent years. Like other seven-transmembrane receptors, chemokine receptors couple to G-proteins. Many chemokine-induced signaling events are inhibited by *Bordetella pertussis* toxin (PTX), suggesting that chemokine receptors are linked to G-proteins of the Gai class (18,19). In cotransfection experiments it has been shown that CXCRl and CXCR2 couple to Gαi2, Gαi3, Gα14, and Gα16 (20). Physical association between Gαi and several chemokine receptors has been documented (21, 22). In some studies, PTX did not completely block the calcium response, suggesting the chemokine receptors may couple to other G-proteins such as Gq or Gα16. In addition, it has been suggested that the specificity of the coupling may be cell type specific (23, 24).

Chemokine receptor activation leads to the generation of a complex cascade of cellular events, including the generation of inositol triphosphates, the release of intracellular calcium, and the activation of protein kinase C. The release of the Gαiβγ subunits from Gai has been described as an essential step in this process (25). However, the release from Gas or Gaq does not result in induction of chemotaxis, suggesting that the βγ subunits are necessary but not sufficient to induce cell migration and that Gai itself plays some role in the process (26).

Activation of the chemokine receptor leads to rapid activation of phosphoinositide-specific phospholipases, which leads to inositol-1,4,5-triphosphate formation and a transient rise in intracellular calcium (18). Phospholipase C (PLC) isoforms that are involved in chemokine receptor activation become activated by direct interaction with the βγ subunits. In addition to its interaction with PLCs, the βγ subunits also interact with the type I, phosphoinositol 3 kinase γ (PI3Kγ), and activation of this enzyme results in the formation of PtdIns(3,4,5)P3 (17). Mice that do not express PI3Kγ have severely impaired chemokine-stimulated signal transduction, and PKB is not activated, suggesting an important role for this pathway in the chemotactic process (27–29). Although leukocytes isolated from these mice showed a decrease in cell chemotaxis, the response is not completely lost, and under conditions of complete PI3Kγ inhibition, neutrophils can still chemotax in response to chemokines (30).

Receptor dimerization upon ligand binding has been described mostly for the growth factor receptor; however, recent reports have also suggested heterodimerization for seven-transmembrane receptors, including chemokine receptors (31–33). It has been proposed that che-

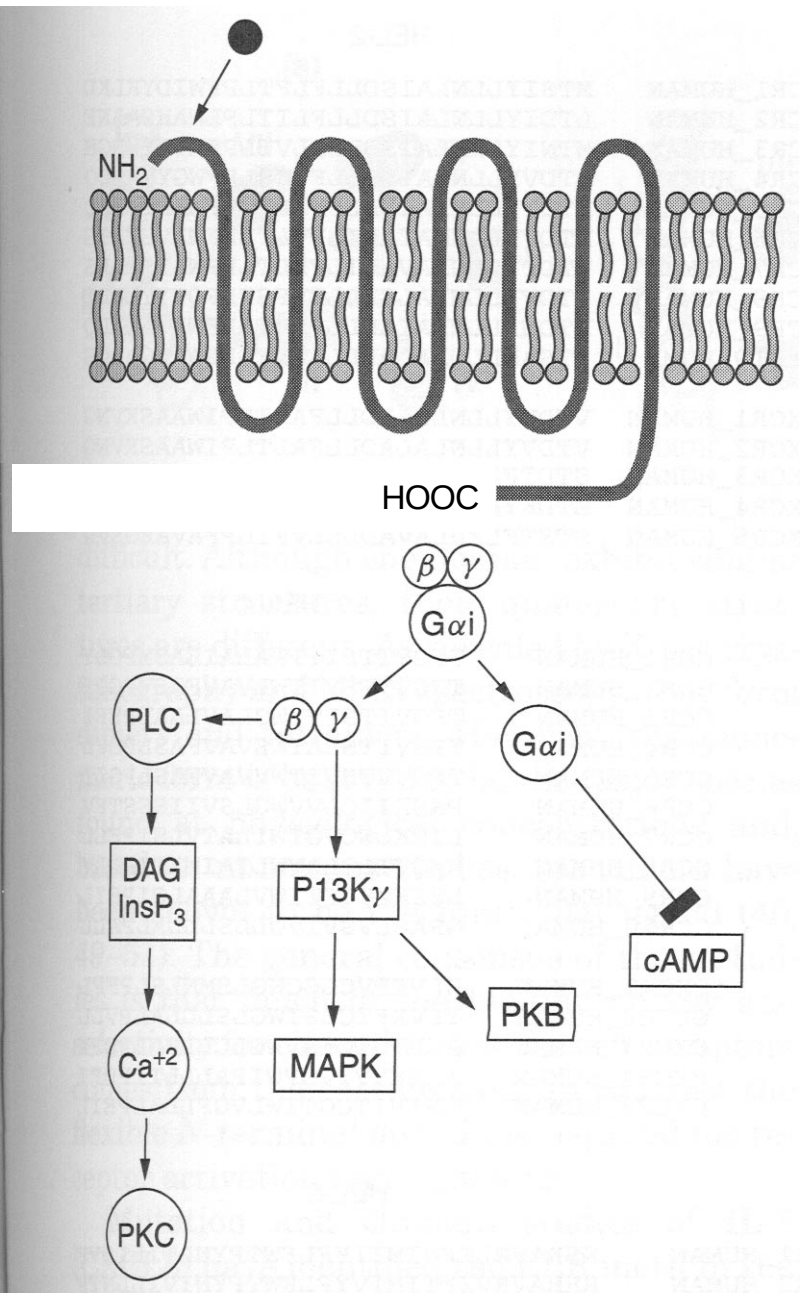

Figure 4.3. Schematic diagram showing the proposed chemokine receptor signaling pathways. This diagram does not represent the complete pathway. Ligand binding induces the exchange of GDP for GTP on the G protein and this causes dissociation of the subunits. Gαi decreases the levels of cAMP and Gβγ causes the activation of PLC and PI3Kγ. PLC, phospholipase C; DAG, diacylglycerol; IP3, inositol triphosphate; PKC, protein kinase C; MAPK, mitogen-activated protein kinase; PI3Kγ, phosphoinositol 3 kinase γ; PKB, protein kinase B.

mokine receptor dimerization results in the activation of the Janus kinase-signal transducers and activator of transcription (JAK-STAT) signaling pathway (34). Figure 4.3 shows a schematic of the pathways involved in chemokine receptor signaling.

4 CHEMOKINE RECEPTOR STRUCTURE

It had been speculated **20** years ago that GPCRs have a seven-transmembrane (TM) helical motif that traverses the cell membrane (Fig 4.3) (35–37). The recent elucidation of a high resolution, three-dimensional (3D) X-ray structure of rhodopsin has confirmed the seven-transmembrane domain topology (38). GPCRs are classified into four families (A-D), with chemokine receptors belonging to the type A (e.g., rhodopsin-like). These single-chain, membrane-bound proteins have an extracellular N-terminal (NT) domain and three extracellular loops (ECL1-ECL3) that act in concert to bind a chemokine ligand (Fig. 4.2). The cytoplasmic domain of the receptor is composed of three loops and a C-terminal segment, which also act in concert to transduce the chemokine signal. The core of the receptor is defined by seven-transmembrane spanning helices. Based on the rhodopsin X-ray structure, the TM-a helices vary in length and can extend beyond the lipid bilayer. The orientation of the TMs impose a stereo and geometric specificity on a ligand's ability to enter the TM binding domain. The TM core is mainly formed by transmembrane a-helices 2, 3, 5, 6, and 7. Chemokine receptors, like other A-family GPCRs, have the conserved signature amino acid sequence in all seven TMs (TM1 GN, TM2 D, TM3 DRY, TM4 P, TM5 P, TM6 W, and TM7 NP) (Figs. 4.4a, 4.4b, 4.4c, 4.4d, and 4.5). There are also conserved amino acid residues that are a unique signature of chemokine receptors. These residues are the Cys-Cys motif in TM7, an acidic Glu residue (E) in TM7, and cysteines in both the N-termini and ECL3 that form a disulfide bond (Fig. 4.2).

5 CHEMOKINE LIGAND STRUCTURE

Remarkable progress has been made in solving the molecular structure of chemokines by high resolution X-ray crystallography and NMR spectroscopy (39–48). Regardless of the low degree of sequence homology among the chemokines, they all exhibit a common folding pattern that adopts a three-stranded antiparallel β-sheet, to which a C-terminal short α-helix is obliquely attached (Fig. 4.6). Intramolecular disulfide bonds stabilize this structure. The N-terminal amino acids preceding the first conserved cysteine have a large degree of movement, which renders structural studies

HEL-1

```
CCR1_HUMAN   FGAQLLPPLYSLVNIGLVGNILWLVLVQ
CCR2_HUMAN   IGAQLLPPLYSLVFIFGFVGNMLVVLILIN
CCR3_HUMAN   LMAQFVPPLYSLVFTVGLLGNVVVVMILIK
CCR4_HUMAN   FGELFLPPLYSLVFVFGLLGNSVVVLVLFK
CCR5_HUMAN   IAARLLPPLYSLVFIFGFVGNMLVILILIN
CCR6_HUMAN   FSRLNPIAYSLICVFGLLGNILWITFAF
CCR7_HUMAN   FKAWFLPIMYSIICFVGLLGNGLVVLTYIY
CCR8_HUMAN   NGKLLLAVFYCLLFVFSLLGNSLVILVLVV
CCR9_HUMAN   FASHFLPPLYWLVFIVGALGNSLVILVYWY
CCR10_HUMA   FSRAFQPSVSLTVAALGLAGNGLVLATHLA

CXCR1_HUMAN  LNKYVVIIAYALVFLLSLLGNSLVMLVILY
CXCR2_HUMAN  INKYFVVIIYALVFLLSLLGNSLVMLVILY
CXCR3_HUMAN  FDRAFLPALYSLLFLLGLLGNGAVAAVLLS
CXCR4_HUMAN  FNKIFLPTIYSIIFLTGIVGNGLVILVMGY
CXCR5_HUMAN  FKAVNPVAYSLIFLLGVIGNVLVLVILER
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HEL-2

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CCR1_HUMAN   MTSIYLLNLAISDLLFLFTLPFWIDYKLKD
CCR2_HUMAN   LTDIYLLNLAISDLLFLITLPLWAHSAANE
CCR3_HUMAN   MTNIYLLNLAISDLLFLVTLPFWIHYVRGH
CCR4_HUMAN   MTDVYLLNLAISDLLFVFSLPFWGYYAADQ
CCR5_HUMAN   MTDIYLLNLAISDLFFLLTVPFWAHYAAAQ
CCR6_HUMAN   MTDWLLNMAIADILFVLTLPFWAVSHATG
CCR7_HUMAN   MTDTYLLNLAVADILFLLTLPFWAYSAAKS
CCR8_HUMAN   ITDVYLLNLALSDLLFVFSFPFQTYYLLDQ
CCR9_HUMAN   MTDMFLLNLAIADLLFLVTLPFWAIAAADQ
CCR10_HUMA   PTSAHLLQLALADLLLALTLPFAAAGALQG

CXCR1_HUMAN  VTDWLLNLALADLLFALTLPIWAASKVNG
CXCR2_HUMAN  VTDWLLNLALADLLFALTLPIWAASKVNG
CXCR3_HUMAN  STDTFLLHLAVADTLLVLTLPLWAVDAAVQ
CXCR4_HUMAN  MTDKYRLHLSVADLLFVITLPFWAVDAVAN
CXCR5_HUMAN  STETFLFHLAVADLLLVFILPFAVAEGSVG
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HEL-3

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CCR1_HUMAN   DAMCKILSGFYYTGLYSEIFFIILLTIDRYLAI
CCR2_HUMAN   NAMCKLFTGLYHIGYFGGIFFIILLTIDRYLAI
CCR3_HUMAN   HGMCKLLSGFYHTGLYSEIFFIILLTIDRYLAI
CCR4_HUMAN   LGLCKMISWMYLVGFYSGIFFVMLMSIDRYLAI
CCR5_HUMAN   NTMCQLLTGLYFIGFFSGIFFIILLTIDRYLAV
CCR6_HUMAN   NATCKLLKGIYAINFNCGMLLLTCISMDRYIAI
CCR7_HUMAN   VHFCKLIFAIYKMSFFSGMLLLLCISIDRYVAI
CCR8_HUMAN   TVMCKVVSGFYYIGFYSSMFFITLMSVDRYLAV
CCR9_HUMAN   TFMCKVVNSMYKMNFYSCVLLIMCISVDRYIAI
CCR10_HUMA   SATCRTISGLYSASFHAGFLFLACISADRYVAI

CXCR1_HUMAN  TFLCKVVSLLKEVNFYSGILLLACISVDRYLAI
CXCR2_HUMAN  TFLCINVSLLKEVNFYSGILLLACISVDRYLAI
CXCR3_HUMAN  SGLCKVAGALFNINFYAGALLLACISFDRYLNI
CXCR4_HUMAN  NFLCKAVHVIYTVNLYSSVLILAFISLDRYLAI
CXCRS-HUMAN  TFLCKTVIALHKVNFYCSSLLLACIAVDRYLAI
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HEL-4

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CCR1_HUMAN   TFGVITSIIIWALAILASMPGLY
CCR2_HUMAN   TFGWTSVITWLVAVFASVPGII
CCR3_HUMAN   TFGVITSIVTWGLAVLAAI  I
CCR4_HUMAN   TYGVITSLATWSVAVFASI  L
CCR5_HUMAN   TFGVVTSVITWVVAVFASLPGII
CCR6_HUMAN   PRSKIICLVVWGLSVIISSSTFV
CCR7_HUMAN   LISKLSCVGIWILATVLSIPELL
CCR8_HUMAN   RMGTTLCLAVWLTAIMATIPLLV
CCR9_HUMAN   LYSKMVCFTIWVLAAALCIPEIL
CCR10_HUMA   GRAHLVSVIVWLLSLLLALPALL

CXCR1_HUMAN  HLVKFVCLGCWGLSMNLSLPFFL
CXCR2_HUMAN  YLVKFICLSIWGLSLLLALPVLL
CXCR3_HUMAN  ARVTLTCLAVWGLCLLFALPDFI
CXCR4_HUMAN  LAEKVVYVGVWIPALLLTIPDFI
CXCRS-HUMAN  LSIHITCGTIWLVGFLLALPEIL
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HEL-5

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CCR1_HUMAN   KLFQALKLNLFGLVLPLLVMIICYTG
CCR2_HUMAN   NNFHTIMRNILGLVLPLLIMVICYSG
CCR3_HUMAN   RHFHTLRMTIFCLVLPLLVMAICYTG
CCR4_HUMAN   KVLSSLEINILGLVIPLGIMLFCYSM
CCR5_HUMAN   KNFQTLKIVILGLVLPLLVMVICYSG
CCR6_HUMAN   KLLMLGLELLFGFFIPLMFMIFCYTF
CCR7_HUMAN   FITIQVAQMVIGFLVPLLAMSFCYLV
CCR8_HUMAN   KIFTNFKMNILGLLIPFTIFMFCYIK
CCR9_HUMAN   KSAVLTLKVILGFFLPFWMACCYTI
CCR10_HUMA   KGASAVAQVALGFALPLGVMVACYAL

CXCR1_HUMAN  RMVLRILPHTFGFIVPLFVMLFCYGF
CXCR2_HUMAN  RMLLRILPQSFGFIVPLLIMLFCYGF
CXCR3_HUMAN  RTALRVLQLVAGFLLPLLVMAYCYAH
CXCR4_HUMAN  VVVFQFQHIMVGLILPGIVILSCYCI
CXCRS-HUMAN  WFTSRFLYHVAGFLLPMLVMGWCYVG
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HEL-6

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CCR1_HUMAN   KSKAVRLIFVIMIIFFLFWTPYNLTILISVF
CCR2_HUMAN   RHRAVRVIFTIMIVYFLFWTPYNIVILLNTF
CCR3_HUMAN   KYKAIRLIFVIMAVFFIFWTPYNVAILLSSY
CCR4_HUMAN   KNKAVKMIFAVVVLFLGFWTPYNIVLFLETL
CCR5_HUMAN   RHRAVRLIFTIMIVYFLFWAPYNIVLLLNTF
CCR6_HUMAN   RHKAIRVIIAVVLVFLACQIPHNMVLLVTAA
CCR7_HUMAN   RNKAIKVIIAVVVVFIVFQLPYNGVVLAQTV
CCR8_HUMAN   KTKAIRLVLIWIASLLFWVPFNWLFLTSL
CCR9_HUMAN   KHKALKVTITVLTVFVLSQFPYNCILLVQTI
CCR10_HUMA   RRRALRVWALVAAFWLQLPYSLALLLDTA

CXCR1_HUMAN  KHRAMRVIFAVVLIFLLCWLPYNLVLLADTL
CXCR2_HUMAN  KHRAMRVIFAVVLIFLLCWLPYNLVLLADTL
CXCR3_HUMAN  RLRAMRLVVVVVVAFALCWTPYHLVVLVDIL
CXCR4_HUMAN  KRKALKTTVILILAFFACWLPYYIGISIDSF
CXCR5_HUMAN  RQKAVRVAILVTSIFFLCWSPYHIVIFLDTL
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HEL-7

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CCR1_HUMAN   LAVQVTEVIAYTHCCVNPVIY
CCR2_HUMAN   QATQVTETLGMTHCCINPIIY
CCR3_HUMAN   LVMLVTEVIAYSHCCMNPVIY
CCR4_HUMAN   YAIQATETLAFVHCCLNPIIY
CCR5_HUMAN   QAMQVTETLGMTHCCINPIIY
CCR6_HUMAN   YTKTVTEVLAFLHCCLNPVLY
CCR7_HUMAN   IAYDVTYSLACVRCCVNPFLY
CCR8_HUMAN   YATHVTEIISFTHCCVNPVIY
CCR9_HUMAN   ICFQVTQTIAFFHSCLNPVLY
CCR10_HUMAN  VALLVTSGLALARCGLNPVLY

CXCR1_HUMAN  RALDATEILGFLHSCLNPIIY
CXCR2_HUMAN  RALDATEILGILHSCLNPLIY
CXCR3_HUMAN  VAKSVTSGLGYMHCCLNPLLY
CXCR4_HUMAN  KWISITEALAFFHCCLNPILY
CXCR5_HUMAN  VAITMCEFLGLAHCCLNPMLY
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Figure 4.4. Sequence alignment of human CC and CXC chemokine receptors. For clarity, only the predicted seven (I-VII) transmembrane helical domains are shown.

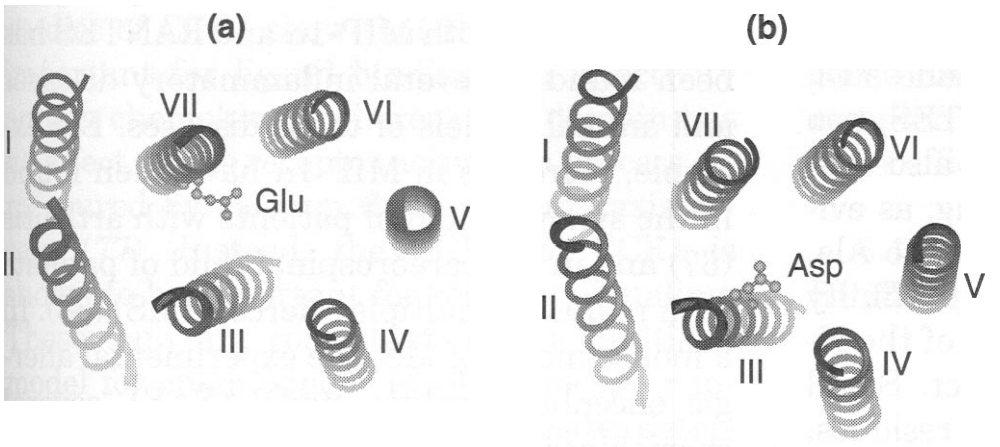


Figure 4.5. Top view of a chemokine binding site model (a), showing only the helical backbone of residues in transmembrane domains I-VII; the side chain for the conserved Glu in TM (VII) is shown. Top view of a biogenic amine binding site model (b), showing only the helical backbone of residues in transmembrane domains I-VII; the side chain for the conserved Asp residue in TM (III) is shown.

difficult. Although chemokines exhibit similar tertiary structures, their quaternary structures are different. As revealed by X-ray crystallography and NMR spectroscopy, they form dimers and tetramers. However, the monomeric form is believed to be the major species found at physiological concentrations and, based on numerous studies, monomers have been shown to be the functional ligand (40, 49–51). The general consensus of these studies is that much of the binding energy and selectivity for chemokines with their receptors comes from the core domain. In contrast the flexible *N*-terminal domain is required for receptor activation and signaling.

Mutation and chimera studies of IL-8 (CXCL8) have identified the ELR motif in the N-terminal domain of IL-8, MGSA (CXCL1), and NAP-2 (CXCL7) as essential for activation of CXCRl and CXCR2 (13). Similar studies with SDF-1 revealed that Lysl and Pro2

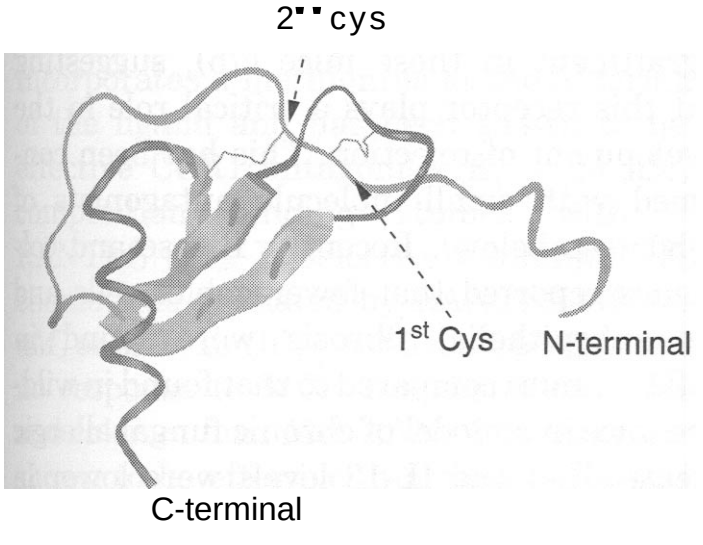


Figure 4.6. Monomer structure of the monocyte chemotactic protein 1 (MCP-1). The structure was retrieved from the Brookhaven data bank (http://www.pdb.bnl.gov/) and displayed by Insight software (Molecular Simulations, San Diego, CA).

are required for activation of CXCR4 (52). Furthermore, although the N-terminus of RANTES (CCL5) contributes significantly to CCR5 activation, it adds little to the receptor-binding affinity (53). Considering that one can modulate signaling without significantly interfering with binding, Simmons and coworkers made a chemically derivatized form of RANTES (AOP-RANTES), which retains nanomolar receptor-binding activity yet serves as a CCR5 antagonist (54). In a similar study it has been shown that deletion of eight or nine residues at the N-terminal of MCP-1 (CCL2) reduced the level of CCR2 binding by less than seven times, and yet this truncation completely inhibits chemotactic response (55–57).

For most chemokines much of the binding energy comes from the core domain, and certain surface residues have been identified as contributing significantly to their binding affinity. The critical residues in RANTES and IL-8 are F12 and 110, respectively. Mutation of these to alanine caused 5000-fold reductions in affinity for CCR3/CCR5 (RANTES receptors) and 100-fold reductions in affinity for CXCRl (IL-8 receptor) (58). Of the surface-exposed residues in MCP-1, Y13 contributes the most to MCP-1 binding affinity (55). In contrast, the corresponding binding contribution of this aromatic residue in eotaxin is much less than the corresponding residue in other CC chemokines (59). Thus, residues immediately following the first two cysteines may in general be a common recognition element in chemokines (58).

Additional hydrophobic residues between the second cysteine and a 3–10 helix in the "N-loop" have also been shown to be important for chemokine binding. The N-loop in

IL-8 and MGSA is important for receptor binding and for determining the specificity (60). The key residues in IL-8 include Y13, F17, F21, and I22 (60, 61). In RANTES, two hydrophobic residues (I15 and L19) also contribute significantly to CCR5 binding, as evidenced by mutation of these residues to Ala, causing a >5000-fold reduction in affinity (53). For MCP-1, mutation of many of the N-loop residues had little or no effect. Small changes were observed for two basic residues, R18 and K19. It can be concluded that the N-loop may be a common recognition element involved in chemokine-receptor interactions, and the nature and distribution of key residues may impart specificity. In MCP-1 two clusters of primarily basic residues (R24, K35, K49, and Y13), separated by a 35-Å hydrophobic groove, reduced the level of binding by 15- to 100-fold. Together these data suggest a model in which a large surface area of MCP-1 contacts its receptor (CCR2), and the accumulation of a number of weak interactions results in the 60-pM affinity observed for the wild-type (WT) protein. In contrast, an Ala scan of eotaxin (CCR3 ligand) has shown that residues contributing to receptor-binding affinity and those required for triggering receptor activation are relatively diffuse and distributed throughout the N-terminal and N-loop regions (59). The receptor-binding sites for variety of chemokines cover both similar and yet significantly different sites, and these studies may provide insight into the issue of receptor specificity.

6 CC RECEPTORS

6.1 CCRl

CCRl was the first isolated CC chemokine receptor. Originally identified as the RANTES (CCL5)/MIP-1α (CCL3) receptor (62, 63), it was subsequently shown that MCP-3 (CCL7) also binds with high affinity to CCRl (64). CCRl is expressed on various leukocyte cell types, including monocytes, T-cells, and eosinophils (see Table 4.1). In mouse, but not human, CCRl is also expressed on neutrophils (65). CCRl mRNA has also been detected in human dendritic cells (66).

A significant increase in CCRl ligand concentration for both MIP-1α and RANTES has been found in several inflammatory diseases and animal models of those diseases. For example, increases in MIP-la have been found in the synovial fluid of patients with arthritis (67) and in the cerebrospinal fluid of patients with relapsing multiple sclerosis (MS) (68). In a mouse model of MS, the experimental allergic encephalomyelitis model (EAE), several chemokines are upregulated, including MIP-la and RANTES (69). In this model, RANTES and MIP-la are produced exclusively by infiltrating leukocytes (70, 71). Moreover, neutralization of MIP-la significantly blocks the development of the initial and the relapsing paralytic disease (72, 73). In spite of this strong evidence regarding the role of MIP-1α in this disease, it has been difficult to establish which receptor(s) mediates the MIP-la response in this pathology. As shown in Table 4.1, this chemokine can bind with high affinity to both CCRl and CCR5.

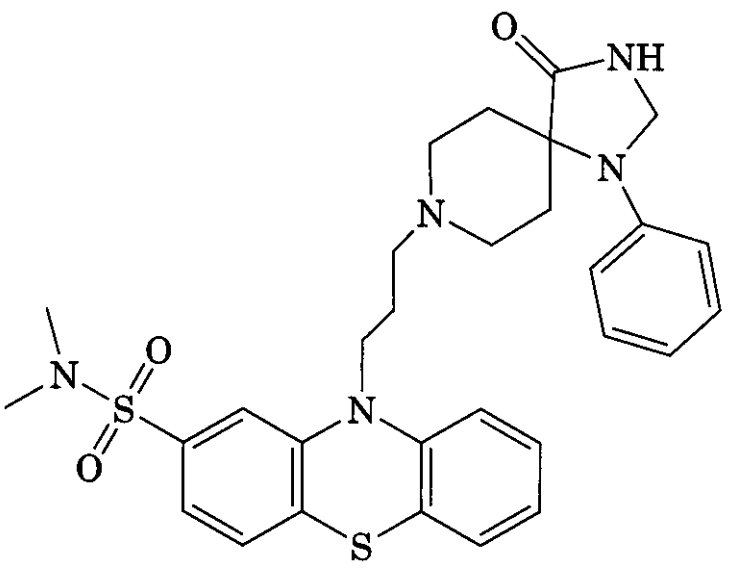
$CCR1^{-/-}$ mice show a normal distribution of leukocytes, although trafficking and proliferation of myeloid precursors are altered (74). These mice are protected from pulmonary inflammation secondary to pancreatitis. Evidence involving CCRl in transplant rejection has also been obtained using knockout mice, and these mice show a prolonged survival after heterotropic heart allografts. Low doses of cyclosporin A, which would not be sufficient to prolong graft survival, produced permanent engraftment in these mice (75), suggesting that this receptor plays a critical role in the development of rejection. This has been confirmed with small molecule antagonists of CCRl (see below). Recently, Blease and colleagues reported that fewer goblet cells and less subepithelial fibrosis were found in $CCR1^{-/-}$ mice compared to that found in wild-type mice in a model of chronic fungal allergic disease. IL-4 and IL-13 levels were lower in the knockout mice but similar changes in leukocyte recruitment and airway hyperreactivity were found in the two groups. Although there was no effect on inflammation, these data suggest a role for CCRl in airway remodeling, an important feature of asthma (76).

6.1.1 CCRl Receptor Structure. Chimera studies of CCRl show that the N-terminus is important for ligand binding and selectivity among chemokines, whereas this domain has no effect on the receptor activation process, as measured by calcium flux or chemotaxis assays (77). Instead, the ECL-3 of CCRl is shown to be important for receptor signaling. These data are consistent with a multisite model for chemokine-chemokine receptor interaction, in which one or more subsites determine chemokine selectivity, but others are needed for receptor activation (78).

6.1.2 CCRl Antibodies. Antibodies to CCRl chemokine ligands such as MIP-la and RANTES have been reported and, although these ligands show some promiscuity based on their involvement with other chemokine receptors, their biological activity is presumed to be through CCR1 based on upregulation of this receptor in disease models. Antibodies to MIP-1α have been used in an EAE model and have demonstrated involvement of the ligand (72). Antibodies to RANTES have been effective in a Lewis rat adjuvant-induced arthritis model, decreasing severity of the ongoing disease (79). A neutralizing anti-CCR1 polyclonal antibody provides protection in a bleomycin-challenged mouse model, reducing both inflammatory and fibrotic changes (80).

6.1.3 CCRl Peptide Antagonists. Modified forms of the chemokine ligands have proved useful in further validating the role of their receptors in disease pathology. Met-RANTES incorporates a methionine at the N-terminus of the ligand and has been shown to be an effective CCR1 antagonist (K_i = 25 nM) in competition binding studies with ^{125}I-RANTES (81). It selectively inhibits THP-1 chemotaxis mediated by RANTES (IC_{50} = 6 nM) or MIP-la (IC_{50} = 0.5 nM), while showing no response to MCP-1-mediated chemotaxis and calcium flux. Met-RANTES has also been shown to be effective in a model of arthritis, effecting a decrease in onset and severity of collagen-induced arthritis (CIA) when administered during induction (82). AOP-RANTES, with an AminOxyPentane that is isosteric with the side chain of methionine, also functions as a CCR1 antagonist. In a rat experimental glomerulonephritis model, AOP-RANTES effectively blocks monocyte infiltration (83). Because RANTES is also a ligand for the CCR5 receptor, it may be that these modified ligands exert efficacy through dual antagonism.

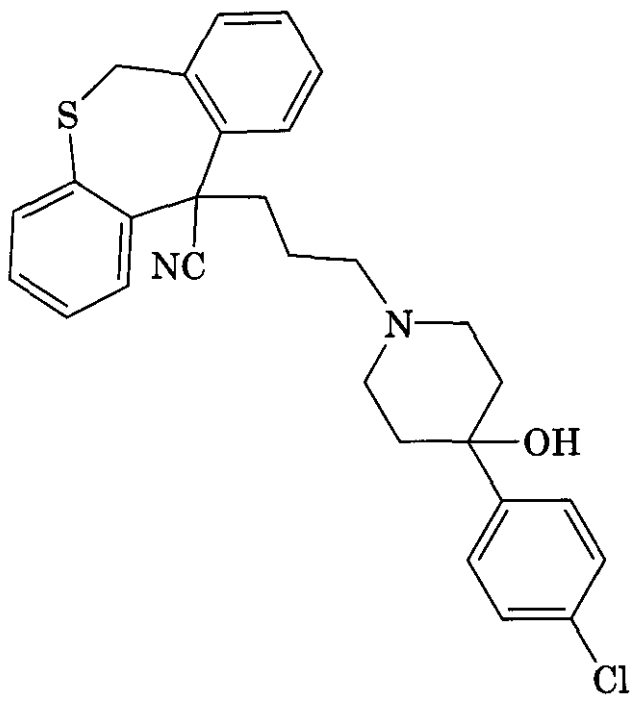
6.1.4 CCRl Small Molecule Antagonists. RP23618 **(1)** is an early example of a nonpeptide small molecule chemokine antagonist (84). This compound inhibits ^{125}I-RANTES binding to THP-1 cell membranes (IC_{50} = 3 μM). The chemokine receptors CCRl and CCR2 have been detected by RT-PCR as being present on this cell type (62). However, because **(1)** inhibits RANTES-induced chemotaxis and has no effect on an MCP-1-stimulated chemotactic response, this compound appears to be CCRl selective. Because **(1)** is not competitive with RANTES, it is presumed to bind at an allosteric site.



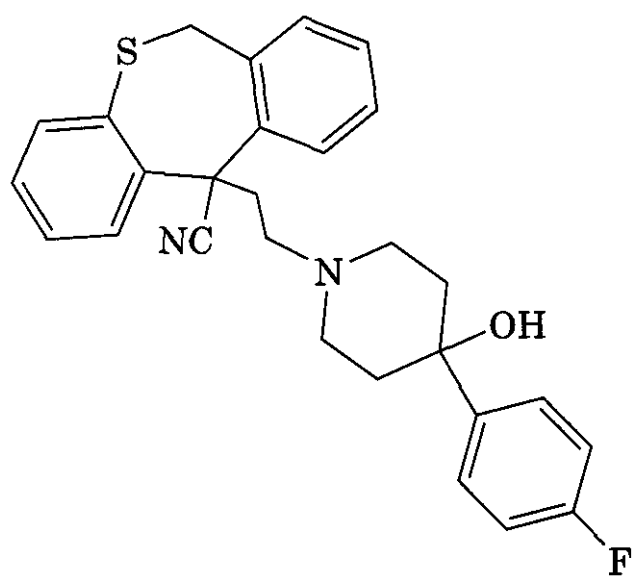
(1)

There is a limited structure-activity relationship (SAR) provided around this series. However, an optimal chain length of three carbon atoms between the phenothiazine and the piperidine is demonstrated. The two-carbon-linked homolog has significantly reduced activity (IC_{50} < 20% at 30 μM) and the four-carbon tether is at least 3 times less active than (1).

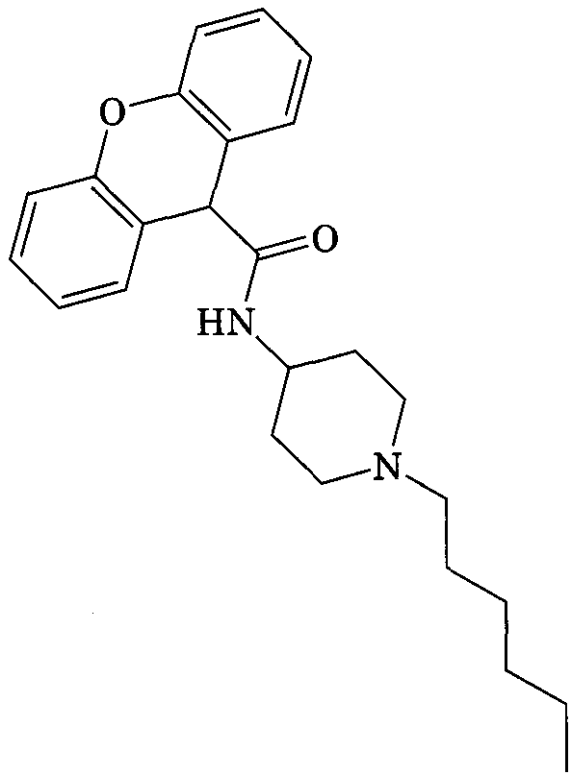
A similar pharmacophore finding was noted for early leads from the Berlex group (85). HEK293 cells containing the CCRl receptor were evaluated for binding with another CCRl ligand, MIP-la. The three-carbon-tethered dibenzothiepine (2) was a potent



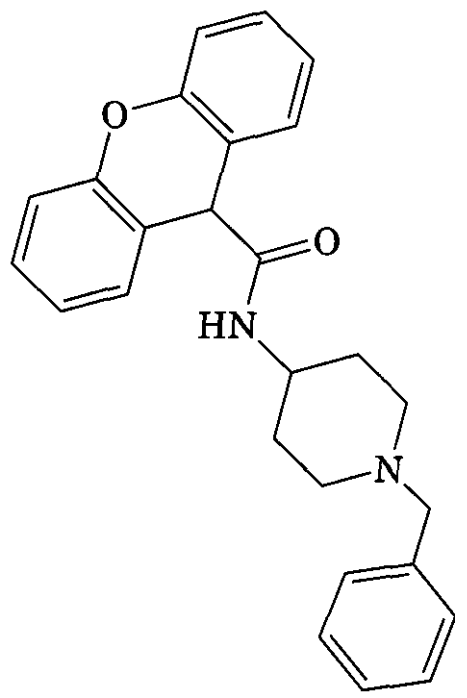
(2)



(3)



(4)



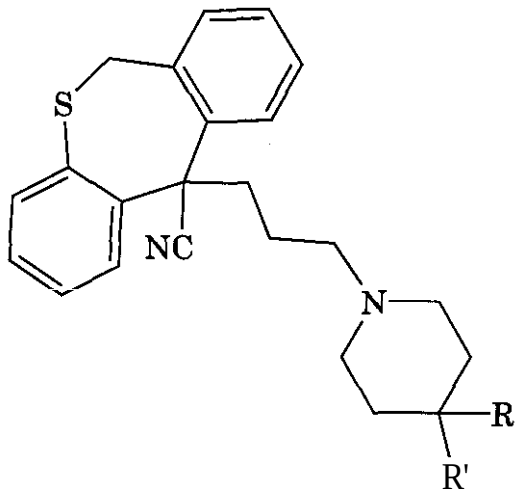
(5)

inhibitor of ^{125}I-MIP-1α binding (IC_{50} = 44 nM), whereas the two-atom-linked analog (3) did not show appreciable inhibition (IC_{50} = 5 μM). In a separate study, the structurally related xanthene carboxamide (4) was identified (86) as a CCRl antagonist for its ability to inhibit ^{125}I-MIP-1α binding to human CCRl transfected in CHO cells (IC_{50} = 510 nM). Replacement of the hexyl group with benzyl (**5**) led to a significant loss of activity (IC_{50} > 10,000 nM). It is interesting to compare (2) versus (4) in terms of a similar linking space between the aryl group and the tricyclic moiety. The conformational shape imposed by the amide and/or the position of the piperidine unit may be important considerations in the SAR of CCRl antagonists.

An initial strategy to optimize the drugability of (2) focused on two fragments with potential liabilities. The 4-hydroxy phenyl piperidine is also present in haloperidol and implicated with neurotoxicity, and the tricyclic dibenzothiepine is a common feature of CNS-active drugs. As indicated in Table 4.2, removal of the 4'-hydroxy led to a complete loss of potency against CCR1.

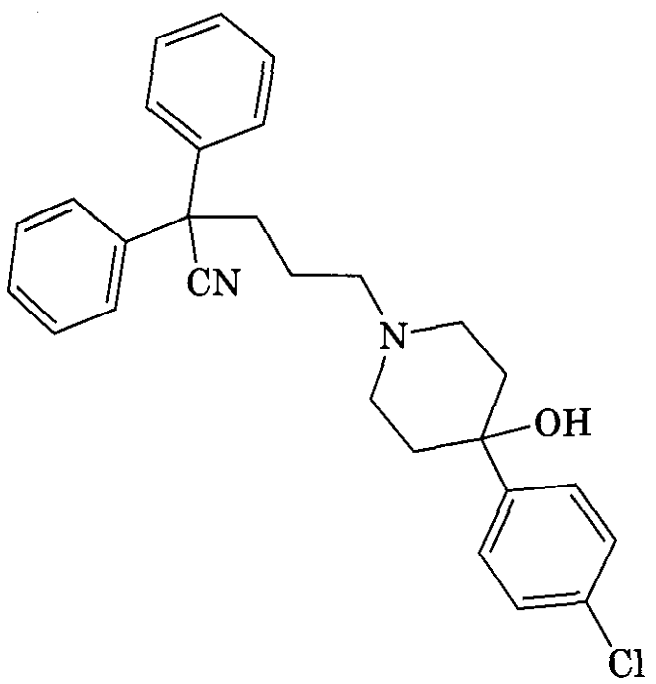
Diphenylmethane is a common tricyclic replacement and this strategy was implemented successfully, leading to (11) (K_i = 52 ± 5 nM;

Table 4.2 Consequence of Replacing Hydroxyl on the 4-Phenylpiperidine Unit in Dibenzothiepine Series[a]



Compound	R	R′	K_i (nM)[b]
(6)	OH	4-F-Ph	44219
(7)	OH	Ph	203±28
(8)	H	Ph	19 ± 5% at 3 μM
(9)	CN	Ph	0% at 3 μM
(10)	$C(O)CH_3$	Ph	0% at 3 μM

[a]From Ref. 85.
[b]K_i values derived from competitive binding on CCRl with ^{125}I MIP-la.



(11)

^{125}I-MIP-1α displacement). Compound (11) appears to be selective for CCR1, in that it has no effect on the chemokine receptors CCR2, CCR5, and CXCR4, or a select panel of biogenic amine receptors (87). There are no in *vivo* data reported for (11), perhaps because of the variable species similarity between human and mouse sequences. The homology between mouse/human CCR1 is 79%. In an effort to compare response of a CCR1 antagonist against the same receptor from different species, HEK293 cells were transfected with marmoset, rabbit, and mouse CCRl (88). Although (11) is efficient in displacement of ^{125}I-MIP-la from human, rabbit, and marmoset, it is ineffective against mouse CCRl (Table 4.3). Species variability is an issue for many chemokine receptors and presents a challenge for evaluation of possible human therapeutics in relevant animal models.

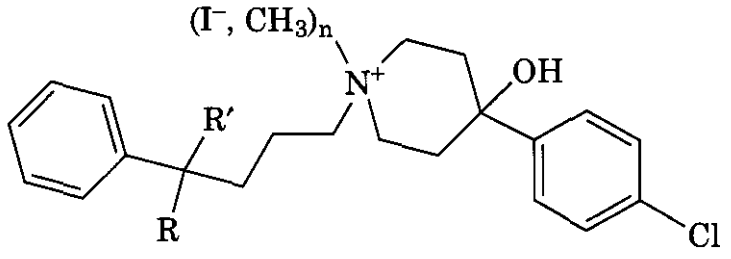
Many of the published chemokine antagonists have a basic nitrogen as a common feature, and it is interesting to speculate how this might serve as an important recognition element. As noted earlier, the protonated nitro-

Table 4.3 Species Selectivity for (11) in Its Ability to Inhibit CCRl from Human, Rabbit, Marmoset, and Mouse[a]

Species	K_d of MIP-1α Binding (nM)	K_i of Inhibition of MIP-1α Binding by (11) (nM)[b]
Human	2 ± 0.2	41 ≥ 6
Rabbit	43 ± 17.8	245 ± 60
Marmoset	81 ± 23	451 ± 191
Mouse	32 ± 3.5	>10,000

[a]From Ref. 88.
[b]K_i of inhibition of ^{125}I-MIP-1α to HEK293 cells expressing CCRl receptors from various species.

Table 4.4 Activity of Quaternary Ammonium Salts versus Nonquaternized Compounds[a]

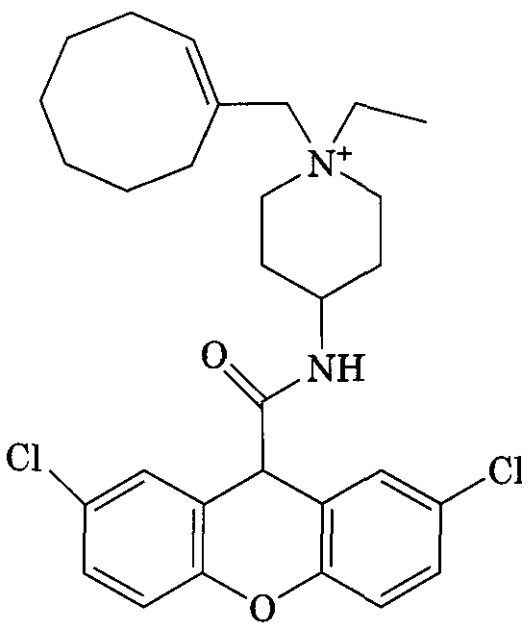


Compound	R	n	K_i (nM)[b]
(**12**)	CN	0	52 ± 5
(**13**)	CN	1	8 ± 2
(**14**)	H	0	116 ± 41
(**15**)	H	1	6 ± 1

"From Ref. 85.
[b]K_i values are derived from competitive binding on CCRl with ^{125}I-MIP-1α.

gen might interact with the conserved Glu in TM7 (Fig. 4.5). This hypothesis is given credence by the finding that quaternary ammonium salts will show enhanced affinity with the receptor, implying a possible ion pairing with a carboxylate (Table 4.4).

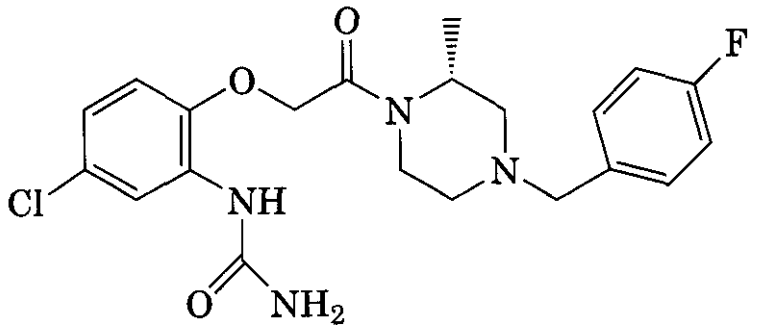
A similar potency enhancement is seen for the xanthene carboxamide quaternary salt (16) (CCR1 IC_{50} = 0.9 nM), where the simple



(16)

nonquaternized parent is 150 times less active (86). Compound (16) can exist as *cis* and *trans* isomers and a stereochemical preference is indicated in CCR1-binding studies (Table 4.5). Additionally, these investigators were interested in identifying a compound that was effective against human and murine CCR1, and therefore they screened against both receptors. The minor isomer, presumed to be the *cis* isomer, was 50 and 100 times less active for the human and murine receptors, respectively. Compound (16) is a functional antagonist of MIP-la-stimulated Ca^{2+} flux in U937 cells (IC_{50} = 0.73 nM). It also shows modest inhibition of CCR3 but is selective against a panel of other chemokine receptors (89).

BX471 (17) is a potent CCRl antagonist that has demonstrated efficacy in animal models of multiple sclerosis and transplant rejection. It has been determined to be a reversible and surmountable antagonist that can effectively displace the radioiodinated CCRl ligands ^{125}I-RANTES (K_i = 2.8 nM), ^{125}I-



(17)

Table 4.5 Species Selectivity for (16) in Its Ability to Inhibit CCRl from Human and Mouse[a]

	IC_{50} **16** (nM)	
Species	Major Isomer	Minor Isomer
Human CCR1	0.9	47
Murine CCR1	5.8	740

"Inhibitory activity against ^{125}I-MIP-1α binding to human and mouse CCR1.

MIP-1α ($K_i = 1$ nM), and ^{125}I-MCP-3 ($K_i = 5.5$ nM) (90). BX471 demonstrates functional antagonism in its ability to block Ca^{2+} flux and inhibit CD11b upregulation in a whole blood assay. Bioavailability has been measured in both dog ($F = 60\%$) and rat (F< 20%). Even though activity against the rat CCR1 is 100 times less potent than against human CCR1, and despite modest bioavailability, the compound was able to demonstrate efficacy in the EAE model when administered subcutaneously (s.c.) (t.i.d.). Additionally, in the rat heterotropic heart transplant rejection model, in which animals are given subtherapeutic doses of CsA, BX471 was able to prolong survival (91).

6.2 CCR2

There are two variants of the CCR2 receptor, CCR2A and CCR2B, differing at their C-terminal tails. These two isoforms are derived from a single gene through alternative splicing (92). CCR2B was found to be the predominant form in monocytes and seems to traffic more efficiently than CCR2A to the cell surface in stably transfected cell lines (93). All four monocyte chemotactic proteins (MCPs) are functional ligands of CCR2 (see Table 4.1). This receptor is expressed on monocytes, T-cells, basophils, and immature dendritic cells. Freshly isolated B-cells also show surface expression of CCR2 (94).

MCP-1 is elevated in several inflammatory diseases, including asthma, rheumatoid arthritis, multiple sclerosis, atherosclerosis, and stroke. Monocyte infiltrates and their cytokine products are characteristic features of inflamed tissue in rheumatoid arthritis (RA). The synovial cells of RA patients, and animal models of the disease, produce elevated levels of MCP-1 (95–99). Peptide antagonists and antibodies implicate MCP-1 as a contributor to disease pathology. $CCR2^{-/-}$ and $MCP\text{-}1^{-/-}$ mice show that this receptor and its ligand play a crucial role in the recruitment of monocytes/macrophages, which are the precursors of the lipid-laden foam cells. A significant reduction in arterial lipid deposition was found for both $CCR2^{-/-}$ and $MCP\text{-}1^{-/-}$ mice in models of atherosclerosis. This reduction was accompanied by fewer macrophages in the atherosclerotic lesions of these mice (100, 101). The relevance of these findings to human disease was suggested by the presence of MCP-1 in human atheromatous plaques (102).

In a mouse model of allergic lung inflammation, MCP-1 mRNA was significantly increased after antigen challenge and appeared to correlate with lung macrophage accumulation (103). Using the same model, it has recently been shown that MCP-1 neutralization drastically diminished bronchial hyperreactivity to methacholine and reduced inflammatory cell infiltration into the lung (104). However, in this type of model a clear discrepancy has been found between $CCR2^{-/-}$ and $MCP\text{-}1^{-/-}$ mice. $CCR2^{-/-}$ mice present an enhanced pulmonary allergic response against Aspergillus *fumigatus* (105) or no difference at all in the response to repeated ovalbumin sensitization and challenges (106). $MCP\text{-}1^{-/-}$ mice, by contrast, show a decrease in T-helper 2 (Th2) type T-cell responses, including a decrease in IL-4 production (107). The mechanism that explains how CCR2/MCP-1 affect T-cell differentiation is not clear, although it seems that they exert their effect at different levels.

As research in the area progresses, it appears that several other chemokine receptors, in addition to CCR5 and CXCR4, can act as coreceptors for the HIV virus (7). One of these receptors is CCR2. A mutation in the CCR2 gene that leads to a conservative amino acid substitution (V64I) has been linked to a decreased rate in the progression of the disease but not with initial susceptibility to infection (108). The mechanism responsible for this is not clear.

6.2.1 CCR2 Receptor Structure. Chimera studies have shown that the N-terminus, ECL-1, and ECL-2 of the CCR2 receptor are essential for MCP-1 binding and receptor-mediated signaling (77). A detailed analysis of the amino acid residues of ECL-1 in CCR2 has shown N104 and E105 as being essential for high affinity agonist binding but not involved in receptor activation. In contrast, the charged amino acid residue H100 does not contribute to ligand binding but is vital for receptor activation and initiation of transmembrane signaling (109).

The role of acidic residues in TM7 and ECL-2 of the CCR2 receptor has been evalu-

ated for their contribution to the binding affinity of MCP-1 and several small molecule antagonists (110). In this study, glutamate 291 (Glu291 or E291), a highly conserved acidic residue in TM7 of most chemokine receptors, has been identified to be critical for binding of certain small molecule antagonists, and to a lesser extent the binding of MCP-1 to its receptor (Fig. 4.5). It is hypothesized that the acidic moiety interacts with the piperidine basic nitrogen of small molecule antagonists (18–20). Because Glu291 is also involved in MCP-1 binding to CCR2, the spiropiperidine blockade of MCP-1 binding occurs by occupation of some of the space that CCR2-bound MCP-1 occupies. A class of antagonists that does not have a basic nitrogen (e.g., 27) is not affected by mutation of Glu291, suggesting a different binding motif for this series.

The acidic residue (Glu) in TM7 is found in most chemokine receptors and is rare in other serpentine receptors (Figs. 4.4 and 4.5). This negatively charged residue may also be significant in the small molecule ligand binding of other chemokine receptors. It is interesting to note that receptor models indicate that Glu291 is inside the ovid-helical bundle (Fig. 4.5) in a mirror image position to the critical binding residue of biogenic arnine receptors, an aspartic acid in TM3. This observation might explain the fact that many of the leads from high throughput screening for the chemokine receptors are compounds originally synthesized for biogenic amine programs.

6.2.2 CCR2 Antibodies. A CCR2 monoclonal antibody (mAb), ID9, was shown to have therapeutic benefit in a primate model of restenosis (111). The inflammatory response after stent implantation involves sustained accumulation of macrophages, resulting in neointimal hyperplasia. The CCR2 mAb ID9 reduced intimal thickening in the area of the stent approximately 60%, as ascertained on day 28. Two CCR2-specific monoclonal antibodies, MCP-1R02 and MCP-1R05, are reported to antagonize MCP-1 activity on CCR2-expressing cell lines and normal human cells (112). These antibodies have been used to map the HIV-1 binding site on CCR2. MCP-1R02 is claimed to be specific for the amino terminus and acts as an agonist; it suppresses HIV-1 replication in M- and T-trophic HIV-1 viral isolates. MCP-1R05 is specific for ECL-3 of CCR2, acts as an antagonist (31), and has no effect on HIV-1 replication. Therefore, the HIV-1 interaction is primarily through the amino terminus.

Neutralizing antibodies to the CCR2 ligand MCP-1 have helped to validate its role in disease pathogenesis of arthritis and glomeruloritis. An antibody against rat MCP-1 has been evaluated in a collagen-induced arthritis model, and was shown to reduce joint swelling of the ankle, and suppresses macrophage infiltration to the synovial tissue (113). In an animal model of impaired renal function, in which MCP-1 was detected in glomeruli, vascular endothelial cells, and tubular epithelial cells of the injured kidney, anti MCP-1 mAbs reduced glomerulosclerosis and improved renal function (114). Anti-MCP-1 monoclonal antibodies have also shown therapeutic benefit in a kidney fibrosis model, through blockade of renal cell proliferation (115), and in a delayed-type hypersensitivity (DTH) model in which T-cell trafficking was inhibited (116). Finally, as part of an effort to define the role of a variety of chemokines in allergic inflammation and airway hyperresponsiveness, antibodies to RANTES, MIP-la, MCP-1, and the mouse-specific chemokine MCP-5 were evaluated in a mouse model of asthma (117). In response to an ovalbumin (OVA) challenge, the neutralizing antibody to MCP-1 reduced bronchial hyperresponsiveness and blocked leukocyte infiltration into the airways.

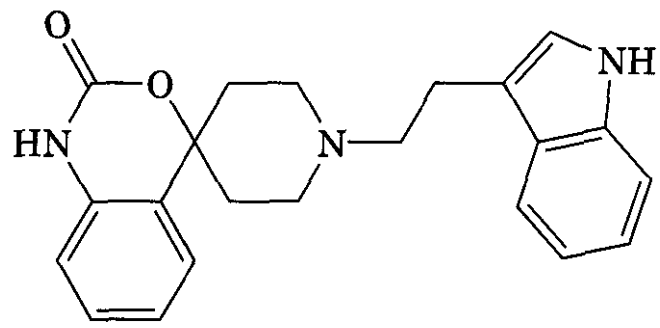
6.2.3 CCR2 Peptide Antagonists. Truncation or N-terminal deletion of select amino acids in MCP-1 results in a protein antagonist that blocks Ca^{2+} flux and chemotactic response (118,119). Truncation of the first nine amino acids ($MCP\text{-}1^{[10-76]}$), or deletion of amino acids 2–8 ($MCP\text{-}1^{[1+9-76]}$, generate functional CCR2 antagonists. These protein antagonists have also demonstrated efficacy *in vivo*. When $MCP\text{-}1^{[9-76]}$ is administered daily in the MRL-lpr arthritic mouse model, there is a significant reduction in the onset of arthritis, as determined by histopathologic evaluation of the joint and reduced joint swelling (57). Even when administered 12 days after disease onset, $MCP\text{-}1^{[9-76]}$ shows a marked

Table 4.6 Selectivity Profile for CCR2 Antagonists (Spiropiperidines) versus Alpha and 5-HT Receptors

	IC_{50} (μM)			
Compound	CCR2[a]	Alpha 1a	Alpha 1d	5HT1a
(18)	11,400	5.6	43	62
(19)	360	130	320	470
(20)	89	72	460	1070

[a]Measured in THP-1 cells using ^{125}MCP-1.

reduction in symptoms and histopathology. This suggests a turnover of cell infiltrate in the lesion and indicates the potential for therapeutic benefit through this mechanism after disease onset. It is worthwhile to note that ligand truncations have the potential to alter receptor selectivity. For example, truncation of the NH_2-terminal amino acid in MCP-1 generates a strong activator of eosinophil chemotaxis (120), presumably acting on CCR3. Further truncation converts the ligand to a CCR2 antagonist with no activity on eosinophils. Although ligand truncations are a common manipulation to profile chemokine SAR, few studies screen for selectivity of these truncated ligands against other receptors.



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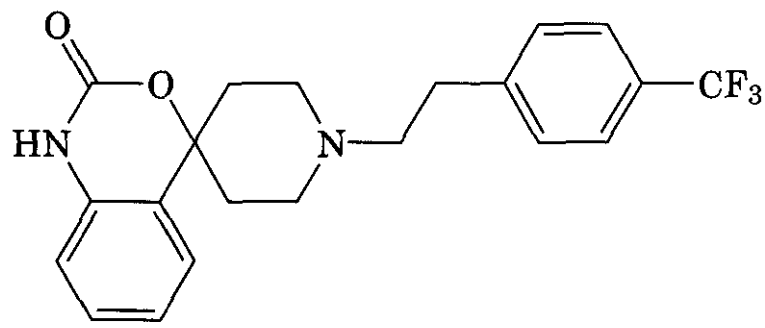

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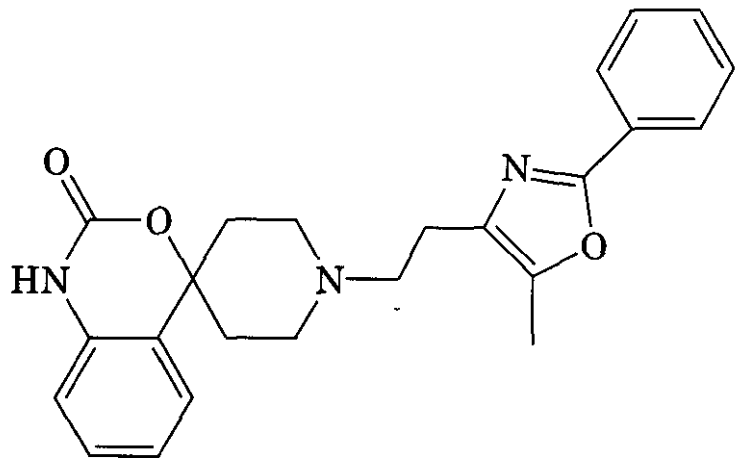


(20)

6.2.4 CCR2 Small Molecule Antagonists. The in *vivo* efficacy seen with anti-MCP-1 antibodies and peptide antagonists prompts the search for small molecule CCR2 antagonists. The spiropiperidines (18–20) are early examples of CCR2 antagonists, as determined by ^{125}I-MCP-1 binding and chemotaxis in THP-1 cells (110). As noted above with the CCR1 antagonists, a basic nitrogen is a common feature within this class of antagonists. Site-directed mutagenesis suggests that this nitrogen may form a critical recognition with an anionic residue (E291) in helix 7. The orthogonal relationship imposed on the phenyurethane and the piperidine ring by the spiro-carbon is urported to be essential for activity.

Selectivity is an issue for the spiropiperidines, given that this class of compounds shows appreciable activity against alpha adrenergic and 5-hydroxytryptamine (5-HT) receptors. Although lead optimization improved CCR2 affinity (18)versus (20), and improved the relative difference in selectivity, the most potent CCR2 antagonist still retained significant Alpha l a adrenergic activity (Table 4.6).

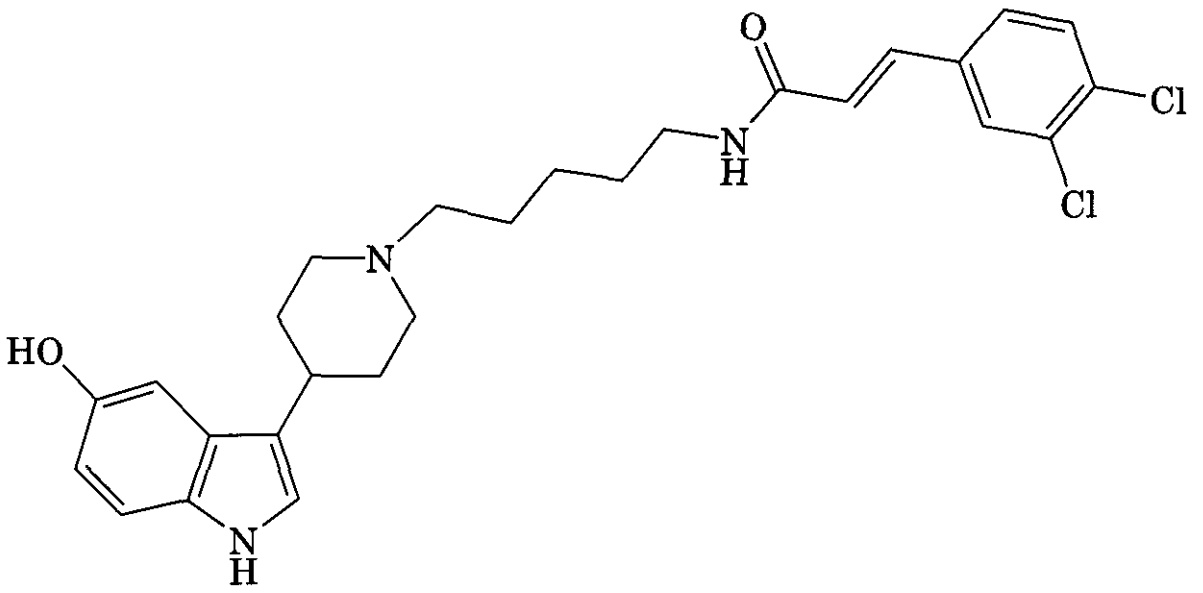
Another example of GPCR selectivity issues for CCR2 antagonists is seen with the indolopiperidines (121) (Table 4.7). As might be anticipated by the initial lead (21), prob-

Table 4.7 Selectivity Profile of CCR2B Antagonists (Indolopiperidenes) versus 5-HT and Dopaminergic Receptors

	pK_i							
Compound	CCR2b	$5HT_{1A}$	$5HT_{1B}$	$5HT_{1F}$	$5HT_{2a}$	$5HT_6$	D_2	D_3
(**21**)	7.3	7.0	7.9	8.5	7.7	7.1	7.8	8.1
(**22**)	6.6	5.9	6.3	5.8	6.8	6.2	6.1	6.7
(**23**)	7.1	6.3	6.7	5.9	7.6	6.2	7.0	7.2
(**24**)	7.4	5.1	6.0	<5.5	<4.7	6.7	5.9	5.6

lematic affinity with 5-HT and dopaminergic receptors was evident. However, impressive improvements in selectivity were effected by increasing the steric bulk around the piperidine nitrogen, leading to a conformationally rigid ring system **(122)** in the form of indolotropane (**22**), or imposing conformational restriction in the linker as with the cis-cyclohexyl derivative **(23).C-2** substituents on the indole ring were not well tolerated, presumably because of the unfavorable influence on the piperidine ring conformation. Incorporation of both the tropane and cyclohexyl moieties simultaneously had a dramatic additive effect in providing a potent **CCR2** antagonist **(24)** with good selectivity profiles.

TAK-779 (25) is recognized primarily as **a** potent **CCR5** antagonist, but it does exhibit



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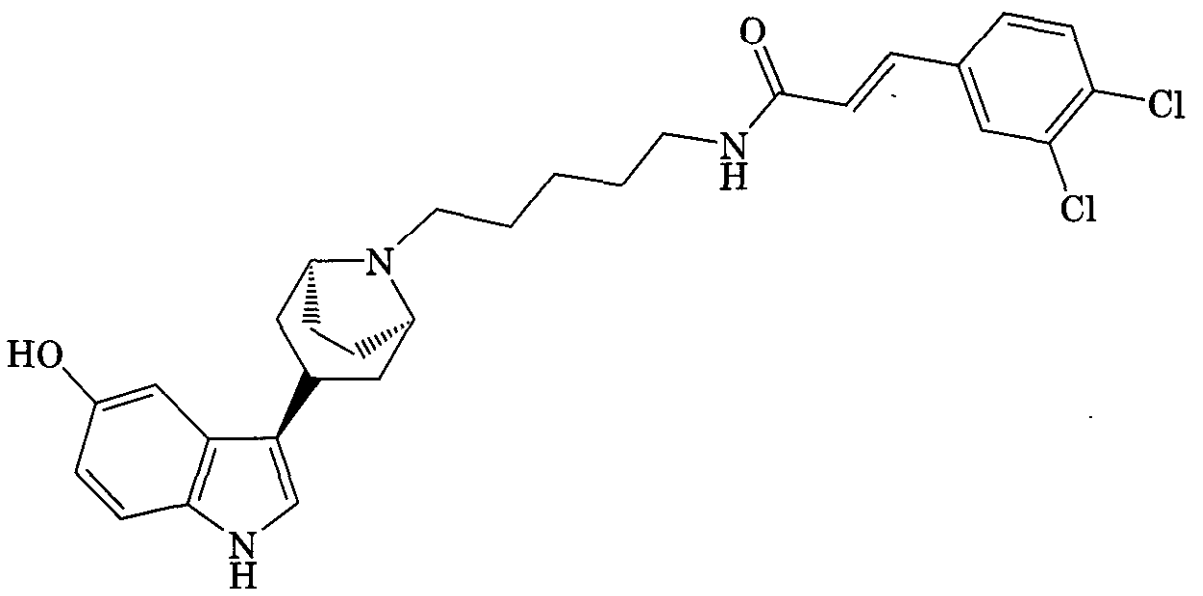

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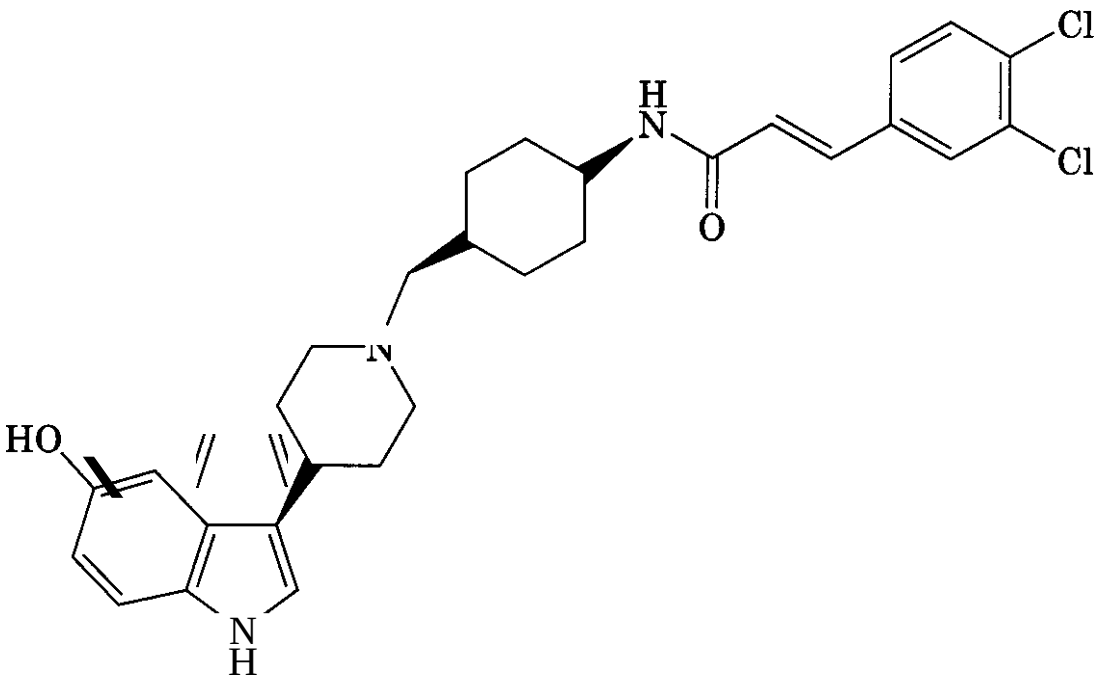
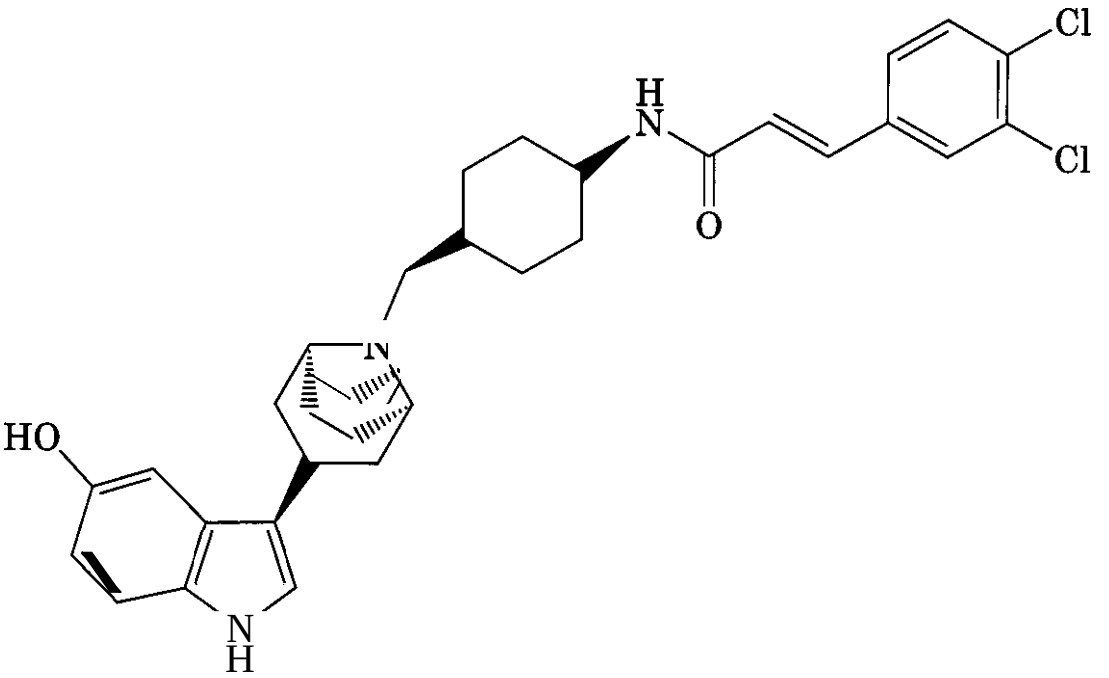


(23)

modest affinity for CCR2 (123). This is not surprising, considering the close homology between the CCR2 and CCR5 receptors (71% amino acid identity). The binding of ^{125}I-MCP-1 to a CHO cell line containing CCR2b was inhibited by TAK-779 (IC_{50} = 27 n*M*), which showed no effect against CCR1, CCR3, CCR4, and CXCR4.

There have been no reports on in *vivo* evaluation of small molecule CCR2 antagonists.



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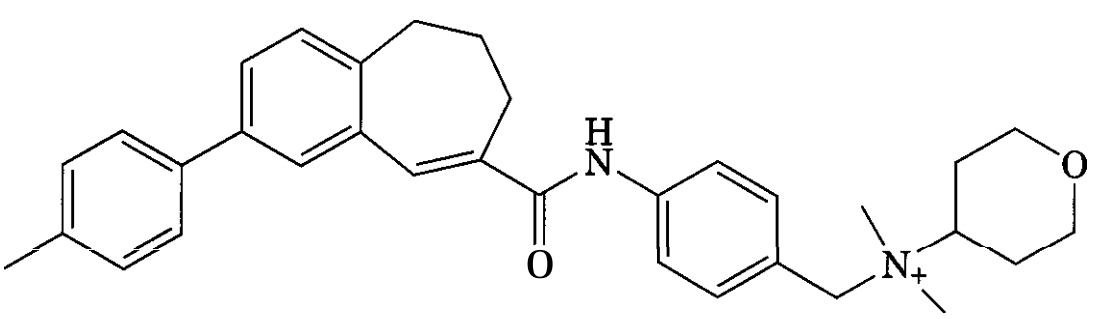

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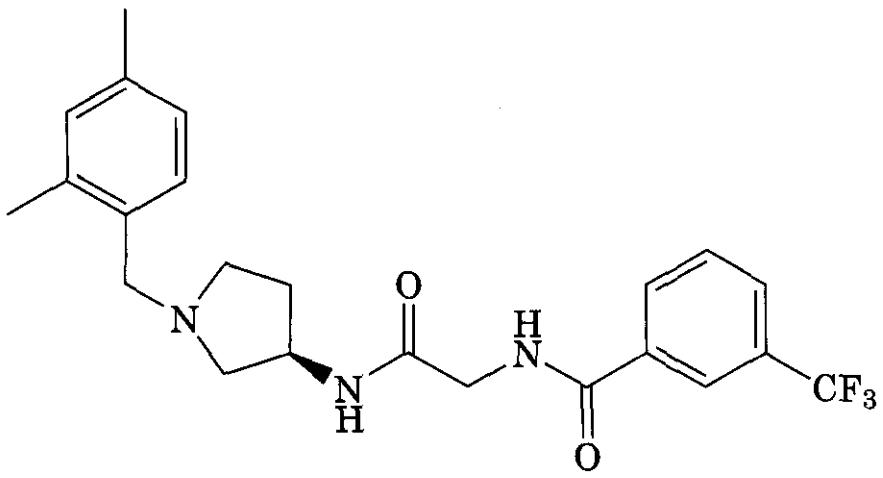
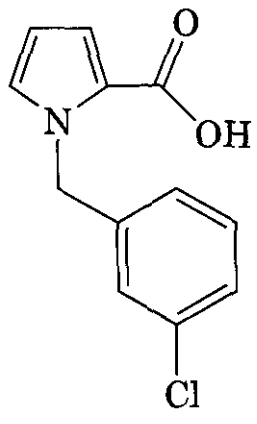
This may be a consequence of dissimilar responses against the human and murine receptors, with these antagonists being less potent against mouse and rat CCR2. This activity discrepancy is noted in the case of (26), in which



(26)

significant potency is demonstrated against the human receptor (binding IC_{50} = 54 nM; chemotaxis IC_{50} = 16 nM), and yet it is approximately 100 times less potent against the murine chemotactic response (IC_{50} = 1400 nM) (124).

Nonbasic CCR2 antagonists have also been reported. The 3-chlorobenzyl 2-pyrrole carboxylic acid (27) is a modest CCR2 antagonist

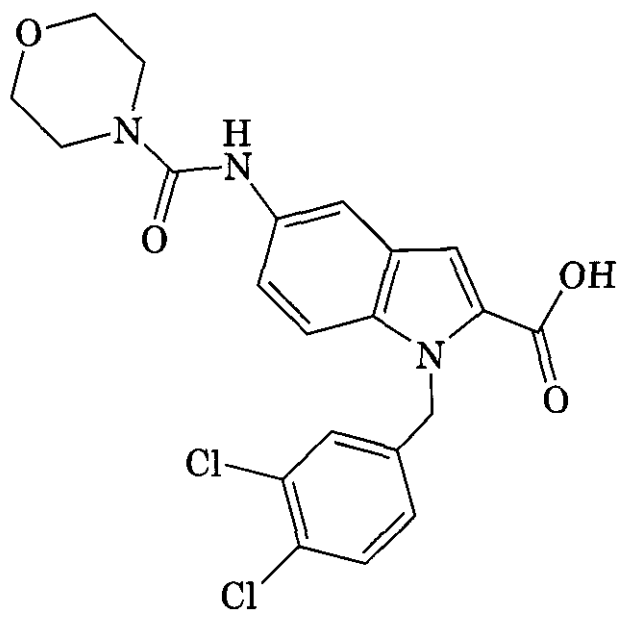


(27)

(IC_{50} = 11.9 μM), and binding does not appear to be influenced by mutation of the conserved glutamic acid in TM7 (110). Consequently, it may bind in a region distinct from the antagonists that contain a basic nitrogen. The closely related indole (28) is also reported to be a modest CCR2 antagonist (IC_{50} = 1.2 μM) (125).

6.3 CCR3

CCR3 was originally reported to be expressed on eosinophils and appears to be the primary chemokine receptor on these cells



(28)

(126). It is also expressed on basophils, Th2 lymphocytes, and mast cells (127–129). Based on its presence in a large array of cell types that are crucial for the induction and maintenance of inflammation in asthma and other allergic diseases, there is a significant effort to discover CCR3 receptor antagonists. In 1993, eotaxin (CCL11) was identified as an eosinophil-specific CC chemokine (130, 131). Subsequently, CCR3 was found to be activated by several other CC chemokines including MCP-3 (CCL7), MCP-4 (CCL13), RANTES (CCL5), eotaxin 2 (CCL24), and eotaxin 3 (CCL26) (126, 132, 133).

In human asthma, increased mRNA and protein expression of eotaxin have been demonstrated, as well as CCR3 expression on local infiltrating eosinophils, as determined by biopsies (134). Results obtained from mice genetically deficient for the eotaxin gene (135), as well as studies using eotaxin-blocking antibodies (117), have demonstrated a significant effect on the course of a lung allergic reaction. By contrast, another report describing the response of eotaxin-deficient mice, in a similar model of allergic lung inflammation, showed no significant difference from that of wild-type (136). The reason for this discrepancy is not understood. $CCR3^{-/-}$ mice have shown that this receptor plays an important role in eosinophil migration into the lung and skin. In intraperitoneally (i.p.) sensitized $CCR3^{-/-}$ mice, eosinophil recruitment to bronchoalveolar lavage fluid and lung is decreased compared to that of wild-type after aerosol challenge. However, airway hyperreactivity (AHR) is enhanced under the same conditions (137). If the mice are sensitized epicutaneously with the same antigen, a diminished AHR response and eosinophil recruitment is observed (138). In the i.p. sensitized mice there is a significant mobilization of mast cells into the airway epithelium, but not into the skin. These results suggest that the mechanism of induction of AHR might be different, depending on the route of sensitization.

6.3.1 CCR3 Receptor Structure. Chimera studies of CCR3 and CCR1 receptors show that the N-terminal segments of CCR3 appear to be important for eotaxin binding (77). However, eotaxin remained an effective agonist at this chimeric receptor in either calcium flux or chemotaxis assays. These data are consistent with a multisite model for chemokine-chemokine receptor interaction.

6.3.2 CCR3 Antibodies. A specific CCR3 monoclonal antibody, 7B11, blocks binding of eotaxin, MCP-3, and RANTES to the CCR3 receptor, and also blocks chemokine-induced chemotaxis and calcium flux in human eosinophils (126). Because the CCR3 receptor is present on basophils, it is not surprising that 7B11 blocks chemotaxis of these cells in response to CCR3 ligands (127). In these same studies, 7B11 was shown to inhibit histamine and leukotriene release mediated by eotaxin. This mAb has also been shown to inhibit the release of reactive oxygen species (ROS) after stimulation with eotaxin-1 or eotaxin-2 (139), and blocks the chemotactic response of eosinophils on HUVEC cells stimulated with TNFα and IFNγ (140). Because CCR3 has been implicated in having a role in HIV-1 pathology, 7B11 has also been used to define the involvement of CCR3 in contributing to HIV-1-specific pathology (141).

CCR3 monoclonal antibodies have also been evaluated in vivo. A rat mAb specific for mouse CCR3 receptor depletes blood eosinophil levels in *Nippostrongyos brasiliensis*-infected mice, and reduced eosinophil levels in lung tissue and bronchoalveolar lavage fluid (BAL) fluid after repeated treatment (142). Surprisingly, this antibody had no effect on CCR3-regulated Ca^{2+} flux. In another report, a guinea pig-specific CCR3 mAb, 2A8, was also evaluated both in vitro and in *vivo* (143). 2A8 blocks the binding of guinea pig eotaxin to guinea pig eosinophils and guinea pig CCR3 transfectants, and shows functional antagonism of guinea pig eosinophils, as measured by Ca^{2+} flux and chemotaxis. In animals pretreated with ^{111}In-labeled eosinophils, 2A8 inhibited accumulation of these eosinophils in response to eotaxin.

6.3.3 CCR3 Peptide Antagonists. As demonstrated for other chemokines, modification of the N-terminal region has been employed as a successful strategy to identify receptor antagonists. Truncation of MCP-3 ($MCP3^{110-761}$) provides a ligand that retains potent receptor binding, yet is unable to induce chemotaxis, enzyme release, or Ca^{2+} flux (144). Dipeptidyl peptidase is able to truncate $RANTES^{[1-68]}$ to $RANTES^{[3-68]}$ and $eotaxin^{[1-74]}$ to $eotaxin^{[3-74]}$, and both have demonstrated functional antagonism in *vitro* (145–147).

RANTES, which can signal through CCR1, CCR3, or CCR5, has been modified to Met-RANTES, as described earlier. Met-RANTES acts as a functional antagonist and blocks human eosinophil chemotaxis, Ca^{2+} flux, actin polymerization, and release of ROS after stimulation with RANTES, MCP-3, and eotaxin (148). Met-RANTES apparently antagonizes

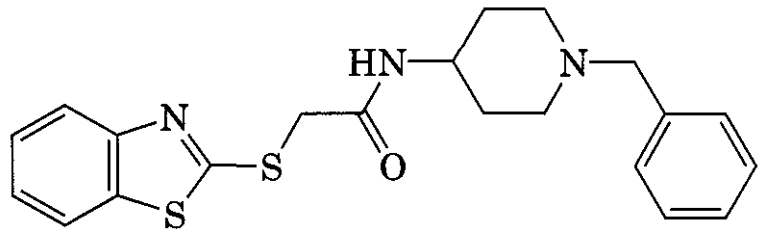
the response of eosinophils through CCRl at low concentrations and through CCR3 at higher concentrations.

Met-chemokine β7 (Ckβ7) is an N-terminal-modified form of macrophage inflammatory protein (MIP4), wherein an alanine is replaced with methionine at the extreme N-terminal residue (149). MIP4 [also known as pulmonary and activation-regulated chemokine (PARC) (150); alternative macrophage activation-associated CC chemokine (AMAC) (151); or dendritic cell-derived chemokine (DCCK) (152, 153)] itself shows some degree of CCR3 antagonistic activity. However, Met-Ckβ7 is significantly more potent as a CCR3 antagonist than MIP4, Met-RANTES, or AOP-RANTES, and blocks eotaxin or MCP4-induced eosinophil chemotaxis at concentrations as low as 1 n*M*. Direct binding of Ckβ7 to CCR3 is evidenced by its ability to displace radioiodinated eotaxin or MCP4 from CCR3.

6.3.4 CCR3 Small Molecule Antagonists. UCB 35625 (**16**), which has already been described as having CCRl activity, also functions as a CCR3 antagonist. This compound inhibits a chemotactic response to transfected cells expressing either CCRl or CCR3 (CCR1/MIP-1α IC_{50} = 9.6 n*M*, CCR3/eotaxin IC_{50} = 93.7 n*M*) (89). Again, this dual activity is not surprising, given the considerable homology between the two receptors. Because (16) is not as effective in ligand displacement binding assay as it is in blocking receptor function, this suggests that it may bind with a common region in both CCRl and CCR3 that are necessary for initiation of receptor signaling.

Another tool for measuring cellular response is a shape-change assay, as measured by gated autofluorescence/forward scatter (GAFS) through flow cytometry (154, 155). Eosinophil shape change in response to eotaxin was effectively blocked by (16) (89).

In an effort to improve CCR3-selective antagonists, carboxamide derivatives based on (16) were synthesized (156). Given that it was presumed that the ammonium salt was forming an important electrostatic interaction with a carboxylate in the receptor, an amine moiety was maintained within the template. The 2-(benzothiazolethio)acetamide (29) showed potency for CCR3 with improved selectivity over CCRl (CCR3 IC_{50} = 750 n*M*, CCRl IC_{50} = 7100 n*M*). Further derivatization of the aromatic groups, believed to be important for binding to hydrophobic pockets, led to (30) (CCR3 IC_{50} = 2.3 n*M*, CCRl IC_{50} = 1900 n*M*). CCR3 functional antagonism was demonstrated by its ability to block Ca^{2+} flux and chemotaxis of human peripheral blood eosinophils stimulated with eotaxin (157).



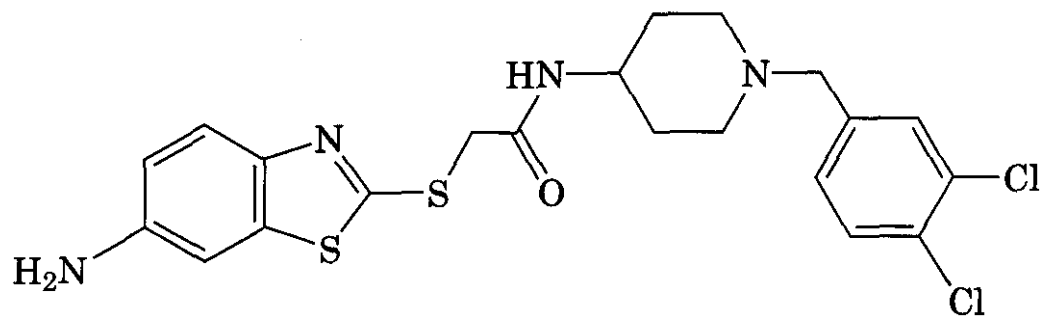
(29)

Other CCR3-specific antagonists are characterized by the piperidine ureas (**31–33**), and the di-substituted pyrrolidine (34) (158). Again, a basic nitrogen, capable of protonation at physiologic pH, appears to be a common feature. It is important to note that the quaternary ammonium salt (32) demonstrates enhanced potency, relative to that of its parent (31) in binding, chemotaxis, and a whole-blood GAFS assay (Table 4.8) (159).

Indolinopiperidines such as (34) were identified through random screening as CCR3 antagonists (IC_{50} = 0.7 μ*M*) (160). An H-bond acceptor at the 3-position of the aryl urea and a six-atom chain linkage were identified as important structural requirements for CCR3-binding affinity. The rigidity of these indolinopiperidines was relaxed by incorporation of a 4-benzylpiperidine in its place, giving (35) (IC_{50} = 0.02 μ*M*). Substitution around the piperidine further improved potency to the nanomolar range, as evidenced by (36) (IC_{50} = 0.001 μ*M*).

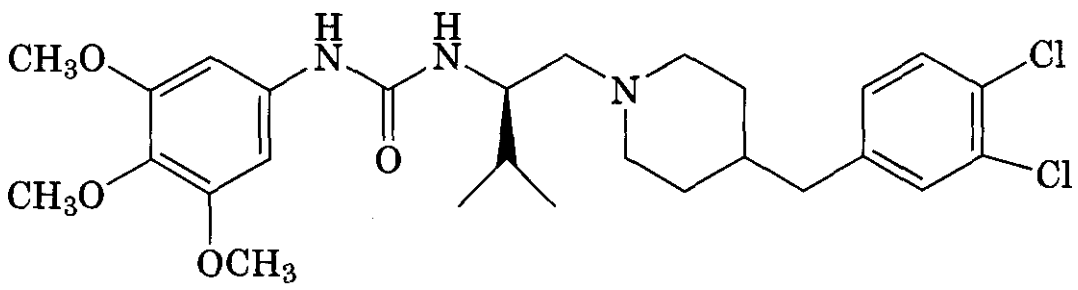
The substituted pyrrolidines (37) and (**38**) have been reported to inhibit the binding of eotaxin in CCR3-transfected HEK cells (IC_{50} ~1 n*M*) (161). These antagonists also inhibit eotaxin-induced calcium signal and eotaxin-induced eosinophilia. It is notable that these relatively compact, low MW analogs are able to provide exceptional binding affinity.

An exception to the requirement of a basic nitrogen in the pharmacophore of a CCR3 antagonist is noted by the phenyalanine deriva-



(30)

tives (39–41). High throughput screening using a FLIPR format to measure intracellular calcium changes identified (39) as an early lead (CCR3 IC_{50} = 535 n*M*) (162). Enantiospecificity appears to be critical for optimal antagonist/receptor interaction as replacement of the L-configuration of tyrosine with the D-amino acid results in greater than 200-fold lower affinity. Lead optimization to (40) demonstrated potent binding affinity (CCR3 IC_{50} = 5 n*M*) and functional antagonism, as measured by inhibition of eosinophil chemotaxis by use of eosinophils from allergic donors (IC_{50} = 15 n*M*). No in *vivo* data are reported for these compounds, presumably because of the hydrolytic lability of the ester. An ester replacement strategy identified 41 with a CCR3 with an IC_{50} value of 45 n*M*. It has yet to be shown whether any of these small molecule CCR3 antagonists are effective in an animal model.



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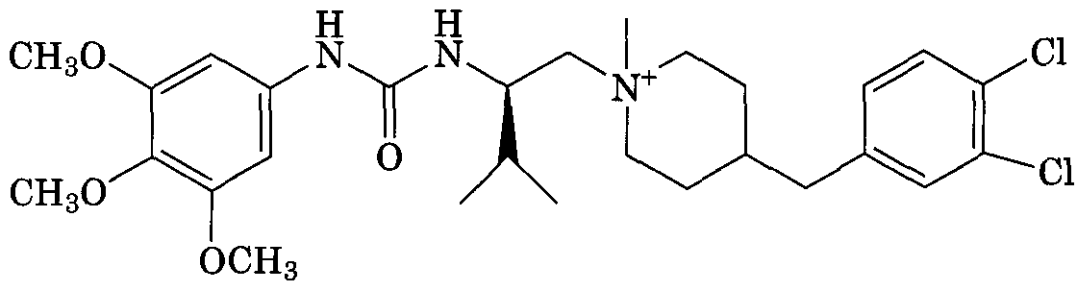

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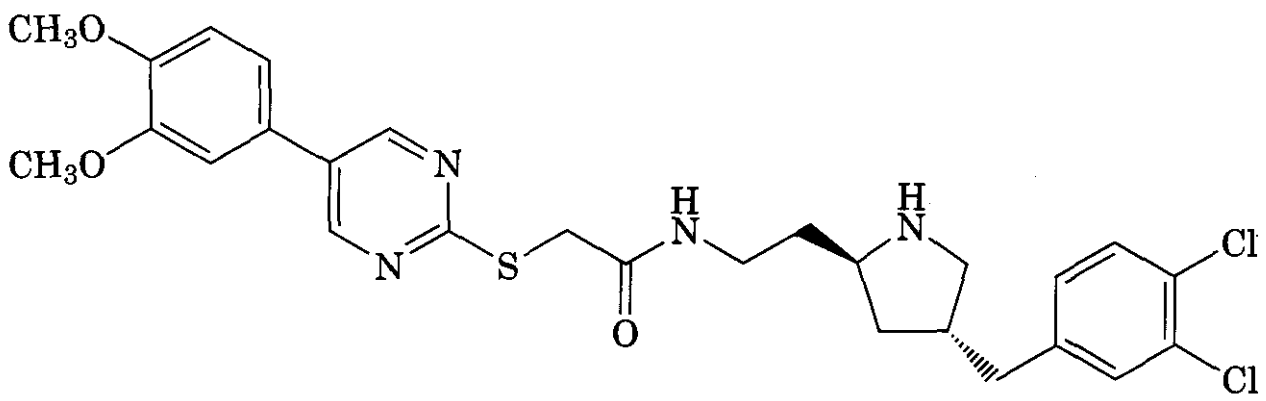

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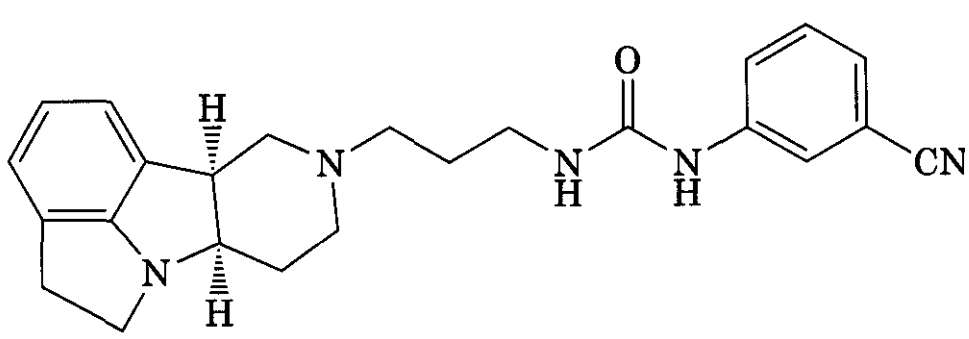
Table 4.8 Inhibition of Eotaxin Binding, Eotaxin-Induced Transfectant, and Eosinophil Chemotaxis

	IC_{50} (μM)		
Compound	CCR3 L1.2[a] Binding	CCR3 L1.2[b] Chemotaxis	Human Eosinophil Chemotaxis[c]
(**31**)	0.059	0.012	0.005
(**32**)	0.001	0.001	0.0004
(**33**)	0.005	0.002	0.003

[a]IC_{50} values derived from competitive binding of ^{125}I eotaxin to CCR3 L1.2 transfectant cells.
[b]IC_{50} values derived from inhibition of eotaxin-induced chemotaxis of the L1.2 transfectant cell line.
[c]IC_{50} values derived from inhibition of eotaxin-induced chemotaxis of human eosinophils.

6.4 CCR4

CCR4 was originally cloned by Power and colleagues from a basophilic cell line (163). RNA for CCR4 is expressed in stimulated T-cells, B-cells, monocytes, platelets, and, to a lesser extent, in basophils (163,164). CCR4 has been linked to Th2 cell polarization (165–167) and the expression of CCR4 was strongly upregulated upon activation through the T-cell receptor or by other cytokines such as transforming growth factor β (TGF-β) (166, 167). Two high affinity ligands have been described for CCR4,



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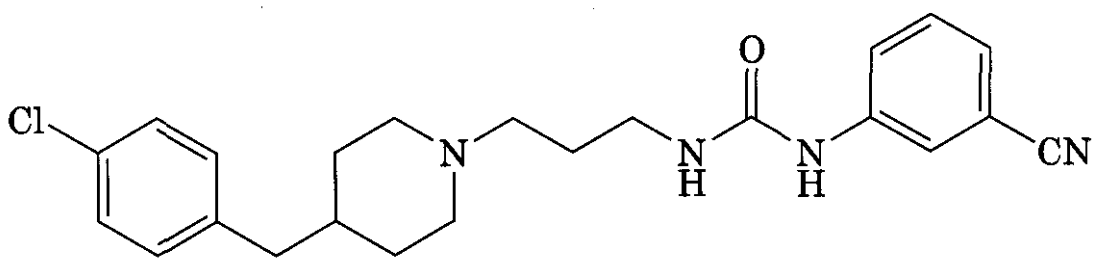

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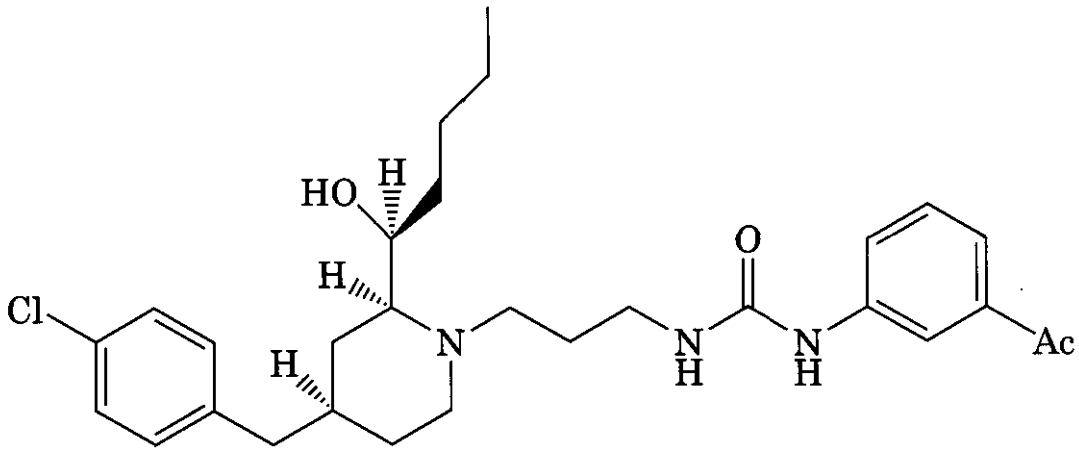

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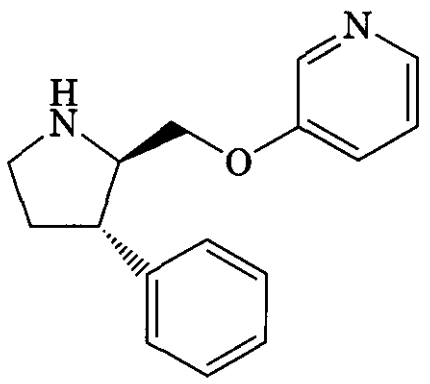

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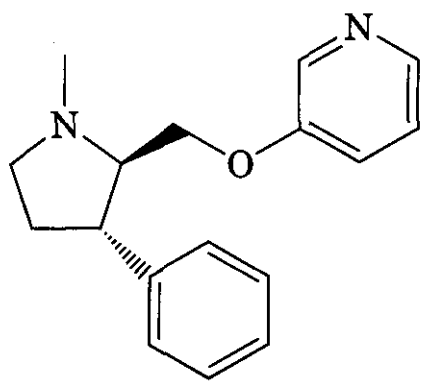

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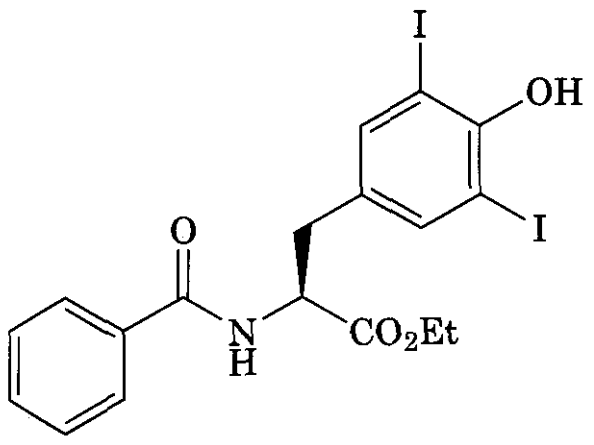

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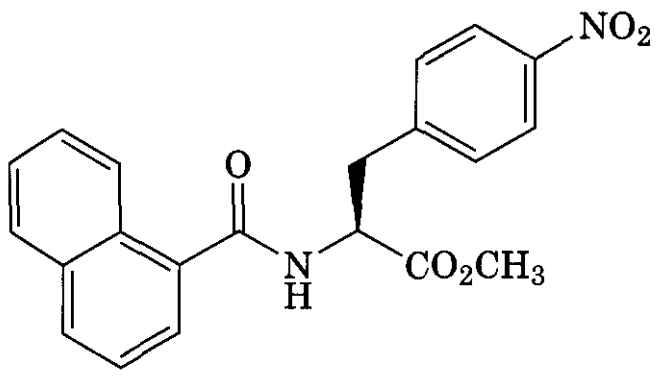

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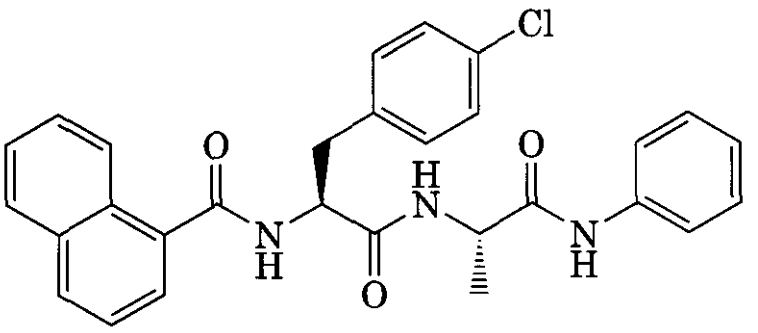
MDC (CCL22) and TARC (CCL17) (see Table 4.1). The role of the mouse ligand analogs has been studied in murine models of asthma. In ovalbumin-sensitized and -challenged mice,



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an anti-MDC antibody inhibits eosinophil recruitment to the lungs and protects against methacholine-induced bronchial hyperreactivity (168). Additionally, an anti-TARC antibody has been shown to attenuate an OVA-induced inflammation and airway hyperresponsiveness (169). In contrast to these results obtained from the neutralization of CCR4 ligands, the receptor knockout does not show any phenotype different from that of wild-type mice in the same models of allergic lung inflammation (170). In human tissues, CCR4 is expressed in all IL-4-positive cells in the bronchial mucosa of asthmatic patients that have been challenged 24 h before tissues were collected (171). In the same study, CCR4 ligands were found to be highly expressed by the airway epithelium in the same patient population. It has also been suggested that CCR4 and its ligands are also involved in other inflammatory diseases such as psoriasis and atopic dermatitis (172–174). These last observations correlate with the fact that CCR4-positive T-cells include all skin-homing cells (cutaneous lymphocyte antigen-positive cells) and other memory T-cells but not intestinal memory or naïve T-cells ($\alpha 4\beta 7$-positive T-cells) (175). This suggests a-role for CCR4 in tissue-specific homing of T-cells; however, the relevance of this tissue-specific homing to inflammatory skin diseases is not yet clear.

6.5 CCR5

This receptor, originally cloned as ChemR13, is part of the family of CC chemokine receptors binding RANTES (CCL5), MIP-la (CCL3), and MIP-1β (CCL4) (176–178). CCR5 is expressed on monocytes and some CD4+ T-lymphocyte subsets and its activation leads to chemotaxis of these cells (7).

CCR5 and CXCR4 represent the most important coreceptors for the HN-1 virus (179). CCR5 is involved in a sequential process, in which the viral gp120 interacts first with CD4, and then associates with the chemokine receptor to allow envelope fusion and viral entry. Strong genetic evidence supports the importance of CCR5 in HIV pathogenesis. Individuals homozygous for the A32 deletion are deficient in the surface expression of CCR5 and constitute the majority of the exposed but uninfected population (180–183). This mutation is represented at a high frequency in several human populations (184).

CCR5 is also expressed in T-lymphocytes found in synovial tissues from rheumatoid arthritis patients. Although CCR5 appears to be preferentially expressed on Th1 lymphocytes (165, 166), a recent clinical study proposed an important role for this receptor in asthma, a classic Th2 disease. This study suggests that individuals carrying the A32 deletion allele are at reduced risk of developing asthma (185). Two subsequent studies, however, suggest that there is no genetic evidence linking asthma and atopy with CCR5 A32 polymorphism (186,187). Further studies in the asthmatic population as well as the use of pharmacological tools would be needed to delineate the importance of CCR5 activation in allergic asthma.

Some correlation has been reported between the A32 mutation and graft rejection. In 21 patients homozygous for the mutation, *a* longer graft survival was observed compared to that for the control group. The results suggest a role for this receptor in transplant rejection (188).

6.5.1 CCR5 Receptor Structure. Many mutagenesis and chimera studies have shown that the N-terminus and the ECL-2 of the CCR5 receptor are involved in ligand binding (189). It has also been shown that the ECL-2 of CCR5 is the major determinant for chemokine binding specificity, whereas the amino-terminal domain plays a more significant role for human immunodeficiency virus (HIV-1) and virus coreceptor function (190). However, a potential involvement for the ECL-2 of CCR5 in HN-1 viral infectivity has been elucidated by mAb studies that use mAb 2D7, in which the epitope maps to a peptide derived from the ECL-2 domain, and inhibits both infection and gp120 binding (191). The overlapping binding site of chemokines and gp120 on the CCR5 amino terminus and ECL-2, as well as the involvement of these residues in the epitopes of mAbs, suggests that these regions are significantly exposed at the receptor surface. More recent studies have focused on the contribution of specific charged and aromatic resides (D2, Y3, Y10, D11, E18, and K26) in the amino terminus of CCR5 and their role in both chemokine and gp120 high affinity binding (192–197).

The role of the transmembrane helical domains of CCR5 receptor has also been evaluated. It has been shown that a hydrophobic residue in TM7, M287, is critical for both MIP-1α binding and receptor activation (Fig. 4.4). However, this mutant remains biologically active in the HN-1 coreceptor fusion assay (198). In another study it has been shown that glycine residue (G163) close to the extracellular domain in TM4, is critical for gp120 binding and infections by all tested R5 isolates of HIV-1 (199). It is interesting to note that chemokine binding can be independent of HIV-1 coreceptor activity and different TM domains can be involved in gp120 and chemokine binding to the CCR5 receptor.

6.5.2 CCR5 Antibodies. CCR5 mAbs have been reported by a number of groups (191, 200–202) and have proved useful in defining structural determinants important for natural ligand binding, chemokine activity and receptor signaling, gp120 binding to CCR5, and epitopes important for controlling HIV-1 entry. The mAbs 3A9, 2D7, and PA12 all inhibit HIV-1 entry and fusion, yet display significant differences in their ability to block chemokine binding, calcium flux, and chemotaxis. The 3A9 mAb was shown to bind to the N-terminus and is ineffective as an inhibitor of MIP-la, MIP-1β, and RANTES binding, and does not block MIP-1β-induced chemotaxis at concentrations up to 100 μM. However, 3A9 blocks infection of PHA-activated PBMCs by M-trophic strains (ID_{50} = 0.5–2.3 μg/mL) (200). The murine mAb PA12, whose epitope also maps to the CCR5 N-terminus, has no effect on RANTES-induced calcium flux and is only a

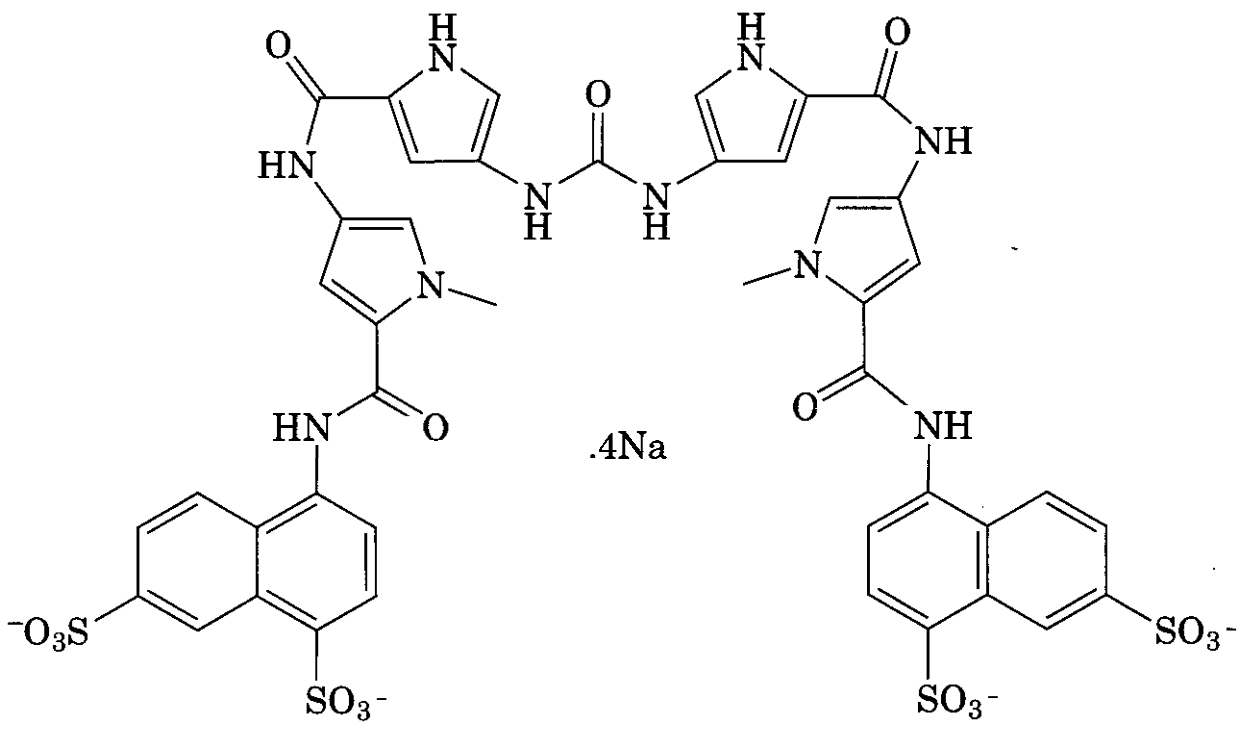
modest inhibitor of HIV-1 fusion and entry, yet strongly inhibits gp120 binding (201). In contrast, the 2D7 binding site is mapped to the second extracellular loop of CCR5 and completely blocks chemokine binding, Env binding, and viral infection (191). MAb 45531 also binds to an epitope in ECL-2 of CCR5, albeit in a different region from that of 2D7 (202). This antibody blocks chemokine binding, but is relatively inefficient in blocking gp120 binding. It would seem then that gp120 and the β-chemokines interact with overlapping but distinct sites on CCR5. Chemotaxis and chemokine signaling are not necessary for HIV-1 entry, and different receptor sites seem to have distinct functions (203–205). As a general rule, gp120 associates with both the N-terminus and ECL-1 of the receptor (192, 195), whereas chemokines favor ECL-2 for binding (206).

6.5.3 CCR5 Peptide Antagonists. Although RANTES, MIP-1a, and MIP-1β have all demonstrated an ability to suppress HIV infection in vitro (54), there has been concern expressed over using therapeutic agents that also function as agonists and have a potential to attract leukocytes to various tissues. Chemokine derivatives such as Met-RANTES, with an additional methionine at the N-terminus, and AOP-RANTES, with an aminoxypentane that is isosteric with the side chain of methionine, are pure and partial antagonists of CCR5, respectively. They inhibit HIV strains containing CCR5 at 10-fold greater potency than that of the natural ligands (54). Combination therapy has also proved effective at inhibiting mixed infections (207). AOP-RANTES (CCR5 antagonist) and Met-SDF1b (CXCR4 antagonist), when used in combination, were much more effective (95–99% inhibition) at blocking R5X4 dual-tropic HIV-1 primary isolates than when used alone (32–61% inhibition).

Truncated peptides have also been evaluated. A truncated version of RANTES ($RANTES^{[8-68]}$) binds to CCR5, yet is incapable of eliciting a chemotactic response (53). This would tend to support the role of the amino terminus in receptor activation. $RANTES^{[8-68]}$ does block HIV-1 infection in cells (208).

6.5.4 CCR5 Small Molecule Antagonists. As a consequence of screening, the National Cancer Institute chemical repository for inhibitors of HIV-1 replication, the distamycin analog NSC65106 (42) was identified as having antiviral activity (209). Its mechanism of action appears to be through involvement of chemokine receptors such as CCR5 and CXCR4. It has also been shown to inhibit CCR1 and CCR3 and is inactive against CCR2 and CXCR2 (210). The monomer (43) and regioisomer (44) were inactive against this panel of receptors.

As part of an effort to identify CCR2b antagonists, the ammonium salt (45) and the



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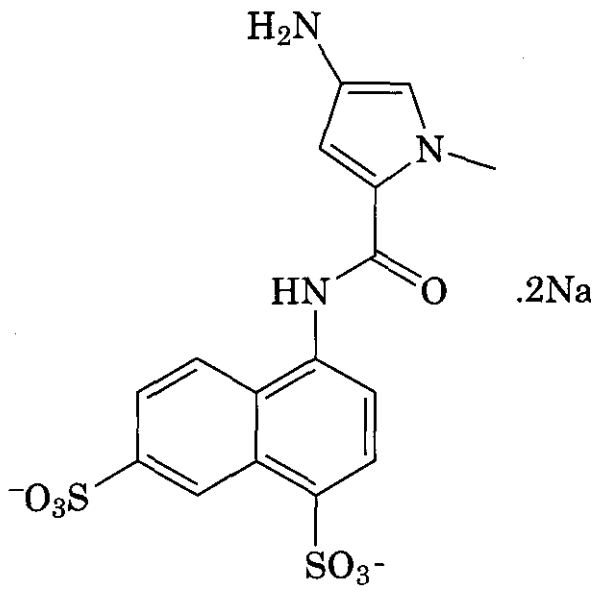

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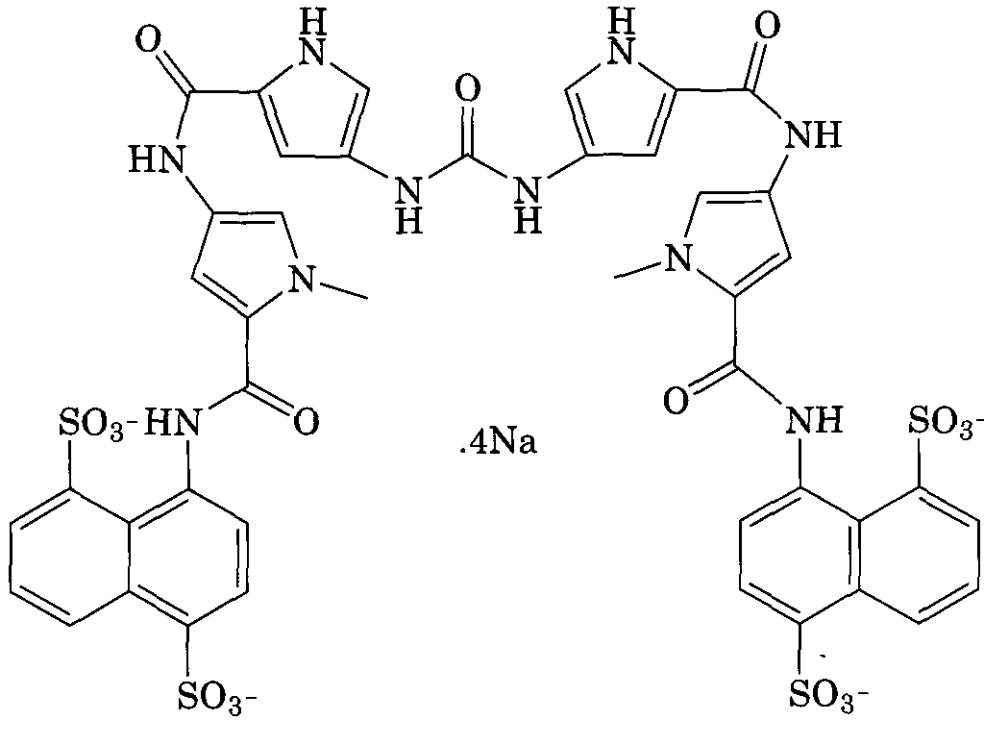
phosphonium salt (46) were found to have fair activity against CCR5 (IC_{50} values of 0.39 and 0.62 μM, respectively) (211). Lead optimization through ring expansion resulted in the quaternary ammonium anilide (25) (CCR5 IC_{50} = 0.0014 μM; CCR2b IC_{50} = 0.027 μM). Also known as TAK-779, (25) is inactive against other chemokine receptors such as CCR1, CCR3, CCR4, and CXCR4 (202).

TAK-779 inhibits the binding of anti-CCR5 mAb 45531 to MOLT-4/CCR5 cells and is therefore presumed to bind to ECL-2 (202). However, it does not inhibit binding of 2D7, which maps to a different region of ECL-2; or of 3A9, which recognizes the amino terminus of CCR5 (123). TAK-779 is an effective inhibitor of HIV-1 in T-cells and blocks R5HIV-1 replication without cytotoxicity to host cells (212,213).

The binding site of TAK-779 has been explored using Ala-scanning mutagenesis on more than 60 residues in the N-termini and extracellular loops 1–3, and more than 35 residues within TM1-TM7 (214). In this study they could not identify specific amino acid residues within the extracellular domains of CCR5 that affected the antiviral action of TAK-779. However, several residues in the TM domains (L33, Y37, T82, W86, Y108,



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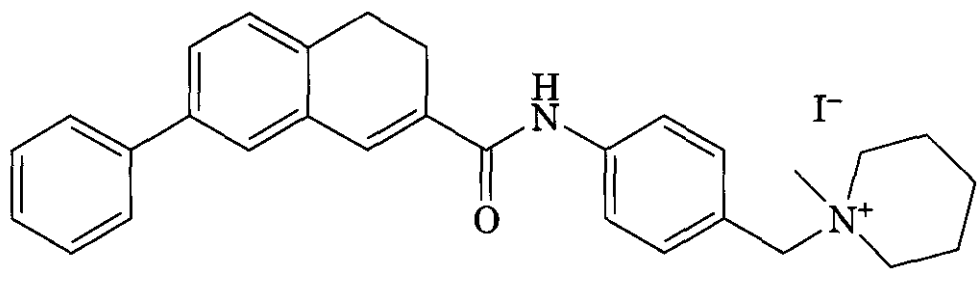

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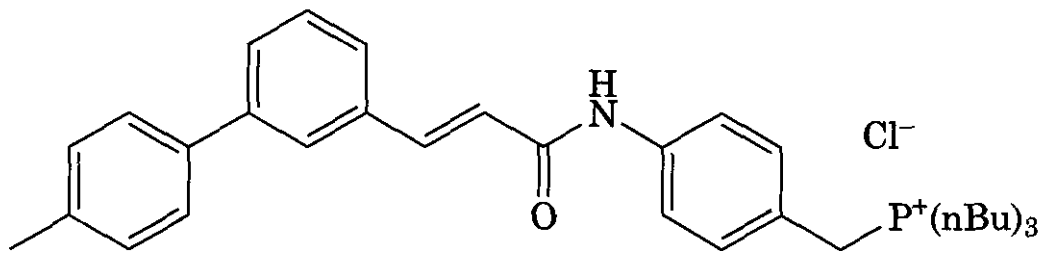
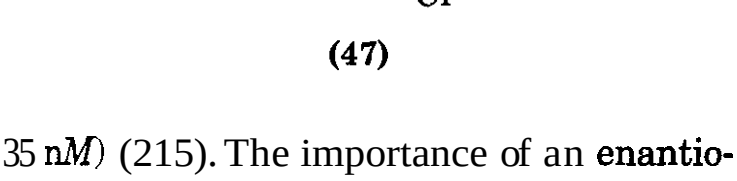


(46)

T123, and E283) were found to interact with TAK-779. The binding site of TAK-779 has been postulated to be within a cavity formed between helices 1, 2, 3, and 7 of the CCR5 receptor (Fig. 4.5).

The sulfonyl spiropiperidine (47) has also demonstrated activity against CCR5 (IC_{50} =

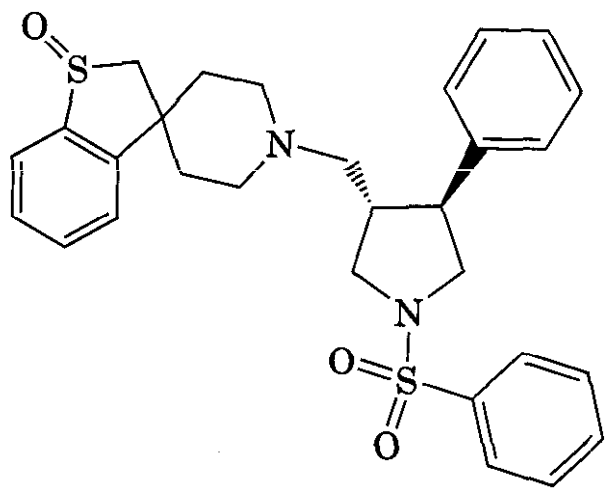


(47)

35 n*M*) (215). The importance of an enantioselective interaction is suggested in that the (2*R*) diastereomer of (**47**) has an IC_{50} value of 870 n*M* for CCR5, and is therefore 25 times less potent than the (2*S*) isomer. HIV-1 viral replication in PBMCs is inhibited by (**47**), with a modest IC_{95} value of 6 μM. The SAR of the C-2 phenyl fragment has also been investigated, with the 3'-C1 derivative proving optimal (CCR5 IC_{50} = 10 n*M*) (216). Oral bioavailability within this series of sulfonyl spiropiperidines has proved to be very poor (F_{rat} < 3%).

Constrained analogs have also been prepmed. The 3,4-*trans*-pyrrolidine (48) shows better potency (CCR5 IC_{50} = 26 n*M*) than the corresponding cis analog (CCR5 IC_{50} = 3500 n*M*) (217).

Diarylsulfones (49) and (50) were identified as early leads in a Schering CCR5 program (218–220). These antagonists had been initially prepared for a muscarinic program and

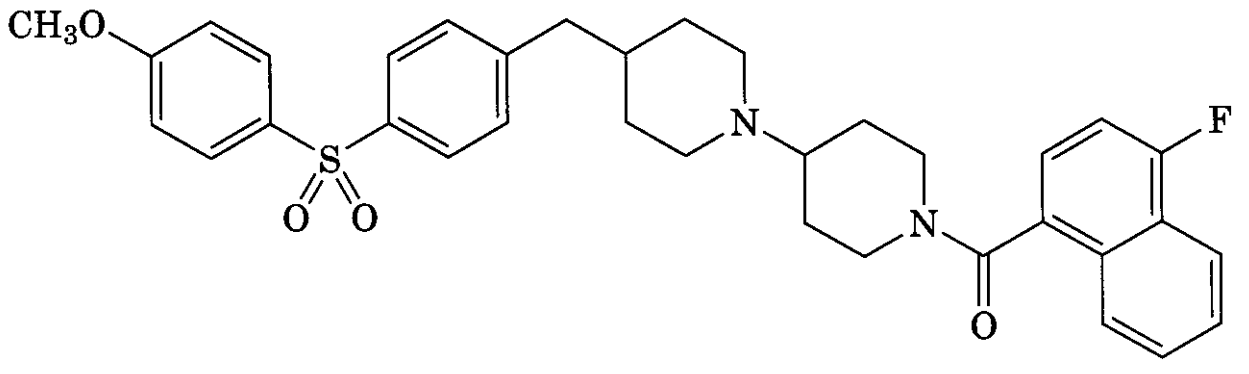


(48)

showed significant activity against the M_2 receptor (**49**): CCR5 K_i = 1.0 @, M_2 K_i = 1.3 n*M*; (50):CCR5 IC_{50} = 440 n*M*, M_2 K_i = 0.8 n*M*).

The initial strategy to improve potency and selectivity focused on substitution on and around the piperazine (218). Chirality of the piperazine 2-methyl was used to improve potency and selectivity, as demonstrated by the preference for the (2s)-methylpiperazine (51) (CCR5 K_i = 28 n*M*, M_2 K_i = 485 n*M*). The absence of any substitution at the 2-position (**52**) resulted in a dramatic loss of activity (CCR5 IC_{50} = 1300 n*M*).

Additional SAR analyses found that replacement of sulfonyl with methylene, as in (**53**), retained potency and selectivity (CCR5 K_i = 18 n*M*, M_2 K_i = 760 n*M*). The next concern was metabolic lability of the methylene dioxy, and the 3'-chlorophenyl analog (**54**) was found to be a suitable alternative (CCR5 K_i = 45 n*M*, M_2 K_i = 1400 n*M*). Both (53) and (**54**) were shown to block viral entry at subcytotoxic levels (IC_{50} values of 1.7 and 12 n*M*, respectively). In addition, (53)inhibited the replication of primary HIV-1 isolates in peripheral blood mononuclear cells (PBMCs) (IC_{50} = 8 n*M*).



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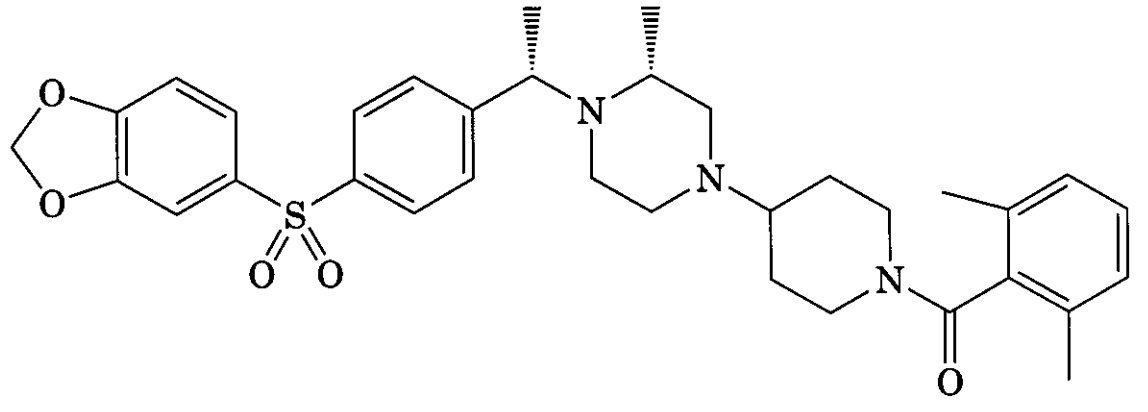
Further exploitation of these leads led to the discovery that the shortened piperidino-piperidine amide analog **(55)** also shows significant inhibition of CCR5 (CCR5 $K_i = 66$ nM, M_2 K_i = 1323 nM) **(220).** However, this compound has poor biovailability, presumably because of metabolism at the benzylic site. To address this shortcoming, ethoxime was intro-



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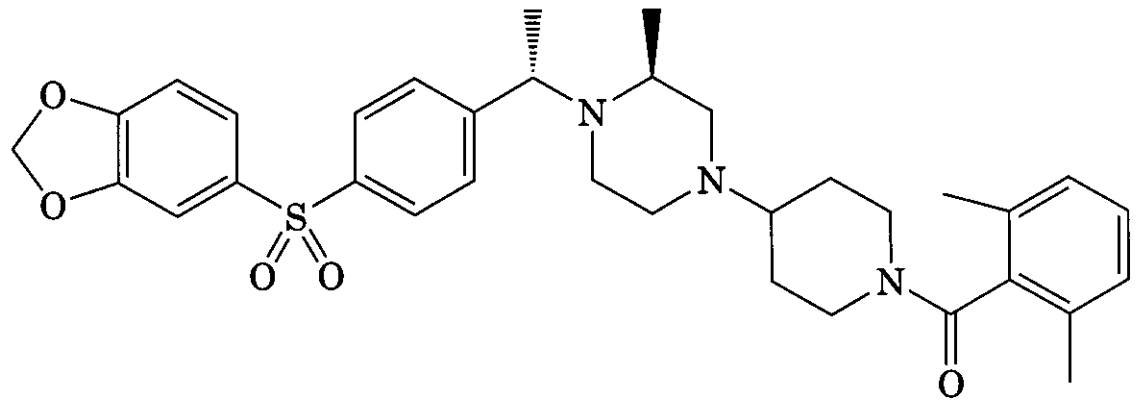

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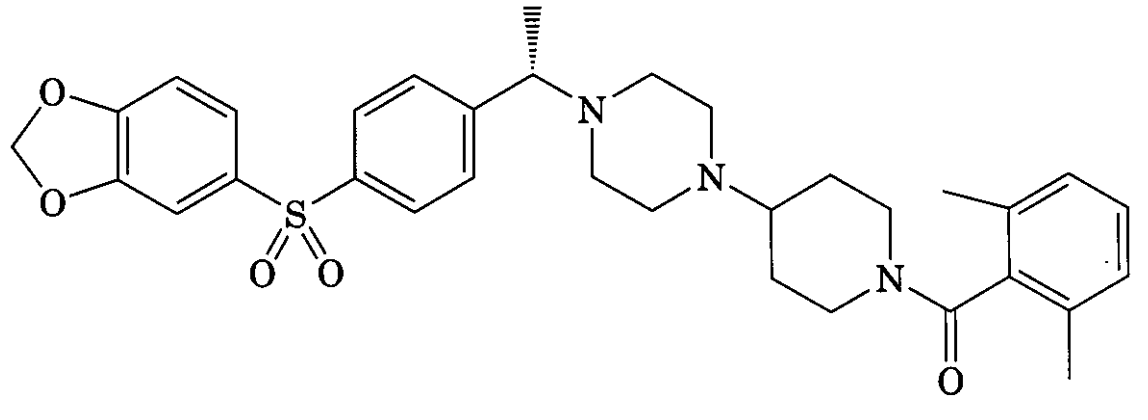

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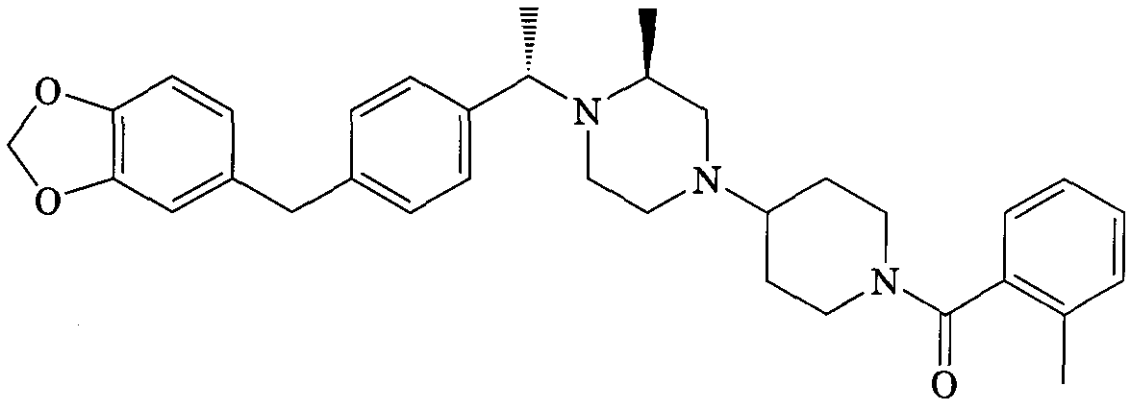

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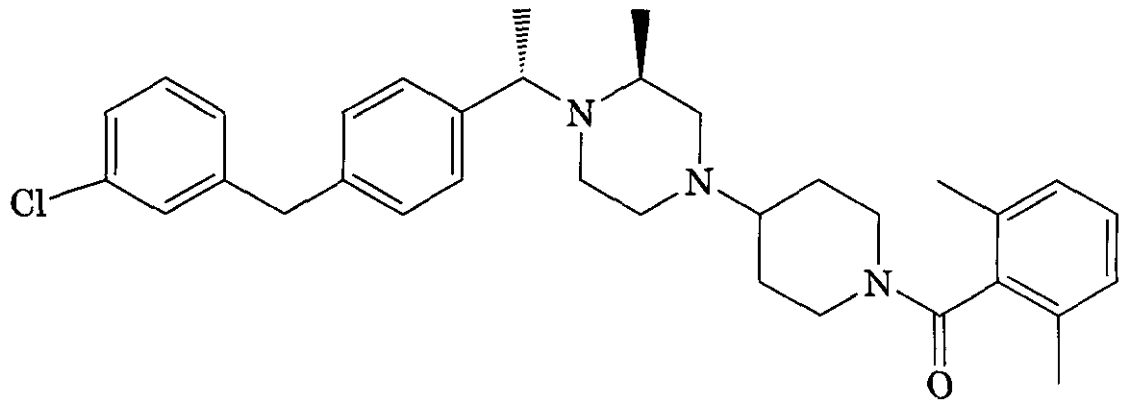

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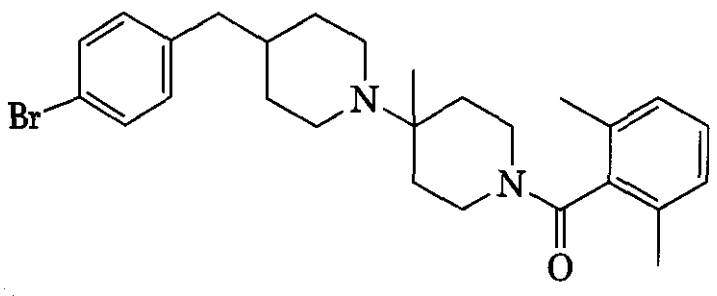
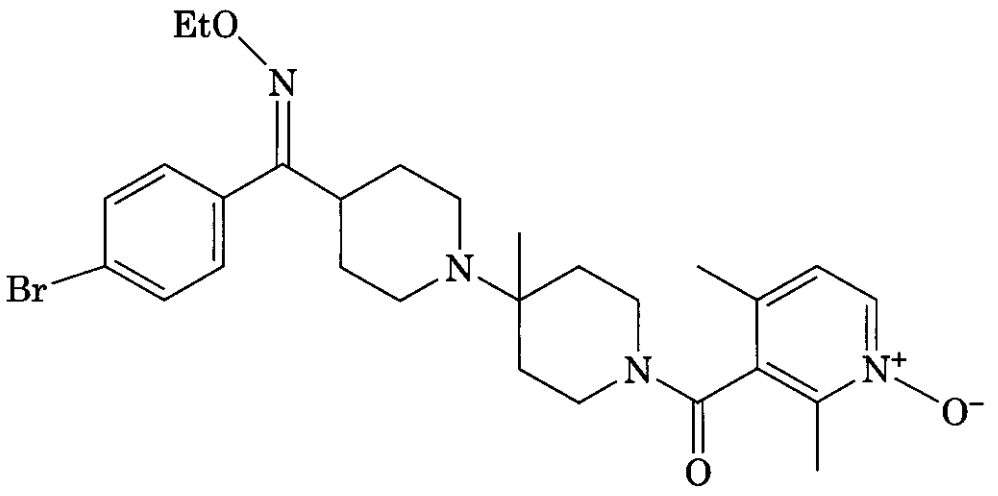


(55)

luced, followed by subsequent replacement of he then labile 2,6-dimethyl phenyl with a yridine N-oxide fragment, to give (**56**). This esulted in significant improvements of rat lasma levels after oral administration (CCR5 ζ_i = 45 n*M*, M_2 K_i = 1400 n*M*; rat pk at 10 ng/kg p.o.: $AUC_{(0-24\ h)}$ = 8200 ng $mL^{-1}h^{-1}$, $F_{(rat)}$ = 63%, $F_{(monkey)}$ = 52%). The piperazine nalog (**57**) also showed improved pharmacoki- netics (CCR5 K_i = 7 n*M*, M_2 K_i = 250 n*M*; rat pk: $AUC_{(0-6\ h)}$ = 9243 ng $mL^{-1}h^{-1}$). Compound (**56**) has also been referred to as SCH-C, and is eported to have entered Phase I clinical trials.

6.6 CCR6

Specific chemokine receptors involved in dendritic cell biology are a topic of current investigation. Originally cloned as STRL-22, CKR-L3, GPR-CY4, or DRY6, CCR6 has only one chemokine ligand, MIP-3α (CCL20) (221–223). More recently, it has been shown that a family of antimicrobial peptides, the β defensins, also bind CCR6, although their affinity for this receptor is significantly lower than that of MIP-3α (224). CCR6 has been shown to be expressed in PBMCs, and in a subpopulation of immature dendritic cells. $CD34^+$-derived dendritic cells, but not monocyte-derived dendritic cells, show CCR6 expression and a significant response to MIP-3α (225). Recently, Yang and colleagues (226) have shown that in the presence of TGFβ, monocyte-derived dendritic cells are also able to express CCR6. In addition, CCR6 has been found to be expressed in a subset of memory T-cells as well as in a subset of B-cells (227, 228). Moreover, it has been described that CCR6 plays an essential role in the arrest of a subset of memory T-cells to activated dermal endothelium under physiologic flow conditions (229). All these results strongly support a role for MIP-3α/CCR6 in dendritic cell migration and in the recall immune response.



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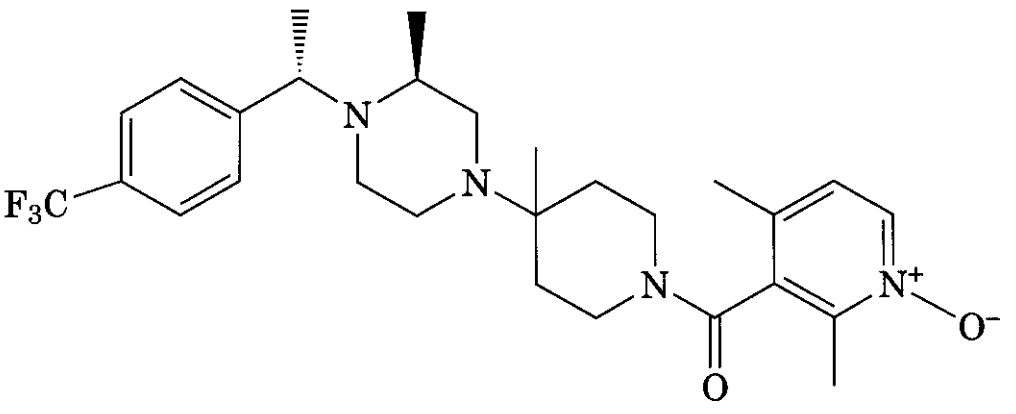
$CCR6^{-/-}$ mice have been recently generated by two different groups. In the Cook study, there was no evidence of gross abnormalities in any major organ, and all major leukocyte populations appeared normal (230). Dendritic cells coexpressing CD11b and CD11c were absent from the subepithelial dome of Peyer's patches. Some decreased humoral response to orally administered rotavirus was observed but no important delay in the viral clearance. In a second study, using a contact hypersensitivity model and a delay hypersensitivity model for the analysis of $CCR6^{-/-}$ mice, Varona and colleagues described a role for CCR6 in the activation/migration of CD4+ T-cell subsets (231). More recently, it has been reported that $CCR6^{-/-}$ mice are protected from developing allergic lung inflammation and airway hyperreactivity in a cockroach model (232).

6.7 CCR7

CCR7 expression has been detected in mature dendritic cells (225, 233, 234), naïve T-cells, and a subset of memory T-cells (central memory T-cells) (235, 236). Two ligands for CCR7 have been identified: MIP-3β (CCL19) and SLC (CCL21) (237). CCR7 and CXCR5 together have been proposed as the main receptors involved in cell recruitment into the lymph nodes, Peyer's patches, and spleen. The constitutive expression of MIP-3β (ELC) and SLC in lymphoid tissue supports a role for these chemokines in the trafficking of lymphocytes to secondary lymphoid organs. MIP-3β is also expressed in interdigitating dendritic cells, whereas the other ligand for CCR7, SLC, is secreted by stromal cells (238–240). Dieu and colleagues (225) have shown an interesting relationship between CCR6 and CCR7 expression in dendritic cells. Although CCR6 is expressed in immature dendritic cells and these cells are responsive to MIP-3a, cell maturation causes CCR6 downregulation and CCR7 upregulation. Concomitant with the expression of CCR7, mature dendritic cells acquire the capability to respond to MIP-3P. Other studies have also observed CCR7 upregulation in mature dendritic cells (233,234). The differential expression of these chemo-



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kine receptors likely accounts for the distinct migration patterns observed for immature and mature dendritic cells.

A natural mutation in mice, the plt mutation, has helped to elucidate the role of CCR7 and its ligands. Mice homozygous for the mutation show impaired homing of T-cells to the lymph nodes and spleen (241). These mice also show a decreased number of interdigitating dendritic cells in lymph nodes. Recently, the plt mutation has been associated with a deletion that results in the lack of MIP-3β and reduced expression of SLC (242). The phenotype of plt mice reflects the role of CCR7 in dendritic and T-cell recruitment.

CCR7 knockout mice show a significant reduction in naïve T-cells in secondary lymphoid tissues as well as important lymphoid tissue abnormalities. When challenged, $CCR7^{-/-}$ mice show an impairment in T-cell-mediated immune response, including impairment of delayed-type hypersensitivity reactions (243). Because of the importance of CCR7 in T- and dendritic-cell recruitment, targeting CCR7 might represent a viable way of interfering with acquired immune responses. However, given that no studies have been reported in which the role of CCR7 has been specifically studied in animal models of human diseases, it is difficult to predict from the in vitro data and the few in vivo studies available, the consequences of inhibiting CCR7.

6.8 CCR8

CCR8 was cloned by several groups as an orphan chemokine receptor (TER1, ChemR1, CKR-L1) (244–246). CCR8 is constitutively expressed at high levels only in the thymus, although low levels of mRNA are also present in the spleen (244). A ligand for human CCR8 has been shown recently to be the CC chemokine I-309 (CCL1). It was the only chemokine, among a large panel, to induce intracellular calcium mobilization and chemotaxis in CCR8-transfected cells (247,248).

Both CCR8 and CCR4 are preferentially expressed by Th2 cells (165–167, 249) and I-309 induces a preferential migration of human Th2-polarized cells in vitro (167, 249).

A murine homolog of CCR8 has been characterized and shows a pattern of expression similar to that of hCCR8 (250). In the periphery, CCR8 mRNA expression was present only in a Th2-polarized subset of CD4+ T-cells. The ligand for mCCR8 has been identified as T-cell activation gene (TCA)-3 (250, 251). TCA-3 induces chemotaxis and activation of neutrophils, macrophages, mesangial cells, and vascular smooth muscle cells (252–255).

CCR8 knockout mice have been generated and analyzed in a mouse model of allergic lung inflammation (256). In models of Th2-type response, including Schistosoma *mansoni*-soluble egg antigen (SEA)-induced granuloma formation as well as ovalbumin- and cockroach antigen-induced allergic airway inflammation, CCR8 knockout mice showed impaired Th2 cytokine production and reduced eosinophil recruitment. By contrast, in a typical Th1 model of secondary granuloma formation, CCR8 knockout mice behaved in a manner no different from that of wild-type mice. These results suggest an important role for CCR8 in Th2 functional responses in *vivo*.

7 CXC RECEPTORS

7.1 CXCRl and CXCR2

Two human receptors for IL-8 (CXCL8) have been identified. CXCRl preferentially binds IL-8 and GCP-2, whereas CXCR2 binds all ELR-containing chemokines, including IL-8, GROα, GROβ, GROγ, NAP-2, ENA 78, and GCP-2 (18, 257–259). IL-8 is primarily regarded as a neutrophil chemoattractant, in spite of the fact that these receptors are expressed on eosinophils, monocytes, and basophils (18). CXCR1/2 activation, in addition to inducing chemotaxis in different leukocytes, also stimulates the release of granule enzymes and respiratory burst in neutrophils (260).

A close association has been described for IL-8 and other CXCR1/2 ligands in acute inflammation, including neutrophil-mediated inflammatory diseases such as ischemia-reperfusion injury, bacterial pneumonia, adult respiratory distress syndrome, and other infectious diseases. Neutralization of IL-8 in rabbits has shown dramatic protection in several models of neutrophil-mediated acute inflammation (261,262).

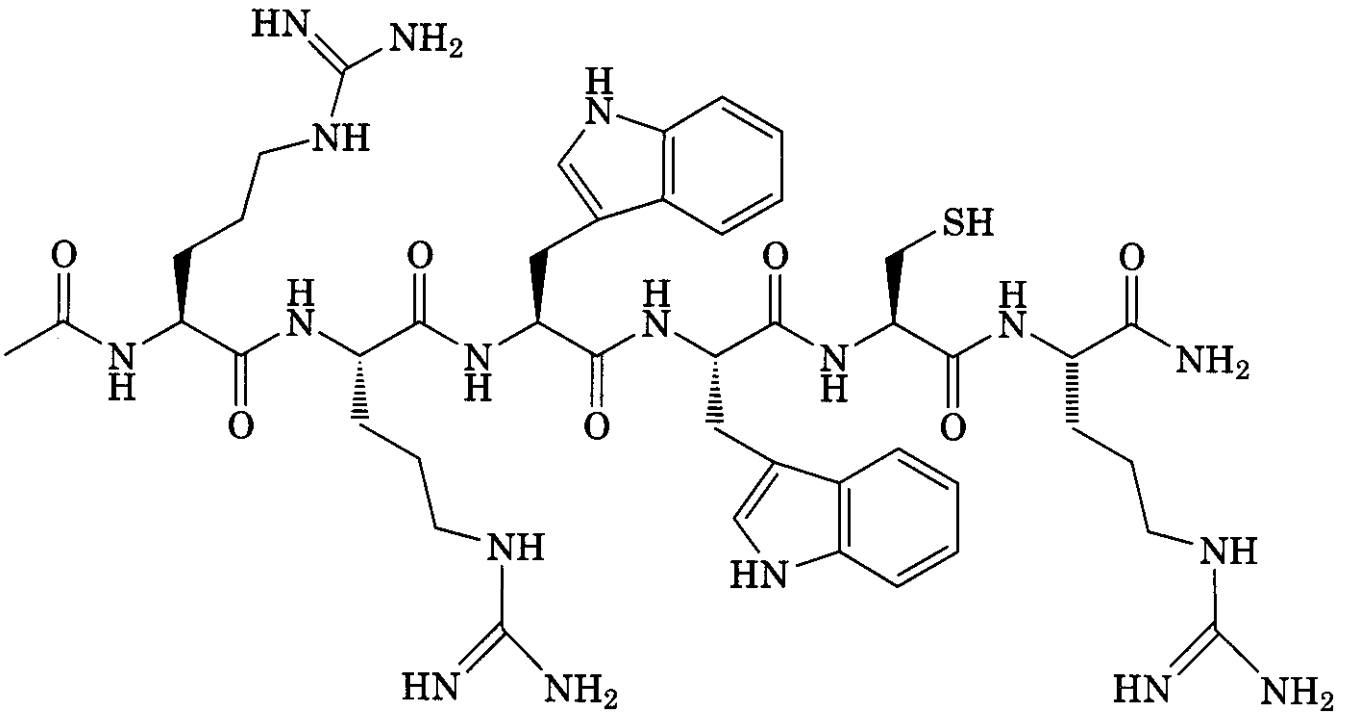
The exact role of CXCRl and CXCR2 has been difficult to define, in part because mice have a single receptor for IL-8-like molecules. Disruption of this receptor gene in mice results in abnormal myeloid and lymphoid development (263). The mice fail to mobilize neutrophils in response to intraperitoneal thioglycolate challenge. In an allergic model of lung inflammation, mice with a targeted deletion of the IL-8 receptor showed a marked decrease in the recruitment of neutrophils into the airways (264). After multiple challenges, knockout mice had increased B-cells and fewer neutrophils compared to those of wild-type (wt) mice. A diminished hyperresponse to methacholine was also observed in the knockout mice, suggesting that the IL-8 receptor can modulate the IgE response and bronchial hyperresponsiveness, in addition to the regulation of neutrophil infiltration. In support of its possible role in airway disease, several studies have shown the presence of IL-8 in the bronchoalveolar lavage (BAL) fluid of asthmatic patients (265–268).

In addition to their role as chemoattractants for neutrophils, CXCRl and CXCR2 ligands have been shown to be potent angiogenic factors, whereas most non-ELR-CXC chemokines (CXCR3, CXCR4, and CXCR5 ligands) are angiostatic (269, 270). Several examples of diseases in which the balance between angiostatic and angiogenic chemokines is altered have been described, including chronic pancreatitis, inflammatory bowel disease, psoriasis, and idiopathic pulmonary fibrosis (271–273).

7.1.1 CXCR1/CXCR2 Receptor Structure. CXCRl and CXCR2 have a high degree of amino acid identity (77%), and yet they show different binding selectivity for CXC chemokines. CXCR2 has similar binding affinity for IL-8, GROa and NAP-2, whereas CXCRl is selective and has high affinity only for IL-8, and a much weaker affinity for GCP-2. It has been shown that the N-terminus and ECL-2 are important for both ligand binding and selectivity among CXCRl and CXCR2 receptors. Ala scanning of the CXCRl extracellular domains shows that several charged residues (D11, R199, R203, D265, E275, and R280) are critical for IL-8 binding (274–277). Similarly, additional charged residues in the N-terminus and ECL-2 of the CXCR2 receptor are also shown to be critical for binding of IL-8, NAP-2, and GRO to CXCR2. Residues important for IL-8 binding are D9, E12, K108, and K120, whereas E7, D9, and E12 significantly contribute to GROa binding (278). In this study it is clear that residues contributing to the high affinity binding site of IL-8 and GROa on CXCR2 have both overlapping and uncommon regions. Notably, mutations of the N-terminus residues (D9, E12) and certain ECL-1 residues (K108, N110, K120) reduce cell activation and receptor signaling to all three ligands (278).

7.1.2 CXCR1/CXCR2 Antibodies. Neutralizing monoclonal and polyclonal antibodies of CXCR2 have been generated to profile the function of each IL-8 receptor (CXCR1 and CXCR2), and important distinctions have been proposed. Changes in calcium flux and signaling for granule enzyme release can be initiated in a chemokine-specific manner, and blocked with antibodies specific to each receptor (anti-IL8R1 and anti-IL8R2) (279). Respiratory burst, activation of phospholipase D (279), and neutrophil chemotaxis (280) are proposed to be stimulated predominantly through CXCRl. Priming of neutrophil NADPH oxidase is also mediated primarily through CXCR1 (281). Understanding the role of each receptor in contributing to a physiologic response of neutrophils will be important in the design of new therapeutics.

7.1.3 CXCR1/CXCR2 Peptide Antagonists. As indicated above, the chemokine N-terminus seems to contain a critical recognition site important for receptor binding and activation. The ELR sequence is essential to CXCR1/ligand interaction and its importance in IL-8 has been established through analog deletion or amino acid substitution within this region (13). Truncation of the first five amino acids in the IL-8 Nt ($IL\text{-}8^{[6-72]}$), or substitution of the first five amino acids with two alanines ($IL\text{-}8^{[AAR7-72]}$) gave high affinity antagonists capable of blocking receptor binding, neutrophil chemotaxis, and respiratory burst (282). An N-terminal truncation of GROa demonstrates CXCR2 antagonism; similar changes in PF4



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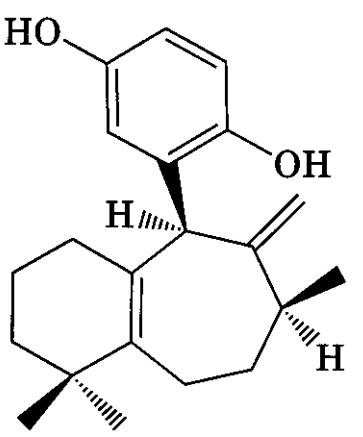
have also been shown to antagonize CXCR2 (283). These NH_2-terminally modified analogs had no effect on IL-&stimulated elastase release or superoxide generation, which are responses presumed to be mediated by CXCR1.

A naturally occurring inhibitor of IL-8 (IL-8INH) has been identified in the supernatant of polymorphonuclear leukocytes. This 52-kDa protein blocks ^{125}I-IL-8 binding to the receptor by binding specifically to IL-8. IL-8INH demonstrates in *vivo* activity by inhibiting neutrophil infiltration to mouse ear (284).

Capped hexapeptides and heptapeptides have also been reported as inhibitors of IL-8. Antileukinate (58) inhibits the binding of IL-8 to both receptors and blocks both neutrophil chemotaxis and activation (285). GROα and IL-8 have been shown to be necessary for growth of lung, stomach, and colon adenocarcinomas, and antileukinate will inhibit the binding of GROα to specific receptors on adenocarcinoma cell lines and block proliferation (286). Antileukinate has also been shown to block Staphylococcal enterotoxin A (SEA)-induced neutrophil infiltration into the lung (287).

7.1.4 CXCR1/CXCR2 Small Molecule Antagonists. There are early reports of natural products identified as CXCRl and CXCR2 antagonists. Extracts from sponge (Dysidea *frondosa*) provided Frondosins A (59) and C (60) as novel sesquiterpenes (288). Lissoclin



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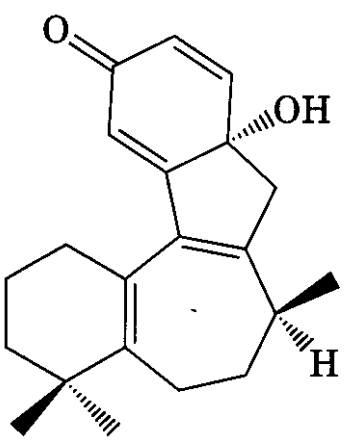

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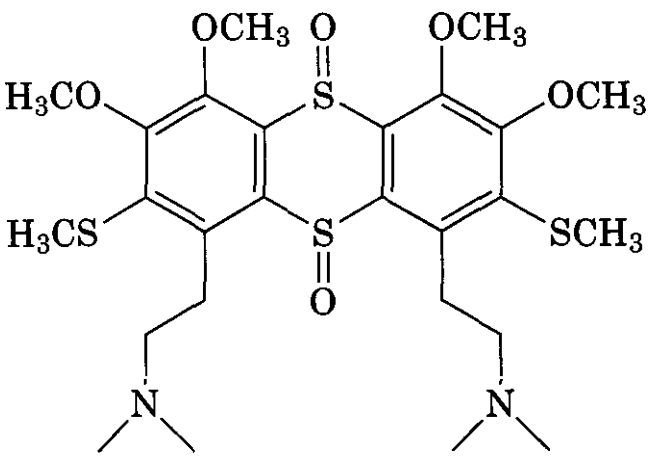
disulfoxide (61) was also extracted from a marine organism, the South African asidian *Lissoclinum* (289). All three natural products (59–61) show modest affinity for CXCR2 (Table 4.9). However, similar activity against PKC was also noted, and thus the activity appears to be nonselective.

Table 4.9 Inhibition of CXCR1, CXCR2, and PKCα with Frondosins A and C, and Lissoclin Disulfoxide

	IC_{50} (μM)		
Compound	CXCR1[a] Binding	CXCR2[b] Binding	PKCα
(**59**)	3.4	3.2	1.8
(**60**)	84	23.6	20.9
(**61**)	0.6	0.8	1.5

[a] IC_{50} values derived from competitive binding with ^{125}I-IL-8.
[b] IC_{50} values derived from competitive binding with ^{125}I-IL-8.

The diaryl urea (62)inhibits ^{125}I-IL-8 binding to CXCR2 on CHO cells (IC_{50} = 500 nM) (290, 291). A simple incorporation of a bromine on one of the aryl groups (63) provided a marked enhancement in CXCR2 affinity (IC_{50}



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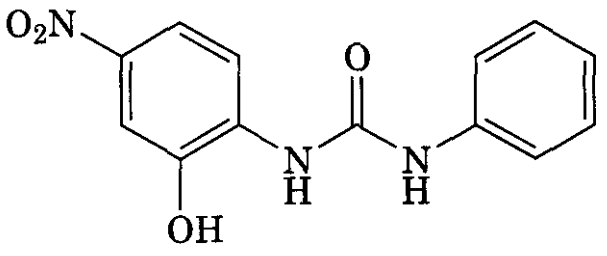

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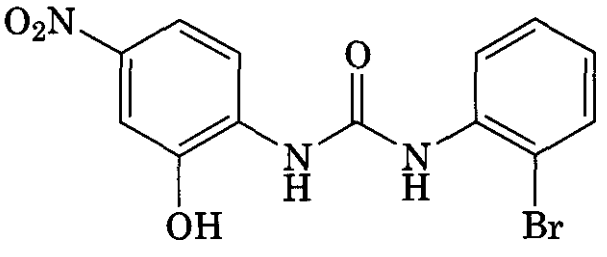
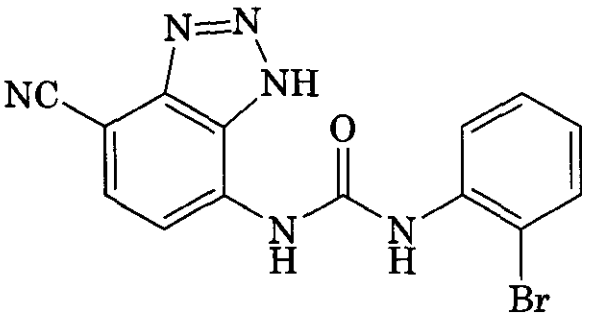


(63)

= 22 nM), with greater than 150-fold selectivity over CXCR1. This compound also inhibits GROα- and IL-&mediated chemotaxis of human and rabbit neutrophils. When administered by i.v. infusion in rabbits, (63) blocks IL-&induced neutrophil margination.

The contribution of the urea acidic group has been evaluated by measuring binding as a function of pH (292). Ureas bind strongly in their anionic form, demonstrating as much as a 10-fold improvement over the non-ionized parent. Because the binding is reversible, the mechanism of antagonism does not appear to proceed through the isocyanate.

In an effort to identify an orally active drug, further optimization in the diaryl urea series led to (**64**), which inhibits ^{125}I-IL-8 binding to



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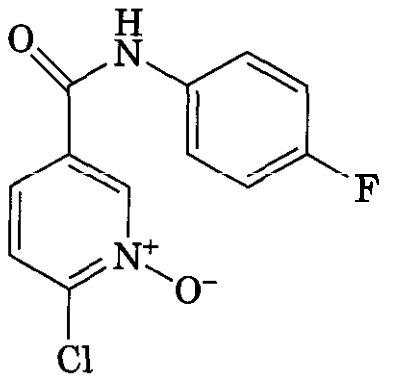
CHO/CXCR2 membranes (IC_{50} = 39 nM) and is selective against CHO/CXCR1 (IC_{50} = 7400 nM) (293). The diarylurea (64) also inhibited GROα-induced Ca^{2+} flux (IC_{50} = 5 nM), which is CXCR2 mediated, while showing weaker inhibition against IL-Smediated Ca^{2+} flux (IC_{50} = 426 nM), which is governed by both CXCRl and CXCR2. Compound (64) was evaluated for efficacy in an LPS-induced airway neutrophilia within rabbits. When given orally 1 h before and 4 h after an aerosol LPS challenge, (**64**), at 25 mg/kg, showed significant inhibition of neutrophilia.

More recently, nicotinamide N-oxides such as (**65**) were discovered to be antagonists of



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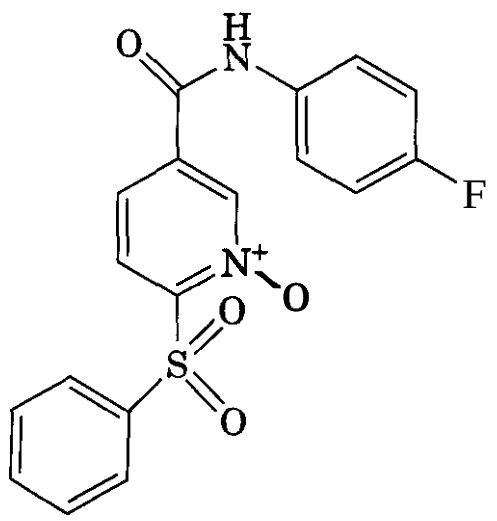
^{125}I-IL-8 binding to CXCR2 (IC_{50} = 1.0 μM) (294). This compound acts as a functional antagonist, as measured by its ability to block both GROα- and IL-8-driven neutrophil chemotaxis.

SAR studies found the 4-fluoroanilide to be an important recognition element. Further lead optimization improved ligand binding antagonism, as indicated by (66) and (67) (IC_{50} values of 0.09 and 0.28 μM, respectively). These compounds varied in their ability to block GROα chemotaxis (IC_{50} values of >20 and 1.0 μM, respectively). The discrepancy between binding and chemotaxis may reflect the different binding interaction of GROα and IL-8 on CXCR2 (277,278).



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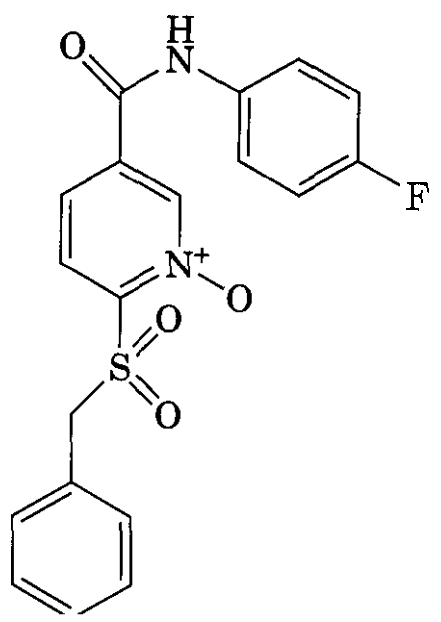

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7.2 CXCR3

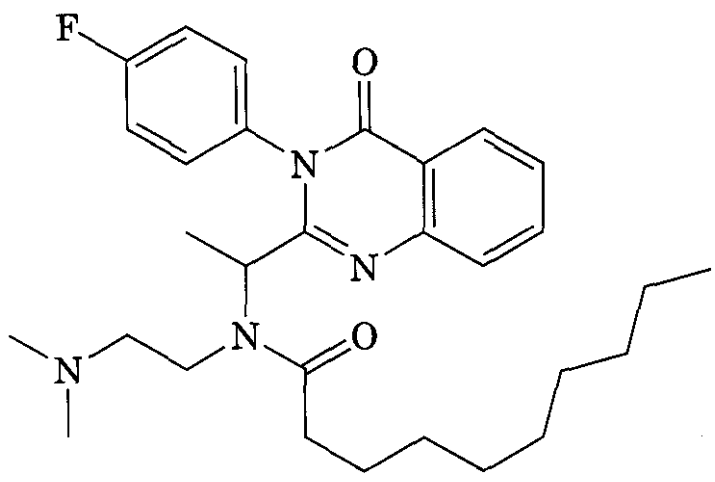
CXCR3 is a receptor for three CXC chemokines, IP-10 (CXCL10), MIG (CXCL9), and I-TAC (CXCL11) (see Table 4.1). This receptor is expressed preferentially on T-cells and it is upregulated upon activation of these cells. The restricted expression of this receptor suggests that its ligands are involved in the regulation of lymphocyte recruitment observed in autoimmune inflammatory lesions. IP-10 was identified as a gene product induced by interferon-y and expressed in delay-type hypersensitivity reactions of the skin (295, 296). More recently, CXCR3 has been described as being present on all perivascular T-cells and astrocytes associated with active lesions in multiple sclerosis. In addition, CXCR3- and CCR5-expressing T-cells are enriched in the cerebrospinal fluid of patients with active disease (297,298).

CXCR3-deficient mice show an impressive response in an allograft survival model. In the absence of any other treatment, allograft survival extended 55–60 days, whereas the normal allograft survival for wild-type mice was around 7 days. Expression of the three ligands for CXCR3 has been reported in this model; however, they show a sequential upregulation, suggesting they may play a role at different times during the development of the inflammatory response (299).

CCR5 and CXCR3 have been identified as being preferentially expressed on Thl cells (300). Accordingly, IP-10 is approximately 10 times more potent on Thl cells than on Th2 cells. Because rheumatoid arthritis is classified as a Thl-type disease, it has been suggested that CXCR3 could play a role in the development of the disease. CXCR3-positive T-cells and CXCR3 ligands have been reported in rheumatoid arthritis in the synovial fluid and synovium from these patients (301,302).

7.2.1 CXCR3 Antibodies. A CXCR3-specific monoclonal antibody (1C6) has been reported (301). It blocks the binding of radiolabeled IP-10 to activated T-cells (IC_{50} = 160 ng/mL), and its epitope is mapped to the first 15 amino acids of the receptor. IP-10-mediated chemotaxis was completely blocked by use of 10 μg/mL of 1C6. Neutralizing antiserum against MIG and IP-10 has been shown to be efficacious in transplant models, as has a blocking anti-CXCR3 mAb in preventing acute rejection (299). Similarly, anti-IP-10 mAbs increase survival times in a cardiac allograft rejection model (303).

7.2.2 CXCR3 Small Molecule Antagonists. To date, there are very few reports on small molecule antagonists for this receptor. The 4-oxo-2,4-dihydro quinazoline (68) is reported to block binding ($IC_{50} < 0.8\ \mu M$) of IP-10 (304). There have been no reports of in *vivo* activity with this type of antagonists.



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7.3 CXCR4

As mentioned earlier, CXCR4 and CCR5 have together been identified as the main coreceptors for HIV, acting with CD4 to enable viral entry. More specifically, CXCR4 has been described as an essential coreceptor for T-tropic HIV-1- and HIV-2-mediated fusion (305,306). CXCR4 was first cloned by several groups as an orphan receptor named LESTR, HUMSTR, or fusin (307). Later, SDF-1α (CXCL12) was identified as the only ligand for this receptor (308,309). These studies have also shown that SDF-la inhibits the infection of CD4/CXCR4-expressing cells by T-tropic strains of HIV.

CXCR4 is widely expressed in different blood cell types including B-cells, T-cells, and monocytes (see Table 4.1). In addition, expression of this receptor has been described in non-immune cells such as endothelial and epithelial cells (307). SDF-1α was originally isolated from bone marrow stroma and it has been shown that it supports the proliferation on B-cell progenitors (310). Subsequent studies demonstrate that SDF-1α is also a potent chemoattractant for lymphocytes and monocytes. Unlike other chemokines, SDF-1α is constitutively expressed and is not regulated by proinflammatory cytokines (307,311).

CXCR4 knockout mice generated by several groups have shown multiple functions associated with this receptor. SDF-1α and CXCR4 knockout mice present very similar phenotypes. Both showed defective lymphopoieis and myelopoiesis in the bone marrow (312, 313), as well as severe heart defects, defective formation of large blood vessels, and derailed cerebellar neuron migration (312–315). Moreover, most $CXCR4^{-/-}$ mice died in utero at 18.5 days of embryonic development and the remainder died within 1 h after birth. This is the only chemokine receptor null mutation described to date that results in embryonic lethality.

7.3.1 CXCR4 Receptor Structure. Rat and human CXCR4 chimeras suggest that ECL-2 of CXCR4 is the major determinant of receptor binding to feline immunodeficiency virus (FIV) (316). Mutation of the DRY motif and the C-terminal tail of CXCR4 did not affect the ability of the molecule to support fusion, suggesting that neither signaling by way of G-protein nor receptor internalization was required for fusion mediated by FIV (316). The contribution of the negatively charged residues in the amino terminal of the CXCR4 receptor has also been evaluated (317). Point mutation studies show that viral entry depends on YDE-rich clusters in both the amino terminus and the second extracellular loop of CXCR4. Different viral isolates vary in their dependency on residues in each domain. The determinants of CXCR4 coreceptor function are therefore more diffuse and isolate dependent than those of CCR5 (317). Residues in CXCR4 required for both SDF-1 binding and

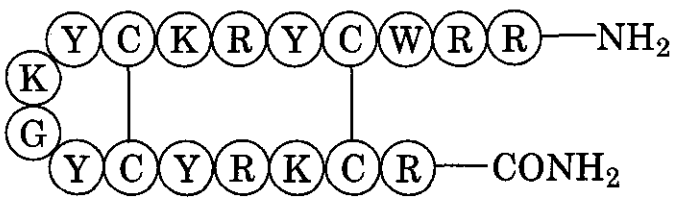
signaling were identified as ECL2 (D187), TM2 (D97), and TM7 (E288). The first residues (2–9) of the receptor N-terminus also seem to be required for SDF-1 binding and signaling. Residues important for ligand binding but not signaling in the N-terminus are E13, E14, and Y21. The coreceptor activity of CXCR4 (gp120 binding) was impaired by mutations of two Tyr residues in the N-terminus (Y7, Y12) and an Asp residue in ECL-2, ECL-3, or TM2 (D193, D262, D97). These acidic residues may engage in electrostatic interactions with basic residues of the gp120 HIV-1 envelope protein in the V3 loop region (318). Ala scanning of CXCR4 indicates that negatively charged residues in the N-terminus (E14, E15, and E32) and D97 in ECL-1 were important for CXCR4 function as a coreceptor. It has been shown that removal of the N-linked glycosylation modifications of the CXCR4 molecule permits the receptor to act as a potential coreceptor for otherwise CCR5-dependent HIV-1 Envs. Similar residues in the ECL-2 of CCR5 were shown to be important for CCR5 coreceptor activity for isolates across several clads. Together, these findings support the hypothesis that there are conserved elements important for coreceptor activity between the CXCR4 and CCR5 molecules. These results highlight a homologous and critical region in ECL5 for both CXCR4 and CCR5 and their respective coreceptor activities (319,320).

7.3.2 CXCR4 Antibodies. A CXCR4 monoclonal antibody (12G5) was developed as the result of efforts to evaluate interactions of cellular molecules and viral envelope glycoproteins. 12G5 was shown to inhibit infection and cell fusion for isolates of HIV-2. After CXCR4 was identified as a CD4-associated accessory factor for T-cell line tropic HIV-1 isolates, 12G5 was shown to react with this protein and showed specific binding for CXCR4 when compared to CCR1, CCR2b, CCR3, CCR4, and CCR5 (321).

7.3.3 CXCR4 Peptide Antagonists. There have been several reports on peptide antagonists that selectively block receptor binding and/or activities of CXCR4. The importance of the N-terminal region of SDF-1a was defined through use of truncated ligands. Short *N*-terminal peptides have significant activity. $SDF^{[1-9]}$ could function as a weak agonist, whereas a P2G mutation of this same truncation resulted in an antagonist (322). The N-terminal residues form an important receptor binding site with Lys-1 and Pro-2 also contributing to receptor activation. Residues 12–17 (RFFESH) are important for receptor binding, but apparently do not contribute to activation. It is presumed then that this sequence is involved in initial docking of ligand to receptor. NMR-conformational analysis of these truncated peptide ligands shows that residues 5–8 and 11–17 present as a β-turn β-αRtype (323).

A 3D structure of the full-length peptide SDF-1 was determined through use of NMR (47), and important differences were noted, when compared to other chemokine ligands, in terms of hydrophobic packing and surface charge distribution. Using a strategy that has worked for other chemokine ligands, the CXCR4 natural ligand, SDF-la, was derivatized with methionine at the N-terminus. Met-SDF-1β caused prolonged downregulation of the receptor (324), and as a single agent, or in combination with other antivirals such as Zidovudine or Nelfinavir, has activity against HIV-1 isolates (325).

T22 (69) is an 18 amino acid analog of a horseshoe crab blood cell-derived peptide. It



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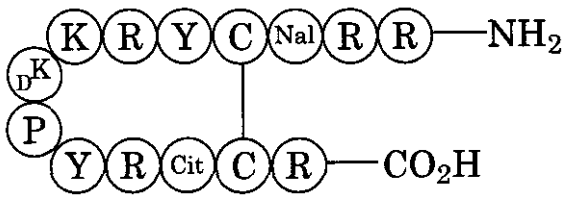
inhibits entry of T-tropic HIV-1 strains into target cells. T22 blocks binding of 12G5 mAb to CXCR4 and inhibits SDF-1-induced Ca^{2+} mobilization. The structure of T22 mimics the rigid structural conformation found in CXC chemokines. The antiparallel β-sheet presentation in SDF-1 is maintained within T22 by virtue of its two disulfide bonds (326).

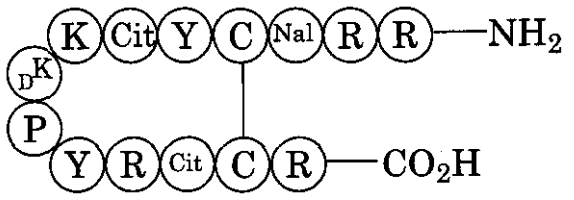
T22 is a cationic peptide characterized by five arginine and three lysine residues. In T22 and its derivatives there appears to be a close correlation between the number of positive

charges and anti-HIV activity (327). Ala scanning shows Arg2, Nal3, Tyr5, and Arg14 to be critical residues for activity (328). An electrostatic interaction of peptide with membrane may contribute to cytotoxicity and a poor selectivity index (SI = ratio of 50% cytotoxic concentration to 50% effective antiviral concentration). Reducing the total positive charge with β-citrilline (Cit) was hoped then to result in less toxicity without a significant decrease in anti-HIV activity. Thus, truncation and Cit-substitution of T22 identified a 14-mer with a single disulfide bond, T140 (**70**), as a more po-



(70)

tent antagonist (EC_{50}=3.3 n*M*, SI = 29,000). In an attempt to further reduce the total positive charge, an additional (Cit) substituent was introduced, resulting in TC14003 (**71**),



(71)

with a decreased net positive charge compared to that of T22 and a greater selectivity index (EC_{50}=4.0 n*M*, SI = 69,000) (328).

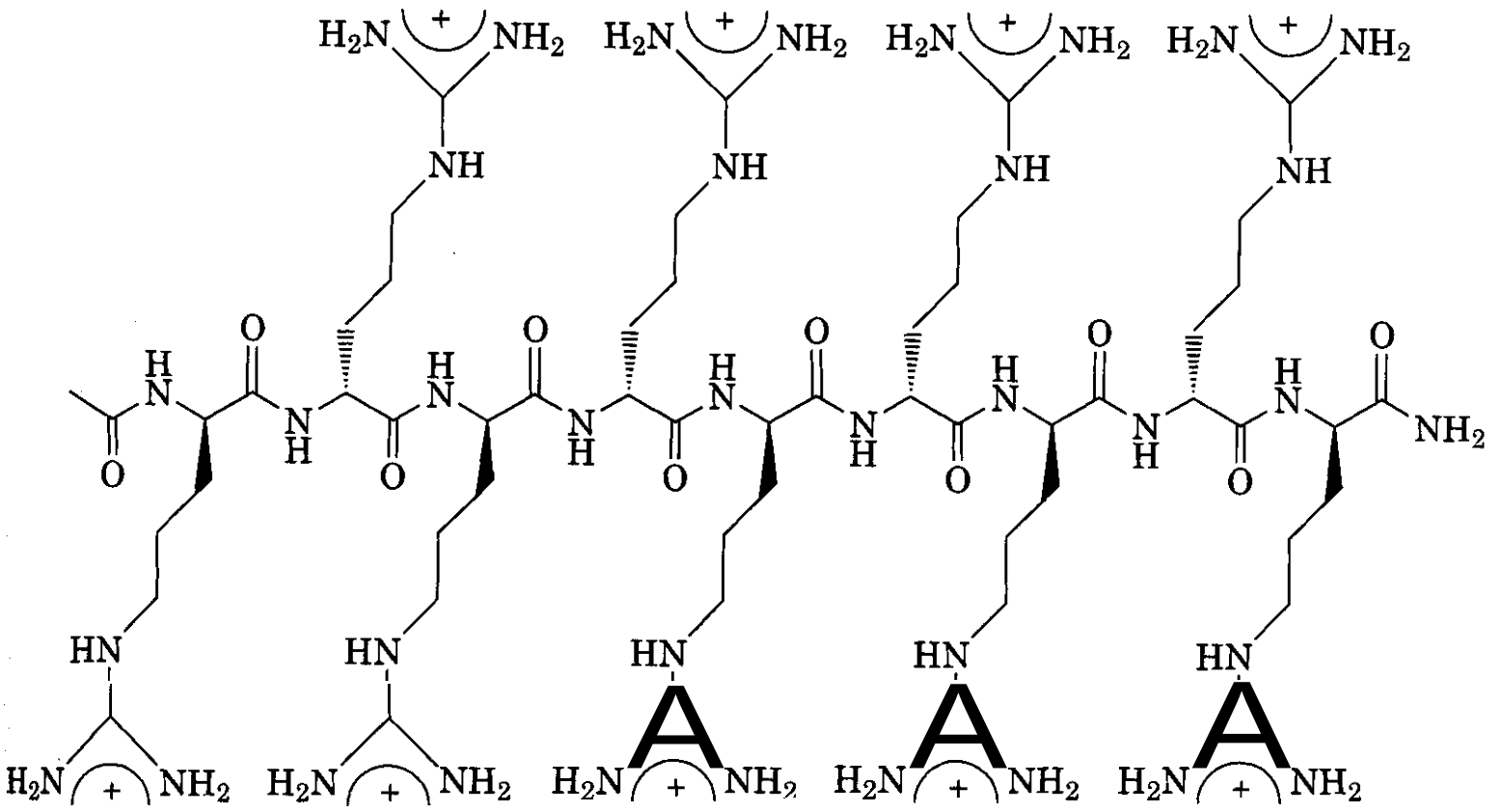
An arginine-rich polypeptide (72) has also been described as an inhibitor of HIV-1 entry acting through CXCR4. Again, the high positive charge on ALX40C is characteristic of the β-sheet region of SDF-1 and these residues are presumed to be a recognition element for CXCR4 (329). The Tat peptide has also been shown to inhibit HIV replication. The peptoid, CGP-64222 (**73**), mimics the basic domain of Tat, and has been shown to interact with CXCR4 (330).

The binding site on CXCR4 is believed to be localized to the second extracellular loop (ECL-2), which contains a strong negative surface charge with many anionic residues (326, 331, 332). The strongly cationic nature of these peptide antagonists probably results in a strong electrostatic interaction with the receptor.

Noting the importance of the arginine, conjugates of L-arginine with aminoglycosides have provided micromolar inhibitors such as (**74**). They interact directly with CXCR4, as evidenced by their ability to inhibit the binding of the 12G5 mAb (333).

7.3.4 CXCR4 Small Molecule Antagonists. In 1992 bicyclams such as (75) were identified as viricidal agents with activity against HIV-1 and HIV-2 (334). The bicyclam AMD2763 (75) was shown to inhibit HIV replication in various human T-cells. Based on time-of-addition experiments, these agents were assumed to interact with virus assembly after virus adsorption and preceding reverse transcription. Their precise mechanism of action was not determined until several years later, when the anti-HIV replication activity of bicyclams was shown to occur through direct interaction with CXCR4 (331,335,336). This class of compounds had no effect against M-tropic HIV viruses (CCR5-dependent). AMD2763 was shown to bind with CXCR4 through competition studies with 12G5 and selectivity was demonstrated by its ability to block Ca^{2+} mobilization stimulated by SDF-la, but not by RANTES, MIP-la, or MCP-3.

A more potent analog, AMD3100 (**76**), inhibits HIV-1 replication at nanomolar concentrations (337). It is not toxic to host cells at 500 μ*M*, and therefore shows a high selectivity index. Compound 76 binds to CXCR4, as determined by inhibition of 12G5, and does not block 2D7 (the CCR5 mAb) (331). AMD3100 inhibits T-cell-tropic HIV-1 infection of PHA-stimulated blasts from PBMCs, with an IC_{50} value of 2–7 ng/mL, depending on viral strain. This is a 10- to 50-fold improvement over SDF1. Further derivatization of the bicyclam series resulted in AMD3329, the py[iso-14]ane N4 dimer (77). Incorporation of heteroaromatics into the cyclam moiety lowered the overall pK_a compared to that of a secondary amine. Compound 77 was determined to be even more potent than AMD3100 in inhibition of binding, Ca^{2+} flux, and suppression of X4 HIV-1 replication. The EC_{50} values against

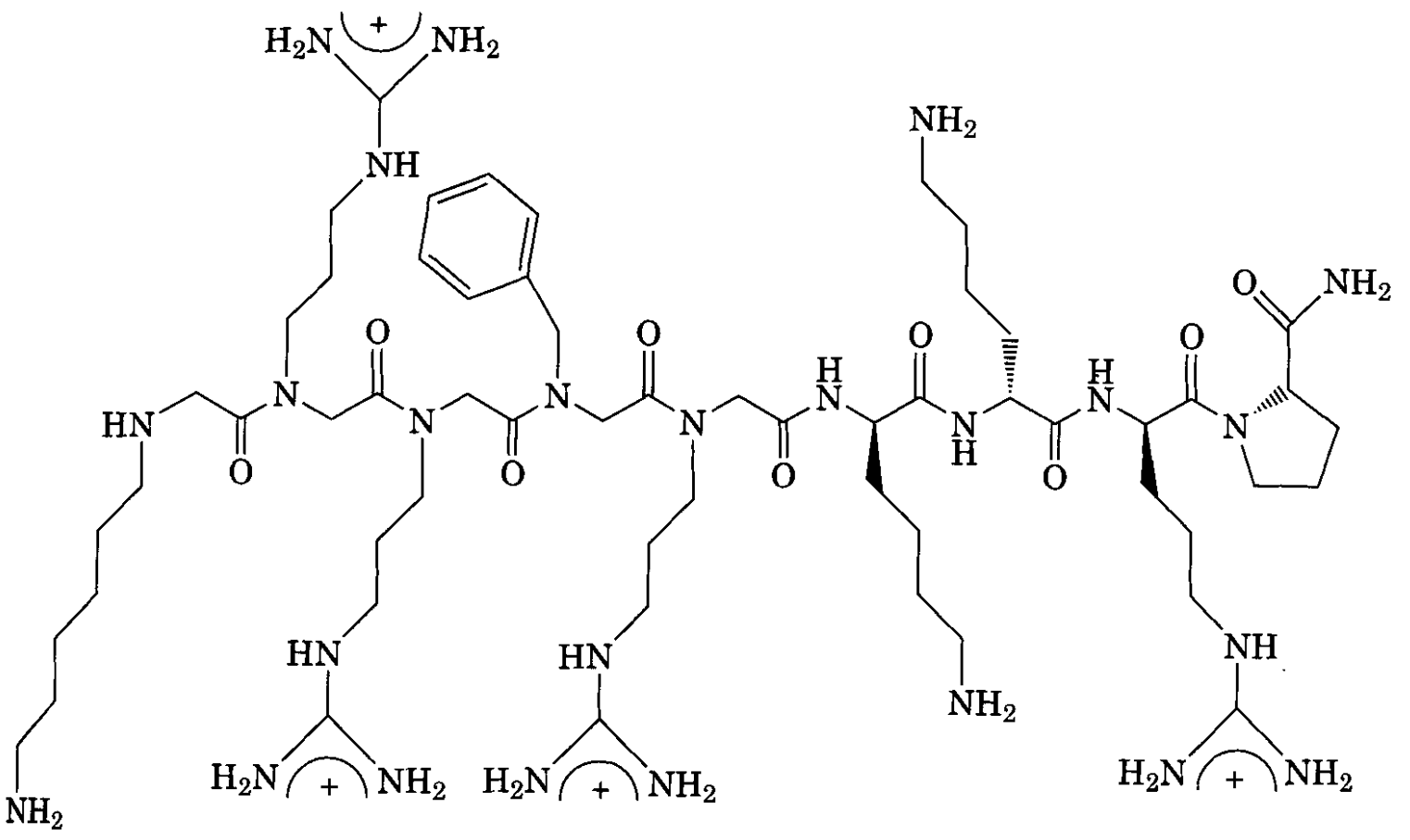


(72)

HIV-1 and HIV-2 replication were 0.8 and 1.6 nM, respectively (338).

In an effort to improve the potential for inhibiting HIV-1 replication, bicyclam-AZT conjugates (78) have also been described (339).

The binding region for AMD3100 has been defined through single amino acid substitutions in the extracellular loops (ECLs) and TM regions of CXCR4. ECL-2 contains five acidic residues that have been implicated as an important recognition site of the V3 domain in



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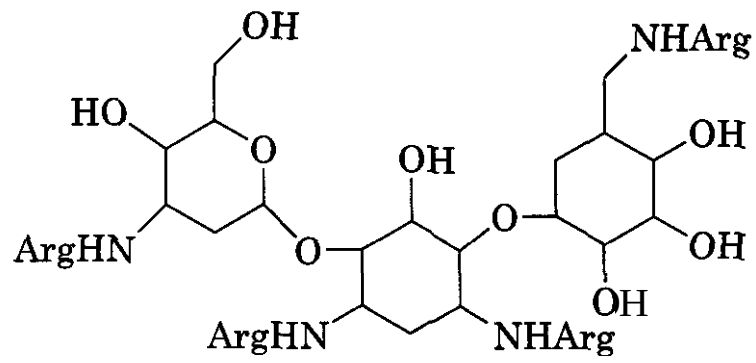

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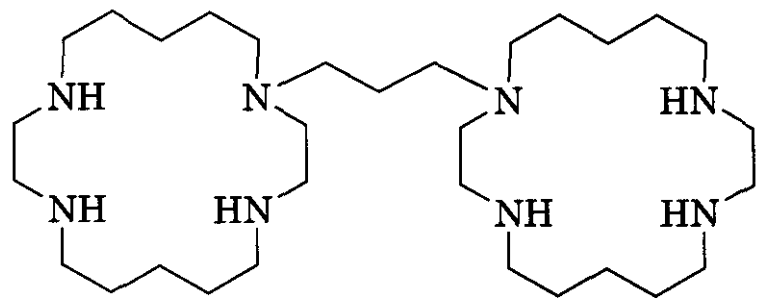

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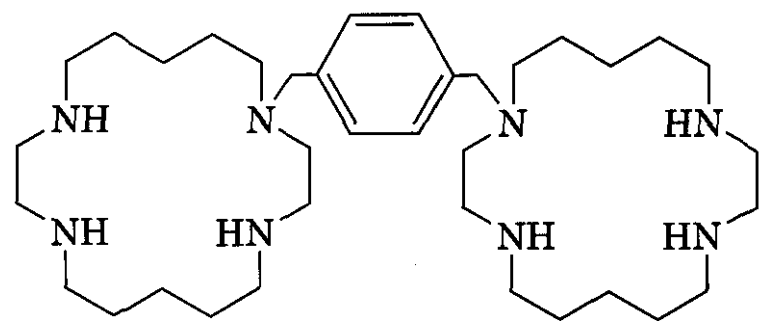


(76)

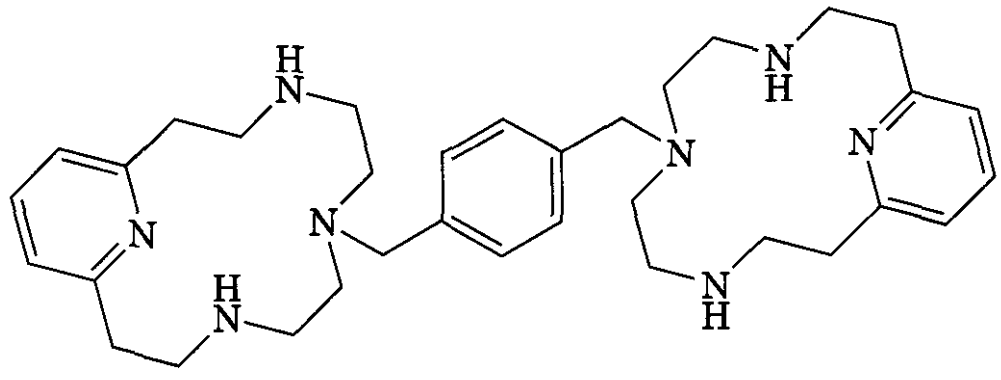
gp120. As part of the mechanism for viral entry, an electrostatic interaction is presumed between the positively charged V3 loop (overall charge of +8) of viral gp120 and the negatively charged ECL-2 (net charge of −9) of CXCR4 (332). Resistance to binding was conferred by single amino acid substitutions in ECL2. The putative binding interaction of AMD3100 with the aspartic acids of ECL-2 is consistent with the cationic nature of the bicyclams and its array of eight proximal nitrogens that will form a multipositive charge (340). The bicyclam unit seems to be very efficient in disrupting this interaction. Through site-directed mutagenesis it has been shown that AMD3100 acts on the CXCR4 receptor through binding to Asp171 in TM-IV and Asp262 in TM-VI with each of its cyclam moieties. Single or double substitution of these aspartates by neutral asparagines resulted in markedly decreased potency of AMD3100 for inhibition of SDF-1 binding to the receptor, SDF-1 induced intracellular calcium signaling, and high affinity binding for certain HIV strains. It is suggested that part of AMD3100's activity is attributed to conformational constraint imposed on the receptor by the rigid connecting phenylene-bismethylene linker (332, 341, 342).

The pharmacokinetic properties of AMD3100 have also been evaluated and it has been shown, not surprisingly, to have very poor bioavailability (343,344). An unsuccessful effort to improve the oral bioavailability through ion-pair formation by use of sodium taurodeoxycholate and sodium taurocholate as counterions has been described (345).

AMD3100 has entered phase I clinical trials as an i.v. formulation, and appears to be well tolerated (346). A 20 mg/kg dose provides a median peak serum concentration of 118 ng/mL, which exceeds its 50% effective antiviral concentration seen in cell culture (335–337).

8 VIRAL CHEMOKINES AND CHEMOKINE RECEPTORS

Herpesviruses, poxviruses, and lentiviruses all encode chemokine and chemokine recep-



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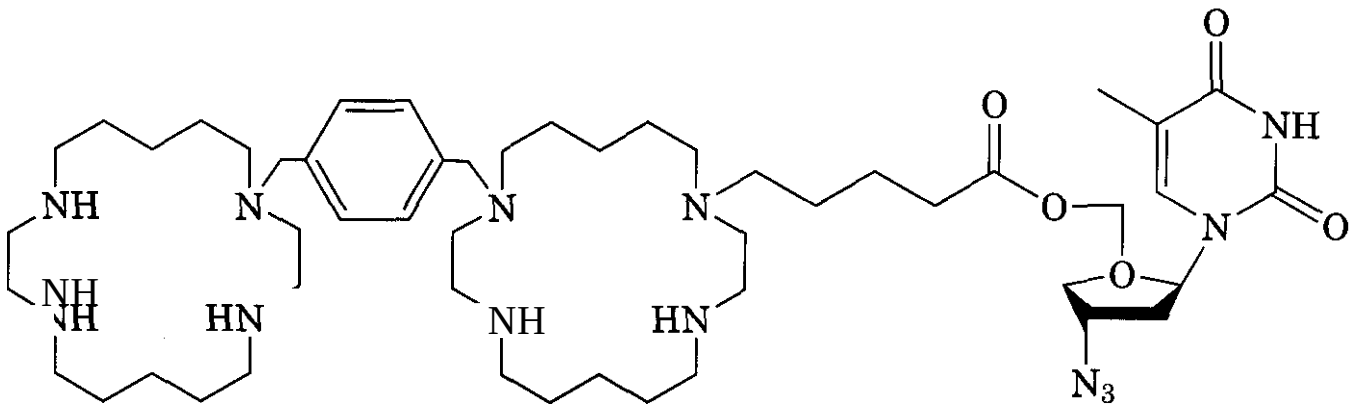


(78)

r-like molecules (347). Some of these molecules are structural homologs of chemokines and chemokine receptors, whereas others are structurally unrelated but bind to either chemokine or receptor and alter the function of these molecules. The selective advantage of expressing these chemokine or chemokine receptors is not yet clear. However, given that chemokines play a crucial role in organizing the host immune response, it is not surprising that viruses have developed different strategies to interfere with the process.

Chemokine homologs are mostly encoded by herpesvirus and include three CC-type chemokines, vMIP-I, vMIP-II, and vMIP-III. vMIP-I is encoded by HHV-8 and binds and induces calcium signals in T-cells through CCR8. This same receptor also shows high affinity for vMIP-II. However, vMIP-I seems to act as an agonist for the receptor and vMIP-II behaves as an antagonist (348). vMIP-II is a broad-spectrum chemokine receptor antagonist. vMIP-III acts as a potent CCR4 agonist and attracts mainly Th2 T-cells.

Molluscum contagiosum virus (MCV) is a human poxvirus that encodes a chemokine homolog named vMCC-I (gene product of MC148R) (349,350). This protein is related to CC chemokines but the mature protein lacks five amino acids in the N-terminus that are critical for receptor activation. Thus, this molecule binds to several receptors such as CCR1, CCR2, CCR5, CCR8, CXCR1, and CXCR2 but is not able to induce signaling, acting instead as an antagonist for these receptors.

In addition to chemokines, viruses also express chemokine receptors. All of them bind multiple chemokine ligands but differ in their specificity as well as in the signal transduction pathways they engage. One interesting example is the HHS-8-encoded chemokine receptor ORF 74. The HHS-8 virus has been implicated in the development of Kaposi's sarcoma (KS). ORF 74 encodes a constitutively active chemokine receptor that binds several host CC and CXC chemokines. It has been shown that this receptor has angionenic, proinflammatory, and oncogenic activities. When expressed as a transgene in hematopoietic cells, it induces lesions that are histologically similar to those of KS (351). This suggests that ORF 74 may play a role in the pathogenesis of KS in humans.

The chemokine binding protein vCCI (produced by pox viruses) is a broad-spectrum CC chemokine scavenger and completely inhibits their function (350,352). Its strong inhibitory properties are attributed in part to the high affinity of vCCI for CC chemokines, having a subnanomolar dissociation constant for all CC chemokines. The affinity of CC chemokines is greater for vCCI than for the native CC chemokine receptors. In a mouse model of allergic lung inflammation, vCCI has been shown to improve pulmonary function and to reduce inflammation (353). vCCI reduced significantly the number of total leukocytes and eosinophils in bronchoalveolar lavage fluid and lung parenchyma. At the same time, a clear reduction in airway hyperreactivity was observed. These results not only show that intrapulmonary inhibition of chemokines by vCCI is a highly effective inhibitor of airway inflammation and hyperreactivity in a mouse model but also suggest a potential use of this agent for the treatment of asthma.

The above examples represent just a small group of all viral chemokine, chemokine recep-

Table 4.10 Cytokine Families and Some Examples (1)[a]

Family	Cytokine	Potential Disease Target
IL-I family	IL-1α	RA, OA, COPD
	IL-1β	RA, OA, COPD
	IL-18	
	bFGF	
	aFGF	
Hematopoietic factors	IL-2	Transplant
	IL-3	
	IL-4	Asthma, allergy
	IL-5	Asthma, allergy
	IL-6	RA
	IL-7	
	IL-9	Asthma, allergy
	IL-11	
	IL-12	Autoimmume diseases
	IL-13	Asthma, allergy
	EPO	
	LIF	
	GMCSF	
	CNTF	
	IFNα	Infectious diseases
	IFNβ	Infectious diseases
	IFNγ	Infectious, autoimmune diseases
TNF family	TNF-a	Arthritis, Crohn's disease, psoriasis
	LT-α	
	LT-β	
	CD40L	
	CD30L	
	Fas-L	

"CNTF, ciliary neurotrophic factor; EPO, erythropoietin; Fas-L, fas ligand; aFGF, acidic fibroblasts growth factor; bFGF, basic fibroblast growth facor; GMCSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; LIF, leukemia inhibitory factor; LT, lymphotoxin; TNF, tumor necrosis factor; RA, rheumatoid arthritis; OA, osteoarthritis; COPD, chronic obstructive pulmonary disease. For a more complete listing of cytokines see Ref. 1.

tor, or chemokine binding proteins. (For more information on this topic, review Refs. 347, 350, 352.)

9 CYTOKINES

Cytokines are extracellular signaling proteins, mostly secreted; however, they could also exist as membrane-bound proteins. They are produced by many different cell types and can have an effect on adjacent cells (paracrine fashion), at a distance (endocrine), or can act on the same cells that produce them (autocrine). Cytokines mediate their action through high affinity surface receptors and can demonstrate a wide variety of actions, including the coordination of inflammatory and immune responses. They are also involved in many stages of the development of autoimmune diseases such as rheumatoid arthritis, inflammatory bowel disease, inflammatory skin diseases, and allergic responses. Table 4.10 shows some examples of the different cytokines and the families to which'they belong.

Small molecule agents that inhibit the production of cytokines have been described. For example, glucocorticoids inhibit the synthesis of several of these molecules by interfering with the transcription factors AP-1 and NFκB (354, 355). Among the cytokines downregulated by glucocorticoids are IL-2, IL-5, IL-12, and TNF-a. Because of the broad effects of glucocorticoids, they have been used in the treatment of many inflammatory conditions such as rheumatoid arthritis, asthma, and transplantation. Other negative regulators of

cytokine production are cyclosporin and FK506. These molecules exert their actions by inhibiting the transcription factor nuclear factor of activated T-cells (NFAT). Cytokines susceptible to inhibition by cyclosporin include IL-2, IL-4, and IL-5. The inhibition of IL-2 production results in an immunosuppressive effect and for this reason these drugs are being used in transplantation therapy. Nonpeptidic small molecule inhibitors that act through direct binding of cytokines and/or their receptors are not common, given the large protein/protein interaction that would need to be disrupted. Cytokine production can also be influenced by modulation of various kinases, such as reducing IL-6 through p38 inhibition. It is beyond the scope of this chapter to discuss all indirect effects on cytokine regulation. In this review we concentrate on cytokines involved in immune responses for which some modulators have been described in either animal models or the clinic.

9.1 IL-1

IL-1 is a proinflammatory cytokine that has multiple roles. It is a central mediator in endotoxic shock and plays an important role in autoimmune diseases such as rheumatoid arthritis. IL-1 is mainly produced by monocytes/macrophages that are activated by T-cells at the site of inflammation. B-lymphocytes and natural killer (NK) cells can also be sources of this cytokine. Two IL-1 receptor agonists have been described, IL-1a and IL-1β. IL-1β is produced as a 31-kDa precursor and is processed to its mature form by IL-1β converting enzyme (ICE) (356). Both IL-1 agonists possess similar biological activities; however, IL-1β is a secreted molecule, whereas IL-1a is primarily cell associated. There are a large number of genes that are regulated by IL-1β. Among them are cytokines and chemokines, cytokine receptors, other inflammatory mediators, growth factors, clotting factors, remodeling factors, oncogenes, and adhesion molecules (for a comprehensive list, see Ref. 357).

IL-1 binds with high affinity to IL-1 receptor I (IL-1RI), resulting in the recruitment of a second molecule, the IL-1R accessory protein (IL-1RAcP). This heterodimer is responsible for the initiation of the signal transduction cascade (358). The interaction between the subunits results in the recruitment of MyD88, an adapter molecule, which is then followed by activation of IL-1R-associated kinase (IRAK) (359). A second IL-1R has been described, IL-1RII. This receptor does not signal; instead it is shed from cells and acts as a decoy receptor (360–362). IL-1RI is found on many different cell types, including endothelial cells, smooth muscle cells, epithelial cells, hepatocytes, fibroblasts, keratinocytes, and T-lymphocytes.

A natural regulatory molecule has been described for IL-1, called IL-1R antagonist (IL-1Ra). Usually this molecule is produced in large excess with respect to IL-1 (363) and binds to IL-1R with near equal affinity compared to that of either IL-1a or IL-1β, but does not signal through the receptor (364). It has been suggested that this molecule plays an important role in the regulation of IL-1β activity *in vivo*.

9.1.1 IL-1 Knockout and Transgenic Mice.

IL-1R knockout mice show a similar phenotype to that observed in IL-1β knockout mice (365, 366). Both mutations have revealed the role that IL-1 plays in IL-6 production and fever. In addition, IL-1R knockout mice had a reduced delayed-type hypersensitivity (DTH) response and acute-phase reaction to turpentine. These mice showed an impaired ability to fight infections by ***Listeria monocytogenes*** (365). In addition, IL-1R is required for the development of inflammatory lesions and clinical symptoms in a mouse model of EAE (367).

9.1.2 IL-1 Modulators/Clinical Data.

Several approaches have been used to antagonize cytokine activity, including antibodies against the cytokine or the cytokine receptor, mutant forms of the cytokine that bind to the receptor yet do not signal, and recombinant soluble receptors. The extracellular domain of the IL-1RI has been used as a decoy receptor in several animal models. For example, in a rat antigen-induced arthritis model, administration of soluble IL-1RI reduced joint swelling and tissue destruction (368). It also increases survival of heterotropic heart allograft in mice (369).

In the clinic, soluble IL-1RI pretreatment before endotoxin administration shows no effect on fever or any other systemic effect. The

lack of efficacy of the IL-1RI treatment is likely attributable to the fact that there was already a decrease in IL-1β and IL-1Ra in these subjects (370). A similar lack of efficacy was observed when soluble IL-1RI was given to rheumatoid arthritis patients. In a double-blind, placebo-controlled trial, no significant improvement in clinical scores was observed, suggesting the inability of this agent to modify the clinical course of the disease (371). In contrast to IL-1RI, IL-1RII has significantly higher affinity for IL-1β than for IL-1Ra. A soluble form of this agent could be more efficacious as an inhibitor of the IL-1 responses, although this would need to be proved.

Administration of recombinant IL-1Ra in several models of chronic inflammation has shown that receptor blockage by this molecule reduces severity of the disease (372, 373). When used in arthritis models, IL-1Ra inhibited joint swelling and suppressed the incidence of collagen-induced arthritis in mice (374, 375). Moreover, IL-1Ra knockout mice present a more severe disease phenotype compared to that of wt mice, and even develop arthritis or other inflammatory conditions spontaneously, depending on the mouse strain (376, 377). This suggests that an imbalance between IL-1 and IL-1Ra may play a role in the predisposition to local inflammatory diseases. Recombinant human IL-1Ra has been clinically evaluated in rheumatoid arthritis patients (378,379). In a 6-month placebo-controlled trial, 43% of the patients showed a 20% improvement compared to 27% in the placebo group. The IL-1Ra-treated patients also had significantly less joint damage. In a study in which IL-1Ra was given together with methotrexate to patients showing active signs of the disease, a similar result was obtained. IL-1Ra did not show significant side effects after 24 weeks of treatment; reactions at the site of injection were the most frequently observed adverse event. In summary, clinical studies show that inhibition of IL-1β results in a significant reduction in signs and symptoms of rheumatoid arthritis as well as reduced joint destruction. Recombinant human IL-1Ra has recently been approved by the U.S. FDA for the treatment of arthritis [Anakinra (Kineret), Amgenl.

9.2 IL-2

IL-2, also known as T-cell growth factor, is produced by antigen-activated T-lymphocytes and has a primary role in regulating T-lymphocyte proliferation and differentiation. Because T-lymphocytes can initiate and regulate the immune response, IL-2 is assumed to have a significant role in antigen-specific acquired cellular immune response. IL-2 also activates NK cells (380) and B-cells (381,382). Although the effect on B-cells is somewhat controversial, it does seem to have a clear role in transcriptional activation of the J-chain and consequent production of IgM (383). Because of its effect on NK cells, IL-2 also has a role in augmenting innate host defense. This cytokine then may have therapeutic utility in boosting immune reactivity in otherwise compromised patients, by promoting generation of both innate and acquired immunity. The role of the IL-2 receptor in human immune function is supported by the finding that patients with X-linked severe combined immunodeficiency (X-SCID) have genetic defects that map to an IL-2 receptor gene locus (384).

IL-2 is a 15.5-kDa protein of 133 amino acids (385). A disulfide bond between Cys58 and Cys105 is essential for bioactivity, whereas glycosylation does not seem to be important (386). A 3D structure for IL-2 has been solved to 3.0-Å resolution (387), indicating that this cytokine is composed of a fourfold bundle of amphipathic antiparallel a-helices. A modification of this structure has been proposed based on secondary structure prediction and comparison with other cytokines (388).

The IL-2 receptor (IL-2R) contains three distinct membrane-associated subunits (p55α, p75β, and p64γ). Each subunit alone will bind IL-2 with low affinity, but heterodimerization and heterotrimerization bind IL-2 with increasing affinity. In addition to being on T-cells, IL-2R is also reported to be expressed by B-cells, although at levels 10 times lower than that of activated T-cells (389).

Signal transduction is linked to the cytoplasmic domains of the βγ subunit (390, 391). Activation of JAK and Src family kinases are initial events for IL-2 signaling. These kinases

will phosphorylate IL-2R and activate the STAT and Ras-mitogen-activatedkinase pathway (392, 393).

9.2.1 IL-2 Knockout and Transgenic Mice. The role of IL-2 in immune response has been clarified in animal models by use of IL-2 knockout mice. Mutated mice that are deficient in IL-2 production, generated by homologous recombination, display normal lymphocyte development, but during the postnatal period become immunocompromised and die within the first few weeks (394,395). In a separate study, IL-2-deficient mice with mutated nonfunctional IL-2 genes were used to examine the role of IL-2 in promoting clonal expansion of cytolytic T-lymphocytes during infection (396). Virus-induced expansion of CD8+ T-cells was inhibited, as was the production of IFNγ by activated T-cells, leading to a reduced ability to clear viral infection. It is worthwhile to point out that mice deficient in CTLA-4, whose expression is IL-2 dependent, display a similar phenotype (397). IL-2 transgenic mice have also been established and do not appear to have an aberrant phenotype (398). Notably, there is no evidence of autoimmune disease in these mice.

9.2.2 IL-2 Modulators/Clinical Data. An IL-2Rα monoclonal antibody has also been developed. It prevents binding of IL-2 to its high affinity receptor and has been used therapeutically as an immunosuppressant in allograft rejection (399, 400).

Other negative regulators include small molecule immunosuppressive agents that exert their effect through inhibiting production or activity of IL-2. For example, glucocorticoids suppress IL-2 production by inhibiting the AP-1 transcription factor *c-jun* (354) and NFκB (355). Leflunamide and mycophenolic mofetil block IL-2 synthesis by inhibiting nucleotide synthesis in T-cells. Cyclosporin, FK506, and rapamycin can suppress IL-2 gene activation through inhibition of calcium-associated signaling events regulated by calcineurin, a protein belonging to the NF-AT pathway. The consequence is immunosuppression through blockade of IL-2 expression and T-cell activation. Many of these small molecule modulators demonstrate therapeutic benefit and protection in patients undergoing organ transplantation.

IL-2 has been evaluated therapeutically as an enhancer of the immune system in the treatment of both cancer and infectious diseases. When a high dose of IL-2 is administered over a 3- to 5-day period, severe systemic toxicity, termed cytokine syndrome, is observed. However, this therapy does provide an antitumor response by an unestablished mechanism in 15% of treated patients (401).

IL-2 may have therapeutic benefit in restoring immune function in AIDS patients. Individuals infected with HIV are characterized by a deficiency in circulating CD4+ T-cells and, consequently, production of IL-2 is compromised. Macrophages infected with HIV-1, and cultured in the presence or absence of IL-2, show a diminished reverse transcriptase activity in those cells treated with IL-2 (402). In addition, the expression of CD4 and CCR5 (HIV-1 receptor and coreceptor, respectively) was downregulated after treatment with IL-2. Clinically, IL-2 has been used in the treatment of HIV-1-infected individuals through either a high dose treatment, as noted above for cancer, or a low dose continuous treatment regimen (403). Using this latter treatment, the concentration of circulating CD4+ T-cells is increased and the risk of opportunistic infection is reduced.

9.3 IL-4

Th2-type cytokines such as IL-4, IL-5, and IL-13 orchestrate a cascade of events during development of an allergic inflammatory response. This is demonstrated both clinically and in preclinical animal models (404, 405). IL-4 plays a critical role in the early commitment of Th0 cells to Th2 cells and regulates IgE secretion by B-cells. It also induces V-CAM expression on endothelial cells, promotes eosinophilic inflammation, and increases airway mucus production.

In asthmatic patients there is an increase in serum and bronchoalveolar lavage fluid IL-4 levels, and atopic individuals have a higher frequency of IL-4-producing T-cells (406, 407). Genetic studies have linked asthma and atopy to the chromosome region 5q31–33, which includes IL-4, IL-5, IL-9, and

the IL-13 genes (408). Thus, aberrant production of IL-4, or excessive response to this cytokine resulting from genetic defects, might contribute to the pathogenesis of asthma.

IL-4 is a 20-kDa secreted glycoprotein and its expression is highly tissue specific. IL-4 is produced by Th2 cells and natural killer cells in response to stimulation through the T-cell receptor (409). IL-4 binds to two types of receptor complexes, type I and type II receptors. Type I receptor complexes are formed by the IL-4 receptor a chain (IL-4Rα) and the common γ chain (γC), which form part of the many other cytokine receptor complexes. The type II receptor consists of the IL-4Rα chain and the IL-13Rα chain. In both cases signaling occurs through the JAK/STAT pathway, more specifically through activation of STAT6 (410).

9.3.1 IL-4 Knockout and Transgenic Mice. In general terms IL-4 and IL-4Rα knockout mice show similar phenotypes. Both develop normally but they show a clear deficiency in the Th2 response (411–413). They are able to mount antibody responses but show a significant decrease in their ability to generate IgE and IgG1. In addition they show a decreased capability to expulse intestinal parasites (414).

Transgenic mice expressing IL-4 in different tissues have been generated. When IL-4 expression is targeted to T-cells, they present increased airway hyperreactivity, inflammation in the eye, and mild B-cell hyperplasia (415). If IL-4 is targeted to the airway epithelium, an enhancement of goblet cell hyperplasia is observed (416). These experiments showed the multiple functions IL-4 can exert over different cell types.

9.3.2 IL-4 Modulators/Clinical Data. Different approaches have been taken to neutralize IL-4 activity, including soluble IL-4 receptor, antibodies against IL-4, and mutated IL-4, which acts as an antagonist of the receptor. In a mouse model of airway inflammation, soluble IL-4 receptor administered intranasally, before allergen challenge, results in a reduction of eosinophil infiltration, V-CAM expression, and mucus hypersecretion (417). This treatment, however, did not change airway hyperreactivity in response to methacholine. In another mouse model of allergic lung inflammation, antibodies against IL-4 administered during sensitization reduce or abolish airway eosinophilia and airway hyperreactivity (418,419). In similar mouse models, a mutant form of murine IL-4 (Q116D/Y119D), acting as both IL-4 and IL-13 antagonist, was found to abrogate the humoral immune response to allergen challenge, and completely inhibited synthesis of allergen-specific IgE and IgG1 (420). Another murine IL-4 mutant (deletion mutant C118), showing similar antagonism against IL-4 and IL-13, inhibited the development of airway eosinophilia and airway hyperreactivity (421). These results suggest that a dual IL-4/IL-13 antagonist could be highly efficacious for the treatment of asthma. Similar mutations have been described for human IL-4 and shown to be efficacious at inhibiting IL-4 and IL-13 responses *in vitro* (422).

As mentioned earlier, soluble receptors are another means to antagonize cytokine activity. The soluble IL-4R (Nuvance, Immunex Corporation) consists of the N-terminal region of the IL-4Rα chain and has been shown to bind IL-4 and sequester this circulating cytokine. Nuvance was tested in a clinical trial with promising results (423). The drug was well tolerated and the placebo group showed a decline in FEV1 not observed in the treated group. The efficacy of Nuvance TM was confirmed by improved asthma symptom scores in the treated group compared to those of placebo. In spite of these promising results, Immunex had announced that Nuvance failed to show efficacy in two Phase II clinical trials. A third trial is still ongoing.

9.4 IL-5

IL-5 is a hematopoietic growth factor cytokine that plays a critical role in differentiation, proliferation, activation, survival, and localization of eosinophils (424). It is not detected at high levels in healthy individuals. IL-5-induced eosinophilia is characteristic of allergy (424), parasitic infections (425, 426), and tumors. Because eosinophils are a characteristic feature of asthma and atopic dermatitis (427), and their numbers are reported to correlate with severity of the disease, IL-5 is believed to have a significant role in the pathogenesis of atopic disease (424,428,429).

In addition to having cell numbers associated with this disease, the biology of the eosinophil also implicates this cell as a major contributor to the disease process. Eosinophils are phagocytic granulocytes that mature in bone marrow, migrate to the blood stream, and eventually localize at the site of injury. They contain highly toxic components that are released upon degranulation, leading to destruction of bronchial epithelium, mucosal edema, and bronchial hyperresponsiveness (430–432).

IL-5 is detectable in bronchial biopsies and bronchoalveolar lavage (BAL) fluid of asthmatics (433, 434). Additionally, inhalation of IL-5 by asthmatics has been shown to cause airway hyperresponsiveness (AHR) and an increase in sputum eosinophils (435).

IL-5 is produced primarily by activated CD4+ T-cells (436, 437), and in lower levels by eosinophils (438), mast cells (439, 440), basophils, B-cells, NK cells (441, 442), and endothelial cells (443). The expression of IL-5 is predominantly regulated at the transcriptional level (444), and can be induced by a variety of stimulants, usually through activation of the T-cell receptor and a second signaling pathway. IL-1α and PMA can induce IL-5 expression; histamine can also increase the production of IL-5 in activated T-cells (445).

Structurally, IL-5 is a disulfide-linked homodimeric glycoprotein between 45 and 60 kDa (446). Glycosylation does not seem to be a requisite for signaling; however, IL-5 must be in native dimeric form for bioactivity (447). Each monomer of IL-5 consists of four α-helices with an antiparallel β-sheet between opposing monomers. The monomers are maintained in dimeric form by cysteine bonds at residues 44 and 86 (448). Mutagenesis shows residues His38, Lys39, and His41 in the second helix; Glu89 and Arg91 in the β-strand; and Thr109, Glu110, TRP111, and Iso112 in the fourth helix as being important contributors to the IL-5 interaction with the hIL-5Rα-chain (449,450). Glu13 on IL-5 has been identified as a contact point for the β-chain of the IL-5 receptor.

The IL-5R a-chain specifically binds IL-5 with low affinity (451). When associated with a signal-transducing β-unit that is also used by other hematopoietin receptors such as GM-CSF and IL-3, IL-5 binds with high affinity (452–454). Both subunits are necessary for signal transduction (455). The pathway for signal transduction includes activation of two Janus kinases (JAK1 and JAK2) and the signal transductionlactivator STAT5 (456,457).

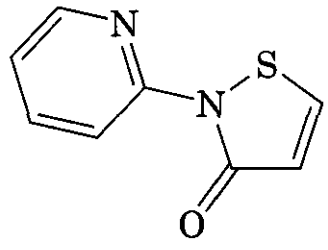
9.4.1 IL-5 Knockout and Transgenic Mice. Animal models have helped define the significance of IL-5 and the eosinophil in the disease process. IL5 administered to mice results in an increase of eosinophils (458). Experiments with IL-5-deficient and IL-5-transgenic mice confirm a role for this cytokine in controlling eosinophilia (459,460). In IL-5 knockout mice, no eosinophils are produced in response to parasite infection or sensitization with ovalbumin, and there is minimal development of lung inflammation or tissue damage. When IL-5 expression is reconstituted in these mice, pulmonary eosinophilia, tissue destruction, and airflow limitation can be observed after allergen challenge (459).

Transgenic mice that have constitutive expression of IL-5 with detectable levels of IL-5 in the serum and persistent eosinophilia have also been described (461). These mice are described as normal, which may suggest that activation and degranulation of eosinophils may be necessary for disease pathology.

9.4.2 IL-5 Modulators/Clinical Data. Modulation of IL-5 can occur by inhibiting its production and synthesis, or through direct binding to IL-5 receptor or ligand. Cytokines can regulate IL-5 levels by inhibiting production. For example, IFNγ and IL-10 have demonstrated they can inhibit IL-5 production in *vitro* (462), whereas IL-12 indirectly modulates IL-5 by biasing toward a Th1 subset population (463).

Small molecule antagonists such as CsA, FK506, and rapamycin all inhibit IL-5 production (464,465). Glucocorticoids, in addition to decreasing bronchial hyperresponsiveness, can also downregulate IL-5 production (466–468). OM-01 suppresses IL-5 protein production, mRNA expression, and transcriptional activity in PBMCs with no effect on either IL-2 or IL-4 (469, 470). (The structure for OM-01 has not been disclosed.)

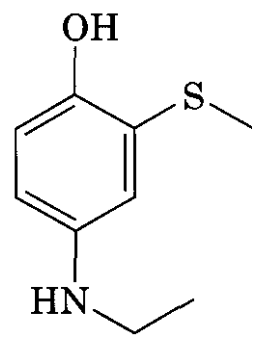
A fusion protein of human IL-5R a-chain and human IgG Cγ3 chain (hIL5Rα-hγ3) was used for screening a library of low molecular weight compounds, and the isothiazolone (79)



(79)

was identified as an IL-5R antagonist. By use of radiolabeled isothiazolone, it was determined that association of (79) with the receptor occurs through covalent modification of a sulfhydryl group on a free cysteine (Cys66) in IL5Rα (471,472).

In a separate study aimed at finding small molecule antagonists, the soluble form of the ligand binding a-chain of hIL-5 receptor was used to identify (80) and (81) as inhibitors of



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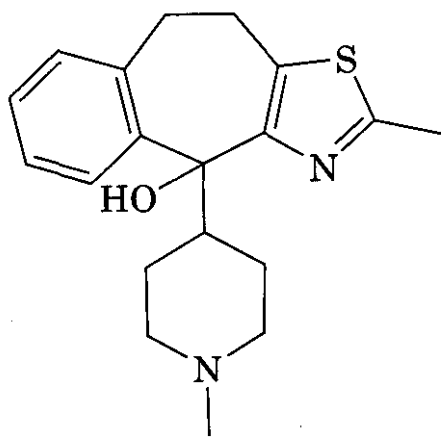

(81)

soluble IL-5R (IC_{50} values of 8 and 11 μM, respectively) (473,474).

These compounds also inhibited interaction of IL-5 with the membrane-bound receptor. They were found to be irreversible inhibitors, again suggesting a covalent interaction. Not surprisingly, they also inhibited interaction of the structurally similar IL-3 and GM-CSF receptors with their corresponding ligands.

Monoclonal antibodies to IL-5 have been evaluated both preclinically and clinically. In both monkey and guinea pig models of allergic asthma, an anti-IL-5 mAb was able to reduce lung eosinophils, airway hyperresponsiveness (AHR), and lung damage in response to antigen (475, 476). In a mouse model of asthma, similar reductions in pulmonary and blood eosinophilia were noted with the anti-IL-5 antibody (TRFK-5) (477). In this study, chronic dosing did not affect normal immune function. Similar results on peripheral blood eosinophil counts were seen when a single injection of anti-IL-5 receptor antagonist was given to IL-5 transgenic mice (458). In mouse models of eosinophilia induced by Nippostrongylus brasiliensis, or Schistosoma mansoni and Strongyloides venezuelensis, an anti-IL-5 antibody successfully reduced eosinophil responsiveness (478–480).

The humanized anti-IL-5 mAb, Sch 55700, when given to Ascaris-responsive primates (0.3 mpk i.v.) also reduces pulmonary eosinophilia (481), and this biological response is sustained for 6 months. In clinical trials, another anti-IL-5 mAb, SB 240563 at 10 mpk i.v., was well tolerated and reduced circulating eosinophils for up to 16 weeks (482). However, there was no effect on the allergen-induced late asthmatic response (LAR) or airway hyperresponsiveness (AHR) to histamine (483). Sch 55700 was also unremarkable in a clinical trial evaluating severe asthma patients (484). These clinical data then suggest no association between the concentration of eosinophils in peripheral blood and sputum with the late asthmatic response or bronchial hyperresponsiveness (BHR) that follows allergen challenge. Taken together with the results of IL-12 in a clinical setting (see below), the role of the eosinophil as a contributor to disease progression in asthma is called into question.

9.5 IL-6

IL-6 is a pleiotropic cytokine implicated in a variety of biological activities, including im-

mune response, acute phase reaction, bone resorption, and hematopoiesis. This cytokine is produced by T-cells, B-cells, monocytes, macrophages, endothelial cells, fibroblasts, mast cells, osteoblasts, smooth muscle cells, glial cells, astrocytes, chondrocytes, keratinocytes, trophoblasts, mesangial cells, islet p-cells, and thyroid cells (485–487), and can be induced by a variety of stimuli including LPS, IL-1, TNFa, IL-2, and PDGF (488–490). Abnormal levels of IL-6 can lead to several disease pathologies and malignancies, including rheumatoid arthritis (RA), osteoporosis, myelomas, and lymphomas.

IL-6 can exist as five different molecular forms, ranging from 21 to 28 kDa, based on different glycosylation and phosphorylation patterns (491). Both the C-terminus and the *N*-terminus appear to be important for eliciting a biological response. As with other cytokines, IL-6 presents itself as a bundle of four α-helices (492).

Similar to the IL-2 and IL-5 receptors, the IL-6 receptor (IL-6R) contains two subunits, an 80-kDa a-chain (IL-6Rα) and a 130-kDa β-chain (gp130). The β-chain can be shared with the IL-11 receptor (493) and is the signal-transducing unit of the IL-6R complex. IL-6 assembles the receptor complex in a sequential fashion, forming first a low affinity interaction with IL-6Rα, then a high affinity complex with gp130 (494). IL-6 then induces a transient tyrosine phosphorylation, which activates the jun B transcription gene (495).

9.5.1 IL-6 Knockout and Transgenic Mice. The pathogenic role of IL-6 in disease is substantiated through the development and evaluation of IL-6 transgenic and knockout mice. IL-6 knockout mice are viable and under normal conditions do not display any obvious phenotypic abnormality. However, in response to tissue damage or infection, IL-6 knockout mice have an impaired inflarnmatory acute response, a reduced IgG response, and impaired germinal center formation (412, 496, 497). Also, in an estrogen-depletion model intended to induce an osteoporotic phenotype, IL-6-deficient mice are protected from bone loss, suggesting that IL-6 has a function in regulating bone turnover (498). More recently, a role for IL-6 in liver regeneration has been established by use of IL-6-deficient mice. When sections of the liver were cut away, the mice would deteriorate and die. However, when the mice were pretreated with IL-6, hepatocyte proliferation returned to normal and liver damage was prevented. Thus, IL-6 therapy may be of benefit in patients undergoing liver transplant, or in those suffering from cirrhosis or chronic hepatitis, which are characterized by liver degeneration. The possible role of IL-6 in autoimmune disease is made apparent with transgenic mice that overexpress the cytokine and show an increase in agalactosyl IgG (499). This immunoglobulin is increased in a variety of autoimmune diseases such as RA, Crohn's disease, type I diabetes, and pulmonary TB (500–502). Transgenic overexpression of IL-6 in the CNS can lead to significant neurodegeneration and subsequent motor uncoordination, ataxia, and tremor (503).

9.5.2 IL-6 Modulators/Clinical Data. Monoclonal antibodies to IL-6 have been described; neutralization of IL-6 with one of these antibodies (MH166) can inhibit human myeloma cell growth in SCID mice (504, 505). In a patient with multiple myeloma, administration of a murine anti-IL-6 antibody to human IL-6 blocked myeloma cell proliferation in the bone marrow (506). An IL-6R antibody (PM1) has also been described and shows a similar response to MH166 in repressing tumor growth in a SCID mouse (507). Anti-IL-6R antibodies are currently in clinical trials for multiple myeloma and RA. The anti-IL-6 receptor antibody, MRA, has shown positive effects in an open-label RA study (508, 509). Decreases in tender and swollen joints were reported and an ACR20 response was noted within 6 weeks of treatment.

Receptor-binding sites on IL-6 have been mapped by mutagenesis studies and critical contact points with IL-6Rα and gp130 have been identified. IL-6 mutants have been prepared based on this information, leading to the generation of "superantagonists" (Sant 1–7) (510).

Small molecules such as Tenidap (511) and retinoic acid (512) have been shown to inhibit production of IL-6. Tenidap, in contrast to other NSAIDs, suppressed production of IL-6 in *vivo* and, in a clinical setting, substantially

decreased the acute phase response in conjunction with lower plasma IL-6 levels (513).

9.6 IL-12

IL-12, originally termed cytotoxic lymphocyte maturation factor (514), is a growth factor for CD4+ and CD8+ T-cells and NK cells (515), and has been shown to modulate the cytolytic and proliferative activity of these cells. In vitro, IL-12 is able to direct T-cells to a Th1 phenotype, and induces production of IFNy and TNFα (516). IL-12 thus has potential to promote an immune response, enhance the host defense response (517), and treat Th2-driven imbalances such as allergy or asthma. Evidence for its role in protective immunity is supported by the finding that individuals who lack the ability to express a component of the IL-12 receptor (IL-12Rβ1) are susceptible to bacterial infection (518, 519).

In contrast, IL-12 may contribute to Th1-associated inflammation and autoimmune diseases such as insulin-dependent diabetes mellitus (520), septic shock (521), multiple sclerosis (522), and rheumatoid arthritis (523). For these diseases, inhibition or neutralization of IL-12 may have therapeutic value (524).

IL-12 is a 75-kDa heterodimer with two disulfide-linked subunits (p35 and p40), and is produced by macrophages (525), dendritic cells (526), and airway epithelial cells (527). Expression of IL-12 receptor (IL-12R) is limited to activated T- and NK cells. Functional IL-12R has β1 and /32 subunits with p40 ligand segment binding primarily to β1, whereas the p35 ligand unit associates with the β2 chain of the receptor (528). The β2 unit appears to be responsible for signal transduction (529).

9.6.1 IL-12 Knockout and Transgenic Mice.

IL-12 ligand knockouts have been developed with mutations generated independently in both the p40 and p35 genes. The mice are viable, displaying normal development and normal levels of T-cells, B-cells, and macrophages in the thymus, spleen, and lymph nodes (530, 531). In response to LPS, however, these mice show an impaired ability to produce IFNy and are unable to mount a Th1 response.

9.6.2 11-12 Modulators/Clinical Data.

Endogenous inhibitors of IL-12 include IL-4, IL-10, IL-13, TGFβ, and PGE,. Small molecules such as pentoxifylline and acetyl salicylic acid (ASA, aspirin) have also been shown to inhibit IL-12 production in PBMCs and monocytes stimulated with LPS. This inhibition is independent of the endogenous inhibitors such as IL-10, TGFβ, and IL-4. Both aspirin and pentoxifylline inhibit accumulation of p40 mRNA and do this through inhibiting the interaction of NFκB in the p40 promoter region, leading to suppression of the p40 gene.

The naturally occurring homodimer IL-12p40 subunit acts as an antagonist of bioactive IL-12 p35/p40 heterodimer. When IL-12p40 homodimer is given to diabetes-prone nonobese diabetic (NOD) mice, diabetes is suppressed (532). This result supports a role for IL-12 in driving Thl-driven autoimmune diabetes.

Preclinically, IL-12 administration demonstrates therapeutic benefit in models of infectious disease (533, 534), cancer (535), and allergic airway hyperresponsiveness (536,537). IL-12's role in host defense is evidenced by the protection that endogenous IL-12 provides against viral infection. It does this through enhancement of NK and cytolytic T-lymphocyte (CTL) activity (533). Recombinant IL-12 also has a curative effect in mice infected with Leishmania major (534). Because of IL-12's ability to enhance NK and CTL activity, as well as its potential to induce IFNγ, this cytokine may have therapeutic benefit as an antitumor agent. IL-12 has shown potent antitumor effects against a variety of murine malignancies (535), and it is believed that this effect is mediated through CD8+ T-cells.

IL-12 has entered clinical trials for cancer (538–541), chronic hepatitis C (542), and asthma (543). Preliminary analysis shows some positive benefit in patients with cutaneous T-cell lymphoma (541). To avoid severe toxicity, an 1L-12 pretreatment regimen appears to be necessary (544).

Administration of IL-12 to patients with mild allergic asthma significantly decreased peripheral blood eosinophils and sputum eosinophils (543). However, in contrast to the mouse model, there was no clinical improvement in airway hyperresponsiveness to hista-

mine, nor was there an effect on the late asthmatic reaction. As with anti-IL-5, which shows a similar clinical nonresponse, this calls into question the role of the eosinophil in this disease, as well as the concept of asthma being governed by a Th2 phenotype.

9.7 IL-13

IL-13, IL-4, and IL-5 together belong to the family of Th2 cytokines and share similar biological activities on human B-cells and monocytes (545). IL-13 is a 17-kDa glycoprotein primarily produced by Th2 cells and, to a lesser extent, by macrophages, dendritic cells, NK cells, mast cells, and basophils.

The involvement of IL-13 in allergic disease is supported by genetic data linking IL-13 polymorphism to asthma. An association between IL-13 promoter polymorphism and increased risk of allergic asthma has been described (546). Another study found an association between a polymorphism in the IL-13 gene and serum IgE levels (547).

This cytokine binds to two membrane-bound proteins, IL-13Rα1 (low affinity) and IL-13Rα2 (high affinity) (548). IL-13Rα1 requires the presence of IL-4Rα to form a high affinity complex. This complex is expressed in a wide variety of hematopoietic and nonhematopoietic cells. IL-13Rα2 alone is a high affinity receptor but there is no clear evidence that this protein is capable of transducing a signal upon IL-13 binding. Because of this, it has been proposed that it may act as a natural antagonist of IL-13 function. Many of the common functions assigned to both IL-4 and IL-13 are probably attributable to the fact that they share the IL-13Rα1/IL-4Rα high affinity complex. Interestingly, IL-13Rα1 is not expressed on T-cells, so it does not act on T-cells directly. However, it could modulate T-cell responses through its effects on macrophages. Binding of either cytokine to the IL-13Rα1/IL-4Rα receptor complex induces the activation of the JAK/STAT pathway.

Both IL-13 and IL-4 have been shown to regulate B-cell function and IgE synthesis. It has also been reported that IL-13 acts on macrophages and regulates cell surface protein expression. More recently, IL-13 has been reported to induce gene expression in nonhematopoietic cells, including fibroblasts, endothelial cells, smooth muscle cells, and epithelial cells (549, 550). Thus, IL-13 seems to have a broader spectrum of actions on airway cells compared to that of IL-4. For example, IL-13 but not IL-4 reduces β-adrenergic responsiveness of human airway smooth muscle cells (551).

9.7.1 IL-13 Knockout and Transgenic Mice.

The role of IL-13 in vivo has been studied by use of transient or constitutive expression of the cytokine. When IL-13 was expressed in an inducible fashion in the lung, a very striking phenotype was observed (552); mucus metaplasia, macrophage, lymphocyte, and eosinophil-rich inflammation and subepithelial fibrosis are characteristics shown by these mice. These same features can also be found in asthmatic patients. These experiments suggest a role for IL-13, not only in the allergic response but also in the remodeling and destruction of the airways. The role of IL-13 in the development of lung allergic inflammation in mice has been recently confirmed by the use of knockout mice. Peripheral T-cells from IL-$13^{-/-}$ mice produce fewer Th2 cytokines because of impairment of Th2 cell development (553). In addition, IL-13 knockout mice do not develop AHR, in spite of the presence of eosinophil-rich inflammation. AHR was restored in these mice by administration of recombinant IL-13. These results indicate that IL-13 is necessary and sufficient for the induction of AHR in mouse.

9.7.2 IL-13 Modulators/Clinical Data.

An IL-13Rα2-IgGFc fusion protein was used to assess the role of IL-13 in a mouse model of allergic lung inflammation (554). The systemic administration of this reagent into mice, before the induction of lung inflammation by ovalbumin, resulted in complete reversal of airway hyperreactivity, even if treatment was given after full development of the phenotype (in contrast with IL-4). Blockade of IL-13 reverses the increase of mucus-containing cells in this model. A human soluble IL-13 receptor is currently being developed by the Genetics Institute.

In a chronic model of Aspergillus *fumigatus*-induced allergic asthma, immunoneutralization of IL-13 has marked effects (555). The

use of anti-IL-13 antibody results in a significant attenuation of airway hyperreactivity in this model. When IL-4 is neutralized, no statistically significant decrease was observed. In addition, neutralization of IL-13 inhibits collagen deposition, subepithelial fibrosis, and goblet cell hyperplasia. None of these effects was observed when neutralization of IL-4 was performed. Again, these results point to a broader spectrum of effects for IL-13 compared to that of IL-4, and for this reason IL-13 may be a more favorable target for asthma.

Other dual modulators for IL-4 and IL-13 are described in the IL-4 section of this review.

9.8 TNFα

TNFa was originally identified because of its antitumor activity. However, later studies have shown that this cytokine plays a major role in autoimmune diseases and is also involved in multiple activities, including metastasis, viral replication, septic shock, inflammation, and fever.

Human TNFa is a protein that exists in both soluble (157amino acids) and transmembrane form (233 amino acids). Soluble TNFa is released from the cell membrane through a TNF-converting enzyme and exists as a homotrimer in aqueous solution (556,557). It is produced primarily by monocytes/macrophages in response to various inflammatory stimuli, but can also be produced by other cell types, including T-cells, NK cells, dendritic cells, and endothelial cells.

TNF mediates its action through two different receptors, referred to as p60 (p55, Type I, CD120a) and p80 (p75, type II, CD120D) (558). In general, most TNF proinflammatory activities are mediated through the p60 receptor, whereas the role of the p80 receptor is less clear. The crystal structure of the p60 receptor bound to TNF has been solved (559). This structure predicts that a ligand trimer brings three receptor chains together to form a complex. The p60 receptor is expressed by all cell types examined to date. In contrast, the p80 receptor appears to be restricted to cells from the immune system and hematopoietic cells. The cytoplasmic domain of the TNF receptors has been shown to bind to distinct serine/threonine kinases and to cause phosphorylation of the receptor (560). Activation of the p60 receptor has been shown to activate apoptosis, the nuclear transcription factor NFκB, and *c-jun* N-terminal kinase (JNK) (561). NFκB activation mediates many of the inflammatory activities of TNF, including the expression of cytokines, chemokines, and cell adhesion molecules.

9.8.1 TNFα Knockout and Transgenic Mice. The role of TNFa in *vivo* is not well understood. It is believed that TNF is required for protection against bacterial, fungal, parasitic, and perhaps even viral infections and other stressful stimuli (562). TNF knockout mice are shown to develop normally. These mice have normal thymus, but their spleen architecture is abnormal and their ability to fight infection is reduced (563). They are also protected from lethal doses of LPS. Knockout studies of the TNF receptor (p60) have shown that mice deficient in this gene are resistant to endotoxic shock but show increased susceptibility to Listeria monocytogenes (564, 565).

9.8.2 TNFα Modulators/Clinical Data. In human rheumatoid arthritis (RA), TNF protein has been observed in the synovial fluid, and TNF mRNA in synovial cells, including monocytes and macrophages (566,567). In animal models of RA, anti-TNF therapy is highly effective. These models include collagen-induced arthritis, adjuvant arthritis as well as streptococcal cell wall arthritis (568, 569). To date, two different approaches have been taken to neutralize TNF activity clinically: recombinant soluble receptor and monoclonal antibodies against TNF. Etanercept (Enbrel, Immunex) is a dimeric fusion molecule composed of two p80 TNF receptor moieties bound to an Fc portion of human IgG1. This molecule is effective in both adult and juvenile RA (570–572) and shows efficacy in controlling signs and symptoms of the disease. In a randomized, double-blind, placebo-controlled trial, 59% of patients responded to Etanercept. The second strategy consists in the generation of mouse monoclonal antibodies against TNF. To decrease immunogenicity of the mouse antibody, the Fc region is replaced by a human Fc domain. Infliximab (Remicade, Centocor) is a chimeric antibody created by use of this strategy and when used in combination with meth-

otrexate (to avoid the development of antibodies), 52–58% response is seen in RA patients (573, 574).

The clinical effects of Etanercept are comparable to those of Infliximab. One advantage of Etanercept is that it is given subcutaneously twice a week, whereas Infliximab is given by slow intravenous infusion every 4–8 weeks. Another advantage is that Etanercept also recognizes lymphotoxin, and this may account for its efficacy in juvenile chronic arthritis. Currently, D2E7, a fully human anti-TNF antibody, is being developed (Knoll). It appears to be effective in RA without the need for methotrexate cotreatment (575).

9.9 IFNγ

Interferon γ (IFNy) is produced mainly by a subset of activated T-lymphocytes and natural killer cells and is the main activator of macrophages (576). An increase in production of IFNy is usually associated with effective host defense against intracellular pathogens and with autoimmune diseases. IFNy is a well-conserved protein among animal species and its biological active form is a 34-kDa homodimer. However, it is heterogeneous in size because of both enzymatic trimming of the C-terminus and variations in glycosylation (577, 578).

The IFNy receptor consists of two subunits: the IFNG1 subunit (IFNy receptor a-chain, 90 kDa), which binds with high affinity to IFNy; and the IFNG2 subunit (IFNy receptor β-chain, 62 kDa), which does not contribute significantly to binding but is necessary for signal transduction (579). Both subunits are expressed ubiquitously on many cell types. Upon stimulation, association between the two subunits is induced and the signaling cascade is initiated. The IFNy receptor activation process is through a JAK/STAT signaling pathway, and JAK1, JAK2, and STAT1 are specifically involved in mediating many IFNy-dependent effects on cells (580, 581). The effects of IFNy are mostly at the transcriptional level. Many genes are induced by treatment with this cytokine. The genes that are induced depend on the cell type and the presence of other cytokines. For example, in macrophages IFNy induces the expression of major histocompatibility complex (MHC) class II molecules, contributing in this manner to antibody formation and development of cytotoxic T-cells (582). In endothelial cells, IFNy augments the production of the adhesion molecule I-CAM, contributing to leukocyte infiltration during inflammation (583).

9.9.1 IFNγ Knockout and Transgenic Mice. The in *vivo* role of IFNy has been elucidated in mice using null mutations for IFNy or the INFy receptors (IFNG1 and IFNG2). All these mice present a common characteristic, in that their ability to resist infection is greatly impaired. In these mice a significant reduction in MHC class II molecules on macrophages was observed (584–587).

INFy or INFy receptor knockout mice have shown that, depending on the animal models, this cytokine can act as either an immunosuppressant or an immunopotentiator. For example, IFNy receptor knockout mice are protected from developing autoimmune diabetes in nonobese diabetic (NOD) mice (588). They are also protected from developing inflammatory bowel disease in an IL-12-induced disease (589). In contrast, these knockout mice are more vulnerable in other disease models, such as the experimental autoimmune encephalomyelitis (EAE) model (590). Furthermore, varied results can be obtained, depending on the agent used to block IFNy actions and the time in which the agent is delivered (see Modulators section).

9.9.2 INFγ Modulators/Clinical Data. Antibodies against IFNy have been widely used in animal models of human diseases. As indicated for the knockout mice, the results of blocking IFNy activity with antibodies depends on the model used. One interesting situation is the collagen-induced arthritis model. When the antibody is given early in the development of the disease, a reduction in the severity is observed. When the antibody is given late, the result is disease aggravation (591, 592). Interestingly, knockout mice are more sensitive to the development of the disease (592).

Other experiments that use antibodies in animal models have shown results similar to those obtained with knockout mice. In disease models of immune diabetes and inflammatory bowel disease, anti-IFNy antibodies alleviate

the disease symptoms (593, 594). In disease models of MS, such as EAE, anti-IFNy antibodies enhance the disease (595–597).

Many research groups have recently identified patients with inactivating mutations of IFNGRl or IFNGR2 who have a severe susceptibility to weakly pathogenic mycobacterial species. Complete deficiency in IFNGRl results in infections with low levels of virulent mycobacteria in early childhood (598).

Attempts to relate polymorphisms in the human IFNy gene with disease association have also been reported. In both Finnish and Japanese populations, distinct IFNy gene polymorphisms are found in insulin-dependent diabetes mellitus patients, suggesting a global disease association (599, 600). Recombinant human IFNy has been used in various trials for the treatment of infectious diseases such as chronic granulomatous disease, visceral leishmaniasis, and multidrug-resistant tuberculosis. In all cases some benefit has been reported after treatment with IFNy alone or in combination with other drugs (601, 602). To date no clinical data have been published regarding the efficacy of IFNy antagonists. However, Protein Designs Labs has a humanized monoclonal antibody in phase I/II trials for autoimmune diseases.

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CHAPTER FIVE

COX-2 Inhibitors and Leukotriene Modulators

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Contents

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1 INTRODUCTION

A number of oxygenated products derived from arachidonic acid, frequently termed *eicosanoids*, have profound physiological and pathological effects. The history of these reviews indicates the expansion of the awareness of the medical importance of different classes of eicosanoids. Two editions from this series (1, 2) contained one chapter listing prostaglandins as the only eicosanoid target. The last volume broadly discussed both thromboxanes and leukotrienes together, while downplaying prostaglandins by lack of detailed discussion. The current volume contains three chapters on eicosanoid targets. Other chapters describe prostanoid receptors and thromboxanes individually. This chapter describes recent efforts in the COX-2 field and updates the leukotriene review of the 5th edition (2).

The previous chapter on thromboxanes and leukotrienes provided a thorough review of both fields. However, a significant amount of work has since been published in the area of leukotriene biology and modulators, thus necessitating our editing and adding to the previous review. In addition, a major breakthrough in prostaglandin research occurred with the discovery of a second cyclooxygenase (PGHS-2 or COX-2) by several investigators. This seminal work provided a new paradigm and new medicinal and clinical research in an area that seemed to be fully mature. The approval of selective COX-2 inhibitors Celebrex and Vioxx are landmarks in eicosanoid research. However, they are perhaps also just first steps with the possibility that second-generation COX-2-selective agents, mixed COX-215-LO, selective PGE, receptor subtype antagonists, or perhaps selective inducible PGE, synthase inhibitors may ultimately provide improved therapy in inflammation, pain, and perhaps cancer.

In this review, we first describe the paths and enzymes leading to prostaglandins, leukotrienes, and lipoxins. We then take each of our topics, separately dealing with leukotriene modulators followed by cyclooxygenase inhib-

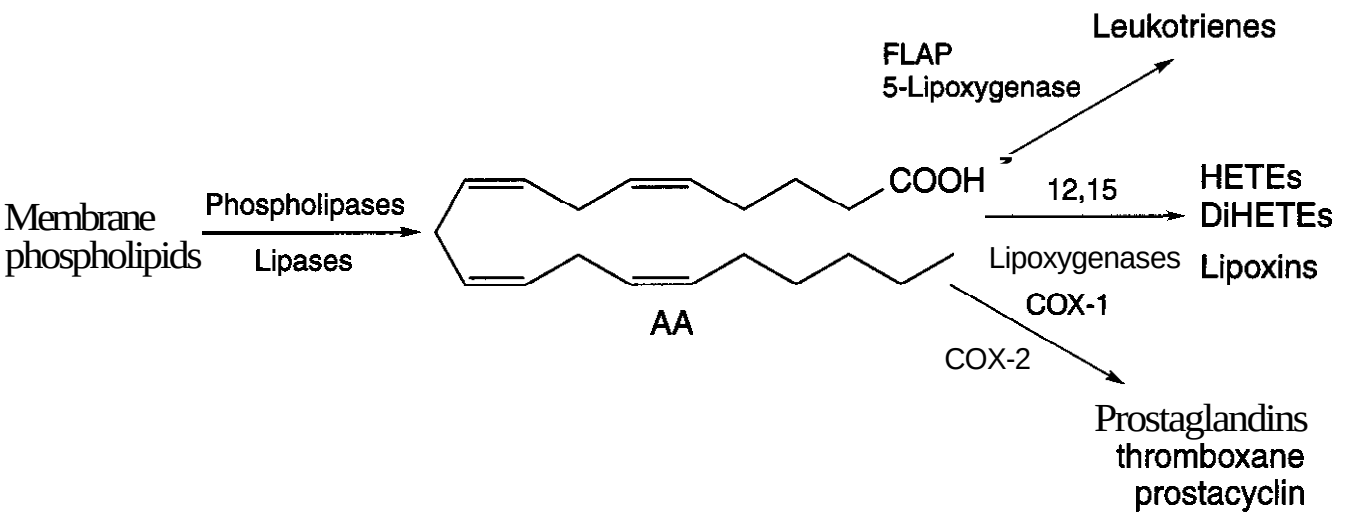


Figure 5.1. Eicosanoid pathway.

itors. Each section contains a brief discussion on the sources and actions of the mediators. The efforts to develop biosynthesis inhibitors and/or antagonists of these classes of mediators are then summarized with emphasis on compounds that have progressed to clinical evaluation. Because of the breadth of the area covered by this chapter, it is impossible for it to be fully comprehensive. Thus, we frequently reference leading reviews in each subheading area.

2 EICOSANOID BIOSYNTHESIS

The eicosanoid metabolic pathway as it is currently understood begins with the liberation of long-chain fatty acids from cell membranes. In mammalian tissues the primary fatty acid released is arachidonic acid (AA, eicosatetraenoic acid, ω6 20:4) (3). Some important exceptions to this generality are well known but are outside the scope of this review. A number of enzymes that release arachidonic acid have been identified and are predominantly phospholipases, although other enzymes clearly are also important under some conditions and in certain cell types (4–9). Of considerable importance is the fact that under most conditions free AA is normally kept very low by the action of various acyl transferases (10). The primary metabolic enzymes using AA as a substrate to form oxygenated products are cyclooxygenases 1 and 2; 5-, 12-, and 15-lipoxygenases; and certain P450 enzymes.

3 BIOCHEMISTRY OF LEUKOTRIENES

After the release of AA from phospholipid stores, the oxidative metabolism of AA occurs through a variety of pathways (see Fig. 5.1). Cyclooxygenase (COX) leads to the prostaglandins (11) and thromboxanes, whereas the leukotrienes are formed through the 5-lipoxygenase (5-LO) pathway. In human cells, there is a family of lipoxygenases, including 5-, 12-, and 15-lipoxygenases, that bear significant sequence similarity to each other and that lead to several series of linear metabolites. The peptide and nonpeptide leukotrienes, various hydroperoxy- and hydroxy-eicosatetraenoic acids (HPETEs and HETEs) are products of the 5-lipoxygenase (5-LO) pathway. Besides these eicosanoids, additional products are made nonenzymatically (12) and some by dual action of 5-LO followed by 15-LO (lipoxins) (13).

3.1 Biosynthesis of Leukotrienes

The committed biosynthetic path to the leukotrienes begins (see Fig. 5.2) with the action of 5-lipoxygenase (5-LO) [EC 1.13.11.34] (14) on arachidonic acid. Purified human 5-LO is an unstable, 78,000-Da protein that has been isolated and cloned (15,16). It contains a tightly bound, nonheme iron that is essential for enzymatic activity (17). Like all known lipoxygenases, 5-LO catalyzes the insertion of molecular oxygen into a 1,4-*cis,cis*-pentadiene unit. The effect of 5-LO on AA is to abstract stereospecifically the pro-*S* hydrogen at position C_7 and to insert molecular oxygen at

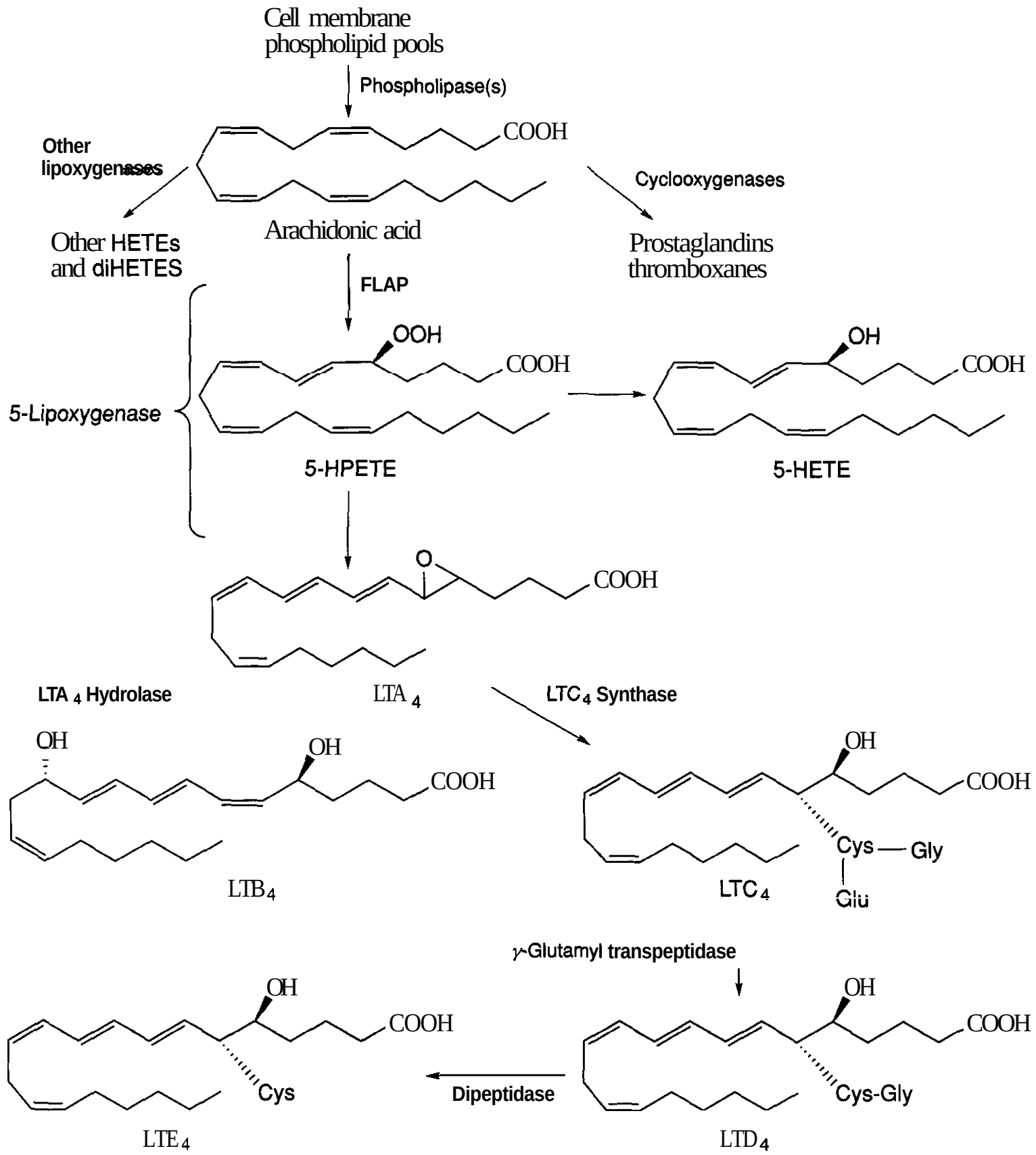


Figure 5.2. Leukotriene pathway.

position C_5 to produce as an intermediate, **5(*S*)-hydroperoxy-6,8,11,14-(*E,Z,Z,Z*)-eicosa**tetraenoic acid (BHPETE). The optimal activity of 5-LO requires the presence of Ca^{2+} (18), ATP (19), and an accessory protein, 5-lipoxygenase-activating protein (FLAP) (20). 5-Lipoxygenase also catalyzes the conversion of 5-HPETE to leukotriene A, [LTA_4, **5(*S*),6(*S*)-oxido-7,9-*trans*-**11,14-*cis*-eicosatetraenoic acid] through the stereospecific removal of the pro-R hydrogen from position C_{11}, radical migration and formation of the **5,6-epoxide** (21, 22). Alternatively, 5-HPETE can be reduced to yield 5-HETE [**5(*S*)-hydroxy-6,8,11,14-(*E,Z,Z,Z*)-eicosatetraenoic acid**].

Conversion of 5-HPETE to LTA_4 was originally thought to be attributed to a distinct **enzyme, LTA_4 synthase,** but sequence cloning studies showed that this protein is identical to 5-LO (23).

A significant amount of work, much of it from Merck and more recently the Peters-Golden laboratory, has indicated that the cellular localization of 5-LO is cell type and stimulus dependent (24–29). Prominently cytosolic in resting neutrophils, a number of stimuli cause a fraction of the enzyme to associate with nuclear membranes. In macrophages the enzyme is in the nuclear cytosol and associates with the nuclear membrane on activation. Control of this movement is associated with FLAP, as implied by inhibitor studies, but no specific binding of 5-LO to FLAP has ever been shown (30). Nuclear responses to leukotriene products are very likely the topic of exciting future research. FLAP has been proposed to present substrate to 5-LO (31).

Further biosynthetic processing of LTA_4 can occur along two distinct pathways. In the first path, it is stereospecifically converted to leukotriene B_4 [LTB_4, 5(*S*),12(*R*)-dihydroxy-6,14-*cis*-8,10-*trans*-eicosatetraenoic acid] (32). This conversion occurs by the addition of water in a 1,8 manner across the conjugated trienyl epoxide of LTA_4, under the influence of the cytosolic enzyme LTA_4 hydrolase [EC 3.3.2.6]. Because LTA_4 is an unstable epoxide, nonenzymatic hydrolysis can be easily accomplished. However, this affords a complex mixture of 5,6- and 5,12-dihydroxyeicosatetraenoic acids that contain no LTB_4. LTA_4 hydrolase is a soluble, zinc metalloenzyme (33), a monomeric protein of molecular weight 70,000, that was first purified from human leukocytes by Samuelsson et al. (34). It has since been cloned and sequenced from several sources (35). The human enzyme is 610 amino acids with a unique sequence compared to that of other known epoxide hydrolases.

Catabolic processing (metabolic inactivation) of LTB_4 occurs predominantly by sequential omega oxidation, first to 20-hydroxy-LTB_4 by a cytochrome P450-catalyzed process (34) and then to 20-carboxy-LTB, through a different, soluble enzyme (36).

Alternatively, a second path for metabolic processing of LTA_4 to the set of cysteinyl leukotrienes begins by conjugation with reduced glutathione (GSH), to form the parent sulfidopeptide leukotriene C_4 [LTC_4, 5(*S*)-hydroxy-6(*R*)-glutathionyl-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid]. This reaction is catalyzed by another integral membrane protein, LTC_4 synthase [EC 2.5.1.37], that has been localized to the perinuclear membrane (37, 38). The purified 18-kDa enzyme is very unstable and requires Mg^{2+} ions and phosphatidyl choline for activity. Initially, it was assumed that LTC, synthase was related to the ubiquitous family of glutathione S-transferases. However, sequence comparison showed little homology between LTC_4 synthase and either the cytosolic or microsomal GSH S-transferases (39). A detailed analysis of protein sequence data showed that there was 31% overall amino acid sequence identity between LTC_4 synthase and FLAP, and that in limited regions these proteins are nearly identical. This observation as well as the fact that the FLAP inhibitor MK-886 inhibits LTC_4 synthase activity ($IC_{50} \sim 3\ \mu M$) caused Lam (40) to speculate: "LTC_4 synthase may represent a member of a lipid-binding protein family rather than classical GSH S-transferases."

Further metabolic progression of LTC_4, first to LTD_4 [5(*S*)-hydroxy-6(*R*)-cysteinylglycyl-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid], and then to LTE_4 [5(*S*)-hydroxy-6(*R*)-cysteinyl-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid], is by the sequential action of either γ-glutamyl-transpeptidase (41) or γ-glutamyl-leukotrienase (42) and cysteinylglycyl-dipeptidase (41).

Deactivation of the cysteinyl (peptidyl) leukotrienes occurs by several pathways. The oxidative burst of activated phagocytes can inactivate the cysteinyl leukotrienes (e.g., LTC_4) by the formation of inactive sulfoxides (43). In this context, it is interesting that the sulfones of LTC_4, LTD_4, LTE_4, and LTF_4 have been synthesized and were determined to be about equally active in *vitro* with the parent sulfides (44). Although isolation of the sulfones from rat peritoneal cells has been reported (45), neither their biological significance nor their distribution is clear. In rat, N-acetylation and omega-oxidation of LTE_4 occur to form the N-acetyl-20-carboxy-LTE, (46). In humans, however, the major excreted cysteinyl leukotriene metabolite is LTE_4 (47).

4 SOURCES AND ACTIONS OF THE LEUKOTRIENES

4.1 Discovery, Structure Elucidation (48), and Cellular Sources of the Leukotrienes (49)

Almost 60 years ago Feldberg, Kellaway, and Trethewie (50, 51) discovered that the addition of cobra venom to perfused guinea pig lungs caused the release of a slow-reacting, smooth muscle contracting substance (SRS) that was distinct from histamine. Early studies on this mediator were usually carried out by use of crude biological extracts and, as such, the samples were contaminated by the presence of other physiologically active agents (especially large amounts of histamine) that interfered with characterization of the SRS. In the late 1950s and early 1960s Brocklehurst worked extensively on the extraction, purification, and characterization of SRS (52). When he noted that SRS could be produced in vitro from the lung tissue of previously sensitized guinea pigs upon antigen challenge, he suggested that it be renamed SRS-A, slow reacting substance of anaphylaxis (53), in recognition that it was generated during anaphylactic responses.

The minuscule amounts of SRS-A available from natural sources and its chemical and biological instability severely hampered efforts to characterize this mediator. In the 1970s a breakthrough occurred with the combined findings from the Piper (54), Parker, and Austen laboratories that: *(1)* under some circumstances cyclooxygenase inhibitors caused an increased production of SRS-A, which implied a link with arachidonic acid metabolism (55); and (2) HPLC could be used to purify SRS-A and that the UV absorbance of the HPLC Samples implied the presence of a triene-containing chromophore.

Samuelsson and Borgeat's subsequent work on arachidonic acid metabolism that led to the discovery of the 5-lipoxygenase pathway (56) and to the assignment of the structure of (5*S*,12*R*)-dihydroxy-6,8,10,14-eicosatetraenoic acid (55, 57) (later named LTB_4) were critical to the efforts on SRS-A. Samuelsson and Borgeat (56, 58) proposed an unstable intermediate (LTA_4) as the precursor to LTB_4. The structure of LTA_4 was correctly assigned in conjunction with Corey (57–60). LTA_4 was recognized as the precursor to SRS-A and LTC_4 was proposed, as a cysteine-containing derivative of 5-hydroxy-7,9,11,14-eicosatetraenoic acid (59), to be the next member of the biological cascade. Through further synthetic efforts glutathione (60, 61), as earler suggested by the Parker laboratory (55), was identified to be the cysteinyl component, and the absolute stereochemistry was assigned (58, 60). LTD, (61–64) and LTE_4 (63, 65) were identified as natural SRS components after they had been synthesized by Corey's group as part of their effort to assign the structure of LTC_4.

The name leukotriene was suggested for these compounds by Sarnuelsson et al. (64, 66) because they were found in leukocytes and had a triene-containing structure. The letters A, B, C, D, and E in part define progress along the biosynthetic pathway and the numerical suffix was appended to describe the total number of double bonds found therein. Although the products from arachidonic acid that are the subject of this review all contain four double bonds, analogous compounds, containing three or five double bonds, may be biosynthesized starting from, respectively, 5,8,11-eicosatrienoic acid or 5,8,11,14,17-eicosapentaenoic acid (65, 67).

The cellular locations of leukotriene biosynthesis are determined by the distribution and location of the enzymes needed for their production. 5-Lipoxygenase is found in only a limited number of specific cell types, predominantly of myeloid extraction. Because of this restricted expression, production of LTA_4 is largely limited to those cells (e.g., basophils, eosinophils, neutrophils, macrophages, mast cells, and monocytes) (66). These cells produce LTA_4, further process it to either LTB_4 or LTC_4, or secrete it.

Neutrophils (67–69), monocytes, and macrophages are the major producers of LTB_4 (70). Once synthesized (e.g., by polymorphonuclear leukocytes), LTB_4 is actively exported by a carrier-mediated process (71). However, LTA_4 hydrolase is a relatively ubiquitous species found in almost all human tissues (72). Because of this broad distribution, conversion of extracellularly synthesized LTA_4 to LTB_4 can occur in a wide variety of cells. This has

been exemplified with erythrocytes, which lack 5-LO, but have the ability to augment LTB_4 production when mixed with neutrophils (73). Also, enzymatic conversion of LTA_4 to LTB_4 in human plasma has been demonstrated (74).

The sulfidopeptide leukotrienes (LTC_4, LTD_4, and LTE_4) are synthesized by basophils (75), eosinophils (67, 68), macrophages, and mast cells. These cells generate the peptidoleukotrienes because they have LTC_4 synthase. Some other cells that lack 5-LO (e.g., platelets) have LTC_4 synthase and can convert imported LTA_4 to LTC_4. Like LTB_4, LTC_4 is actively exported to the extracellular environment (76). In this case export is by a probenicid-sensitive membrane carrier-mediated process that is temperature dependent and saturable (77). Conversion to LTD_4 and LTE_4 occurs extracellularly.

4.2 Biological Properties (78)

The biological effects of both LTB_4 and the peptidoleukotrienes are mediated through specific receptors (79, 80). The effects of LTB_4 are mediated through the BLTl (IUPHAR receptor nomenclature) and BLT2 receptors and the peptidoleukotrienesare mediated through the CysLTl and CysLT2 receptors.

Despite the fact that potent specific antagonists are available for these receptors, some of which are approved drugs, only recently has the molecular identity of LT receptors been discovered. There are two known receptors for LTB_4. These receptors differ in affinity for [^{3}H]LTB_4 by about 150-fold (81). Yokomizo et al. (82) discovered BLTl using a subtraction strategy from HL60 cells. This receptor corresponds to the high affinity receptor and appears to be important in leukocyte chemotactic response to LTB_4. This laboratory also discovered the lower affinity receptor BLT2 (83) as did Kamohara et al. (84). The two receptors differ in expression with the high affinity receptor expressed selectively in peripheral leukocytes, whereas the second receptor is expressed in many tissues. The receptors are structurally similar (45% amino acid identity). Interestingly, the BLT2 open reading frame overlaps the promoter region of BLTl (85). The BLT2 receptor is activated by both 12(*R*)-HETE and LTB_4 (86). Another less examined, but potentially important interaction of LTB_4, is with the nuclear receptor PPAR a (87).

LTB, induces a broad range of proinflammatory responses. It is a potent chemotactic and chemokinetic agent for eosinophils and neutrophils from several species (88, 89). In addition to influencing the directional migration of cells, it upregulates the production of cell adhesion molecules, such as the a,-integrin adhesion protein CD11b/CD18, that are needed for cellular movement (90). It causes accumulation of PMNs *in vivo* and at higher doses can induce their degranulation (91), with the resultant release of a broad array of lysosomal enzymes (e.g., glucuronidase and lysozyme) and the production of oxygen radicals. LTB_4 increases vascular permeability (92), the exudation of mucus, and membrane permeability to calcium (93). McMillan and Foster have hypothesized that LTB_4, in conjunction with other chemotactic mediators, will have a synergistic effect on amplifying the inflammatory response (94).

LTB_4 is viewed as an important mediator of acute and chronic human diseases. It has been hypothesized that agents that either block its production or antagonize its effects might yield an anti-inflammatory agent with improved side-effect profiles compared to those of current agents. Increased production of LTB_4 has been detected in many inflammatory diseases, including adult respiratory distress syndrome (ARDS), arthritis, asthma, contact dermatitis, cystic fibrosis, gout, inflammatory bowel disease, psoriasis, and rheumatoid arthritis (95).

Ironically, it was only after three cysteinyl leukotriene antagonists had been approved for clinical use that the two currently known cysteinylleukotriene receptors were identified molecularly. However, two receptors were proposed earlier by Labat et al. (96). The receptors were proposed to be seven transmembrane G-coupled receptors by several groups (97). Metters, using photoaffinity methods in guinea pig lung preparations, had also identified a molecular size for the CysLTl receptor (98). The groups from Merck Frosst and SmithKline Beecham identified the CysLT1 receptor in 1999. Using similar strategies, the

receptor was identified from screening transiently expressed orphan G-coupled receptors using LTD_4 to stimulate calcium flux (99). The identified receptor expressed in HEK293 cells had the reported preference for LTD_4 versus LTC_4 in calcium mobilization and rank order potency of specific antagonists. Localization studies indicated expression in human lung and bronchus and human peripheral leukocytes as well as several other tissues. The receptor has 337 amino acids and shows some homology to LBT1 (28%) and P2Y receptors (31%). Another receptor, the CysLT2 receptor, was also discovered recently by three different laboratories (100). This receptor was discovered through its sequence similarity to the CysLTl receptor (36% amino acid identity). It had the reported equivalent potency of LTC_4 and LTD_4 and was antagonized by BAYu9773. This receptor has more limited expression that overlaps with the CysLT1 receptor but appears more cardiovascular and endocrine in nature. The gene has been localized to chromosome 13q14 close to a marker that has been associated with atopic asthma in two studies (100). Currently approved drugs have activity only against the CysLT1 receptor. It is unclear what further pharmacological effects an agent antagonizing both receptors would have.

The cysteinyl leukotrienes are potent smooth muscle constrictors both ***in vitro*** and ***in vivo*** in human and animal studies. In humans LTC_4 and LTD_4 are approximately 1000 times more potent than histamine, on a molar basis, in inducing contractions on isolated human bronchus. LTE_4 is about 1/10 as potent as LTC_4 or LTD_4 (100). Inhalation studies in humans have also shown that the leukotrienes are about 1000 times more potent than either histamine or methacholine in producing bronchoconstriction (101). The sensitivity to inhaled LTD_4 is such that the order of responsiveness is asthmatics > allergics > normals (102). Inhaled leukotriene D_4 induces hyperresponsiveness to methacholine (103) and inhalation of LTE_4 induces eosinophil migration into the lungs of asthmatic test subjects (104). Because eosinophilia has been correlated with the degree of asthma severity, it is possible that the leukotrienes play a significant role in converting the initial acute bronchoconstrictor response to a chronic inflammatory response.

Asthma is the disease that has been most strongly linked to production of cysteinyl leukotrienes (105). This statement is supported by (1) the increased levels of LTE_4 found in the urine of asthmatic patients during an attack and *(2)* the efficacy of leukotriene agents in the disease. Urinary LTE_4 can be used as a predictor/indicator for nocturnal asthma (106). Other diseases in which elevated concentrations of the peptide leukotrienes have been found include rhinitis, urticaria, arthritis, ARDS, uveitis, and psoriasis (107).

5 AGENTS INHIBITING LEUKOTRIENE BIOSYNTHESIS

The generation of leukotrienes depends on the formation of LTA_4, the key intermediate leading to either LTB_4 or the peptidoleukotrienes. Thus, the most versatile and thus most widely studied approach to the inhibition of leukotriene biosynthesis involves the inhibition of formation of LTA_4, although attempts to inhibit the enzymes involved in the transformation of LTA_4 also offer interesting alternatives (LTA_4 hydrolase, LTC_4 synthase), which are discussed later.

It is clear that 5-lipoxygenase (5-LO) [EC 1.13.11.34], through a two-step process, is the enzyme responsible for the production of LTA_4. Thus the mechanism of action and, to a lesser extent, the structure of 5-LO have largely influenced the design and discovery of inhibitors of leukotriene biosynthesis. This enzyme has been the subject of intense research since its discovery, as discussed below (Section 5.1), and many inhibitors have been described.

5-Lipoxygenase-activating protein (FLAP) is also involved in the process by which AA is converted to LTA_4 and a number of interesting inhibitors have been described (Section 5.2).

The advantage of inhibiting 5-LO (or FLAP) is essentially to block the effects of all LTs and hence provide agents with therapeutic potential in a wide range of allergic and inflammatory diseases including asthma,

COPD, fibrotic lung disease, allergic rhinitis, rheumatoid arthritis, inflammatory bowel disease, and possibly psoriasis. Furthermore, concerns over the likely heterogeneity of different functions and signaling pathways of LT receptors and agonist-ligand specificities do not affect this approach. The potential disadvantages arise from the possibility that complete LT inhibition could give rise to undesirable side effects, although no indications of any essential physiological role of LTs have appeared since the discovery of these mediators. Indeed, recent work with 5-LO(−/−)-deficient mice, generated by inactivation of the 5-LO gene (108, 109), showed that these animals developed normally with no adverse health effects, which suggests that 5-LO blockade should not cause untoward effects. In addition, studies on Zyflo (see next section) indicate that inhibition of whole body leukotrienes at least to >80% would seem to be broadly safe.

The endeavors to discover such inhibitors and the successes to date are described in the following sections.

5.1 Inhibitors of 5-Lipoxygenase (5-LO)

The mechanism of 5-LO appears to involve the oxidized active Fe^{3+} state and the generation of radical species (2). Known mechanisms of inhibition constitute trapping of radical intermediates, ferric iron ligation, reduction of the nonheme iron, reversible binding at active or other regulatory sites, and combinations of these effects in the same molecule. Consequently, three broad classes of direct 5-LO inhibitors have evolved over the last 15 years: "redox," iron ligand, and "nonredox" inhibitors. First, we will summarize the properties of 5-LO in the light of these different methods of inhibiting this enzyme.

5.1.1 Structure and Mechanism of 5-LO. It should be noted that most of the original drug discovery effort targeting 5-LO inhibitors was done in the absence of any detailed structural knowledge of the enzyme. The amino acid sequence of human 5-LO was not described until 1985 (110), when isolation and cloning showed both it and rat 5-LO to be 78-kDa proteins with 93% homology. In its active form 5-LO is membrane bound; purification, therefore, modifies its natural behavior. In 1991 recombinant methods allowed the preparation of pure enzyme in milligram quantities (110) and allowed the experiments that definitively showed nonheme iron associated with highly active enzyme obtained from a baculovirus expression system (111).

Interestingly, even at that time, a series of five histidine residues was postulated to constitute the potential iron-binding site, in that they are also conserved in other lipoxygenases, although site-specific mutagenesis studies indicated that three of these could be mutated individually in 5-LO without loss of either oxygenase or LTA_4 synthetase activity (112,113). This proposal has now been refined and it is believed that the nonheme iron is bound permanently by His^{372}, His^{550}, and Ile^{673} and that a fourth exchangeable ligand, His^{367}, is replaced by a reaction intermediate during the catalytic cycle (114). This exchange ligation is interesting from the point of view of designing inhibitors because it suggests that compounds in the appropriate oxidation state with affinity for Fe^{3+} could also displace this ligand and thus inhibit catalysis. Indeed, hydroxamate and *N*-hydroxyurea inhibitors may possibly act in this way. Except for other lipoxygenases, 5-LO shows little homology to other proteins. The enzyme has recently been shown to have a SH_3 domain that binds to the growth factor bound receptor protein (115). This interaction may be the site that allows membrane association of the enzyme, although that relationship has not been definitively shown. Isolated 5-LO requires Ca^{2+}, ATP, and phosphatidyl choline for optimal activity but its amino acid sequence has not been found to show any strong homology with other proteins possessing either Ca^{2+}- or ATP-binding sites.

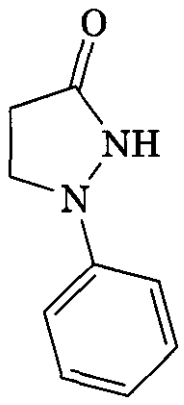
Detailed structural information is still unavailable for 5-LO and thus has not been helpful in the design of 5-LO inhibitors. Initial research attempts were also made difficult by the complex kinetic behavior of this enzyme (116–118).

Elucidation of the actual mechanism of 5-LO has been a difficult and often disputed area. Here, we highlight the essential steps to help clarify the inhibitory mechanism of the compounds described hereafter. After activa-

tion (Fe^{2+}·Fe^{3+}) during the lag phase, Fe^{3+} specifically oxidizes the diene of AA at position 5, to generate a pentadienyl radical cation. A basic amino acid residue in the enzyme-active site abstracts a proton and the ferrous iron is reduced to Fe^{2+}. The pentadiene radical then stereospecifically reacts with oxygen at C_7, forming a 5(*S*)-hydroperoxy radical that undergoes electron transfer and reprotonation to form 5-HPETE. Fe^{3+} is regenerated for recycling. 5-HPETE is subsequently converted to LTA_4 through deprotonation at C_{10} and epoxide cyclization. The participation of radical species has been demonstrated by Mossbauer experiments (119), consistent with pentadienyl radicals being intermediates in the catalytic reaction.

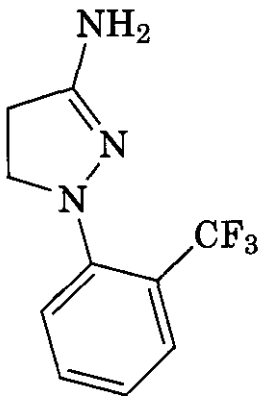
From this reaction mechanism, as mentioned above, it is clear that many types of molecules could inhibit 5-LO ***in vitro,*** especially those with antioxidant properties. Indeed, several hundred patent applications have been filed by over 40 different pharmaceutical companies since work has begun on 5-LO, claiming inhibitors with various degrees of potency and selectivity.

5.1.2 Antioxidants. 5-LO is particularly susceptible to inhibition by compounds with low redox potentials. Phenidone and BW-755C (1, 2, respectively) (120) were among the first



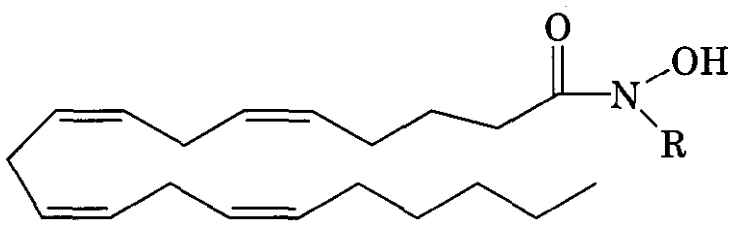
(1) Phenidone

compounds to be identified and other compounds in this class are based around the heteroatom-substituted pyrazole ring. They are loosely termed ***redox inhibitors*** because they may, directly or indirectly, reduce either Fe^{3+} in the active enzyme or one of the radical intermediates to give the inactive Fe^{2+} form. Early redox inhibitors were reviewed by Swingle et al. (121). Although a large number of inhibitors have been examined ***in vitro*** and ***in vivo*** (2), no favorable clinical data have been forthcoming from this approach.



(2) BW 755C

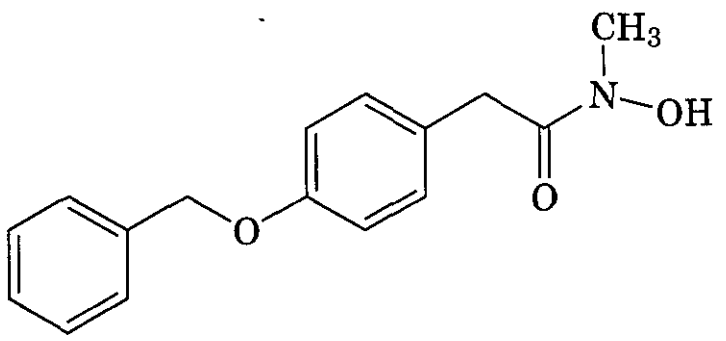
5.1.3 Iron Ligands. Based on the hypothesis that incorporation of functional groups that could chelate to iron would inhibit 5-LO, Corey (122) synthesized arachidonohydroxamic acid and a number of N-alkyl derivatives (3). These indeed proved to be potent 5-LO



(3) R = H, Alkyl

inhibitors (IC_{50} = 0.03–0.1 μM, RBL-1) and rapidly, elsewhere, many hydroxamate inhibitors were prepared that possessed high ***in vitro*** potency.

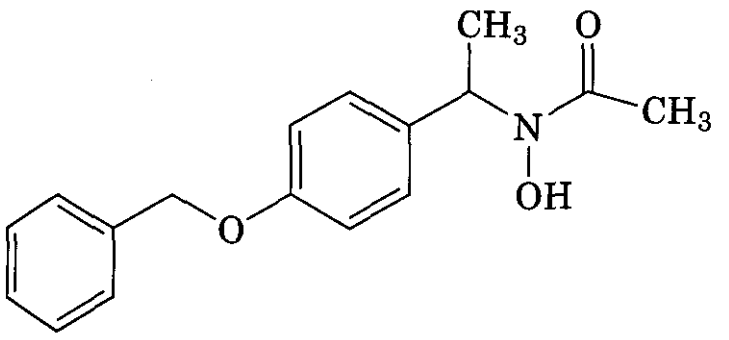
Work at Abbott identified hydroxamates, such as compound (4), by modification and



(4)

simplification of the lipophilic chain. These compounds are very potent ***in vitro*** and are well absorbed; however, they are rapidly me-

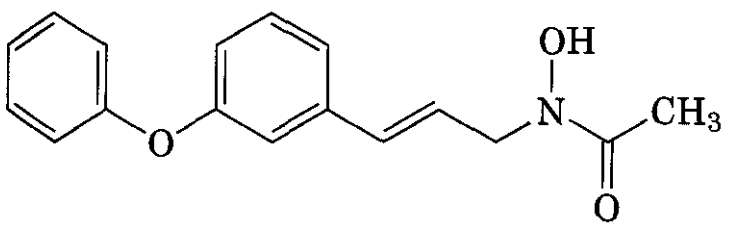
tabolized to inactive carboxylic acids and show short half-lives and low *in vivo* potencies (123). Modification of the orientation of attachment of the hydroxamate group (e.g., A-63162,s) had little effect on *in vitro* activity,



(5) A-63162

but A-63162 showed significantly improved *in vivo* activity (124, 125).

Parallel work at Wellcome revealed similar trends. That group discovered BW-A4C (**6**), a

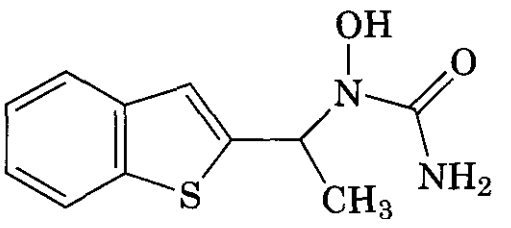


(6) BW A4C

hydroxamate similar to A-63162 in structure, which showed good *in vitro* potency (IC_{50} = 0.14 μM, human PMN) (126).

Despite promising oral activity in animal models, BW-A4C had poor pharmacokinetics in human volunteers. Extensive metabolism of BW-A4C occurred in humans (127), resulting in the accumulation of high concentrations of metabolites. Additional modifications were required to avoid the metabolic problems encountered with hydroxamates to convert this class into orally active clinical candidates.

5.1.4 N-Hydroxyureas. The substitution of the hydroxamate with N-hydroxyurea, first done at Abbott, provided compounds that were quickly shown to have superior *in vivo* behavior in animal models and pharmacokinetics. From a study of several hundred compounds containing the *N*-hydroxyurea moiety, Zyflo (zileuton; A-64077, **7**) emerged as the candidate for clinical trials (128, 129). In this compound the lipophilic benzyloxyphenol

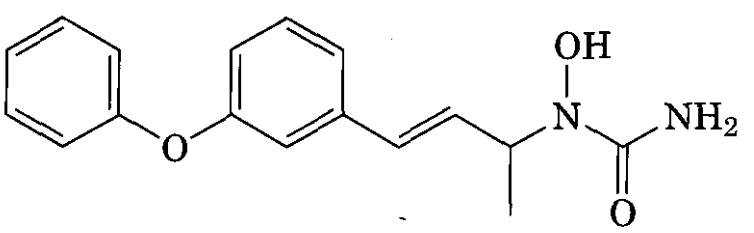


(7) Zyflo (zileuton, **A-64077)**

moiety of A-63162 was replaced by a benzothiophene, although the important a-methyl was retained, with the result that zileuton, like A-63162, contains a stereogenic center.

Zileuton is a potent 5-LO inhibitor *in uitro* and shows approximately 120-fold selectivity over COX-1. Interestingly the R and *S* enantiomers that constitute zileuton were very similar in biochemical potency (130), varying by a factor of only 2–3. These data imply that the a-methyl site binds to an area of the enzyme with considerable flexibility and that specific ligand interaction with iron through the *N*-hydroxyurea occurs in both enantiomers. In contrast, minor modifications of the benzothiophene ring resulted in significant loss in activity (131).

The mechanism of 5-LO inhibition by N-hydroxyureas and hydroxamates is clearly more complex than mere iron chelation. There is no doubt that these compounds bind strongly to iron in solution ($K = 10^{12}$/mol for BW-A4C) and that they are not powerful redox compounds, having relatively high electrode potentials (E_0·2 V for BW-B70C, 8, and zileuton, 7). Mechanisms for the activity of the compounds remain complex (132).



(8) BW B70C

5.1.4.1 ***In Vivo Activities and Clinical Studies with N-Hydroxyurea Inhibitors.*** BW-B70C is efficacious in allergen-induced bronchoconstriction and late-phase lung eosinophil accumulation in sensitized guinea pigs (133). Unfortunately, it produced kidney lesions in the rat, preventing clinical evaluation. Zileuton (Zyflo), however, was approved for use in the

treatment of moderate to severe asthma in 1996 as Zyflo. Several summaries of the clinical studies on Zyflo have since been published (134, 135). Thus we will summarize only the pertinent development events and key clinical findings. Zileuton is an orally active inhibitor of 5-LO in rats, dogs, monkeys, and humans measured by ex vivo inhibition of LTB_4 produced by addition of calcium ionohore A23187 in blood. Preclinical studies with the compound showed it capable of inhibiting leukotriene-dependent inflammation in mice (136) and inflammatory cell influx in rats (137). It is also effective in guinea pig and sheep models of antigen-driven bronchoconstriction (138).

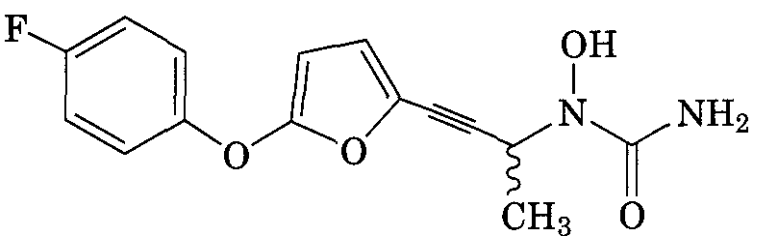
In phase I, volunteer studies showed that the inhibition of ex *vivo*-stimulated LTB_4 in blood correlated with plasma drug levels (139). Pharmacokinetic studies in phase I volunteers determined that the major route of metabolism of zileuton is glucuronidation of the N-hydroxyurea group followed by urinary excretion, and the oral half-life was estimated to be 3 h (140). With this relatively short half-life it was necessary to administer 600 mg four times a day (qid) to maintain ex *vivo*-stimulated LTB_4 inhibition in human blood at approximately 80% returning to control levels after removal of the drug. No untoward side effects were observed.

Consequently, Zyflo was thoroughly evaluated in a number of clinical trials for effects on models of asthma and in the treatment of chronic asthma. Challenge models of asthma with a variety of stimuli (allergen, exercise, cold dry air, or aspirin) were successful. Results in aspirin-induced asthma were dramatically effective, indicating that leukotrienes are the primary mediators of this response. Zyflo also shows remarkable anti-inflammatory effects, as predicted from animal studies (137). Eosinophil influx and albumin leakage were reduced and the urinary increase in LTE, was blocked (86%)(141, 142).

Zyflo was also found to be effective in chronic asthma, as evidenced by two large placebo-controlled trials (143, 144). Improved FEV1 and reduced bronchodilator use were the primary clinical endpoints. Interestingly, Zyflo appeared to be most effective in moderate to severe patients. Additional trials indicated that Zyflo is also steroid sparing (145).

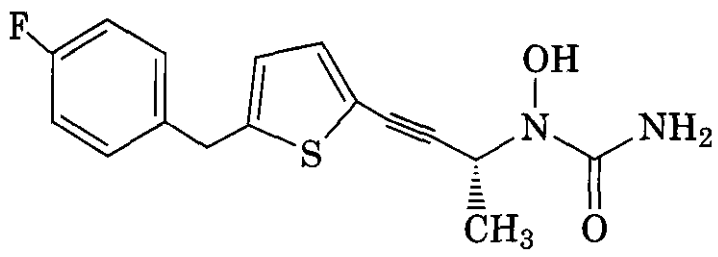
5.1.4.2 Second-Generation N-Hydroxyureas. During these trials it was realized that the short half-life (3h) and high clinically effective daily dose of Zyflo (2400 mg) needed to be improved. At Abbott, the search for more potent, longer-acting N-hydroxyurea inhibitors focused on reducing the glucuronidation rate of the N-hydroxyurea function. Various in vitro models were used, particularly microsomal preparations from human or monkey liver, to assess new inhibitors (146). In this way, structure-activity relationship (SAR) studies indicated that the lipophilic heteroaryl template and the linking group to the N-hydroxyurea could be profitably modified (131). Introduction of an unsaturated linking group was shown to modulate inhibition and metabolism. The spatial orientation of the lipophilic template with respect to the pharmacophore had a dramatic effect on pharmacological properties. Acetylene linking groups significantly decreased the rate of glucuronidation (GT) (2, 139).

Inhibition of leukotriene formation in human whole blood was also improved. There was little difference in in vitro activities between A-78773 and its enantiomers (9); however, the glucuronidation rate in monkey microsomes was lower for *R*(+) A-79175. The relative rate correlated with in *vivo* elimination half-lives in cynomologus monkeys [4.7 h for the racemate, 1.8 h for *S*(−) A-79176 and 9.0 h for *R*(+) A-791751, prompting a Phase I clinical study in which *R*(+) A-79175 exhibited an apparent half-life of 6.5 h for a single 600 mg p.o. dose, twice that of Zyflo (147,148).



(9) (±) A-78773
R(+) A-79175
S(−) A-79176

Further chemical modification led to *R*(+) ABT-761 (10), which was found to be even more resistant to in vitro glucuronidation (149). Its elimination half-life in monkeys was 16 h and consequent phase I studies in hu-

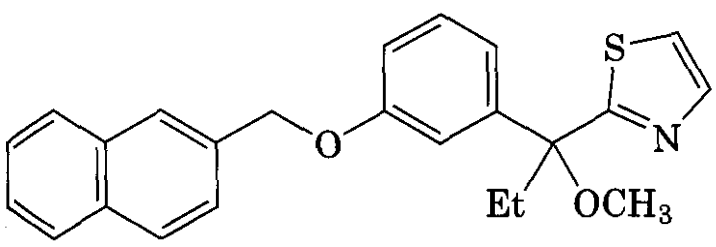


(10) ABT-761

mans showed excellent bioavailability and extended duration of plasma levels. Significantly, a single 200 mg dose provided >95% inhibition of *ex vivo*-stimulated LTB_4 formation for up to 18 h. ABT-761 showed significant protection against exercise and adenosine-induced bronchoconstriction in asthmatics (150, 151). However, idiosyncratic liver toxicology precluded the further development of this potent, effective 5-LO inhibitor.

5.1.5 Substrate Inhibitors. A number of groups also searched for orally active drugs with mechanisms of action different from the inhibitors just described. At Zeneca, this work was begun in the late 1980s where "active-site-directed" inhibitors were designed to fit 5-LO selectively without the need for redox or strong iron ligand properties. This was particularly difficult in the absence of structural data on 5-LO; however, based on the hypothetical mechanism discussed in Section 5.1.1, which implies the presence of an Fe^{3+} atom and a basic group adjacent to a lipophilic pocket in the active site, lipophilic imidazoles were initially synthesized to interact with these structural elements.

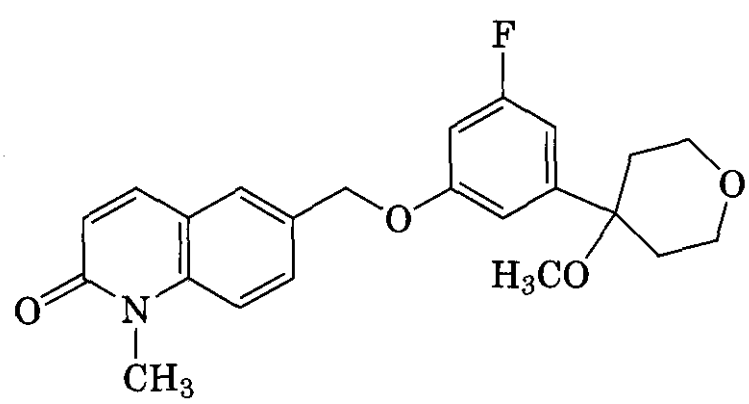
It was discovered that replacement of the imidazole by a thiazole and introduction of confonnational restriction with rnethoxy and alkyl groups gave the *in vitro* potent ZM211965 (**11**) (IC_{50} = 0.1 μM, guinea pig 5-LO; IC_{50} = 0.4 μM, human whole blood LTB_4 inhibition) that importantly showed no



(11) ZM211965

COX-1 inhibition up to 100 μM in human blood (152, 153). Cyclic voltammetry and iron chelation measurements confirmed that this (methoxyalkyl)thiazole series is free from redox and iron-complexing properties. The series differs from redox and the *N*-hydroxyurea series in that it demonstrates enantioselective inhibition of 5-LO both in vitro and in vivo (154). This was the first evidence of 5-LO inhibitors forming enantiospecific interactions with the enzyme. Thus, unlike the a-methylene center in the Abbott N-hydroxyurea series, the stereoselectivity of these ligands indicates a close contact of the stereogenic center and active site of the protein.

A number of steps were required to discover an orally bioavailable inhibitor in vivo similar to that seen with the *N*-hydroxyureas (2). The compound discovered was improved by structural modification of the naphthyl ring, and further introduction of a fluoro substituent to reduce susceptibility to metabolism. This compound was ZD2138 (12) (IC_{50} =



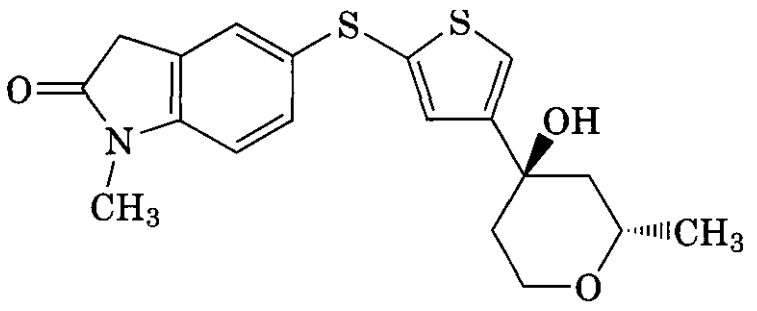
(12) ZD2138

0.02 μM, human whole blood) (155). ZD2138 possesses no redox properties and extensive SAR data and detailed conformational analyses suggest that the (methoxyalkyl)thiazole and tetrahydropyran (THP) series are related (156). ZD2138 shows no inhibition of CO, even up to 20,000 times the levels that inhibit 5-LO in dog or human blood. ZD2138 does not antagonize FLAP.

In phase I studies, ZD2138 was well tolerated up to 1000 mg per volunteer. A single oral 350 mg dose completely inhibited LT synthesis *ex* vivo in stimulated blood for over 24 h and the half-life in humans was estimated to be 12–16 h (157). Phase II clinical trials demonstrated that in aspirin-sensitive asthmatic pa-

tients, 350 mg p.o. given 4 h before challenge caused bronchodilation and inhibited the fall in FEV1 (158). However, in allergen-challenged asthmatics the same treatment with ZD2138 had no effect on the early or late asthmatic response (159). Further development of ZD2138 was suspended (160). A detailed description of the discovery of ZD2138 can be found in Ref. 161.

Extension of the work in the Zeneca group around this series led to ZD4407 (13), which is



(13) ZD4077

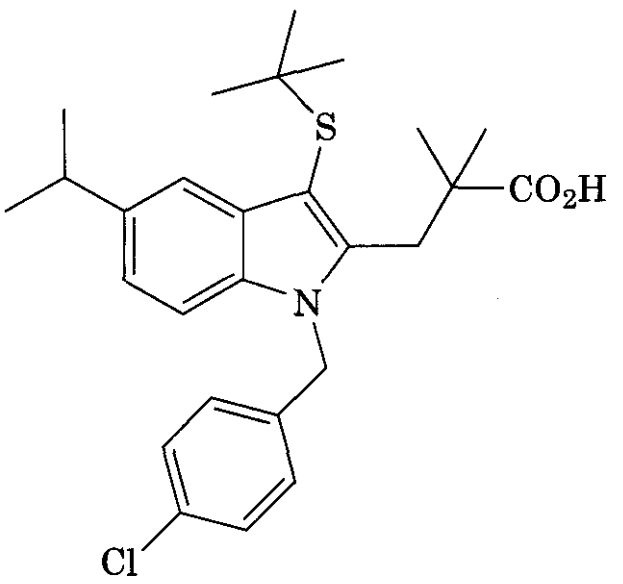
extremely potent (IC_{50} = 0.02 μM, human whole blood leukotriene inhibition) and is devoid of any autoinduction of liver enzymes that had been observed preclinically for earlier members of this series (162).

The methoxytetrahydropyran moiety has been exploited by other groups. Merck Frosst identified a class of lignans derived from a natural product, Justicidin E, as moderately potent redox inhibitors of 5-LO (163). Abbott also described compounds in this arena (164). These compounds were very weak inhibitors of 5-LO in broken cell preparations, as was ZD2138, although many compounds with potent whole blood activity were obtained. These data imply a different mode of action for this series than direct 5-LO inhibition and are inconsistent with a substrate inhibitor.

5.2 Inhibitors of 5-Lipoxygenase-Activating Protein (FLAP)

FLAP inhibitors are a novel class of compounds that inhibit cellular leukotriene biosynthesis without acting directly on 5-LO. Instead, they bind to the 18-kDa membrane FLAP, thus preventing the activation of 5-LO.

Research at Merck Frosst in 1989 (165, 166) led to the initial FLAP inhibitor, MK0886 (14), and consequently to the discovery of FLAP itself. MK0886 was derived from a



(14) MK0886

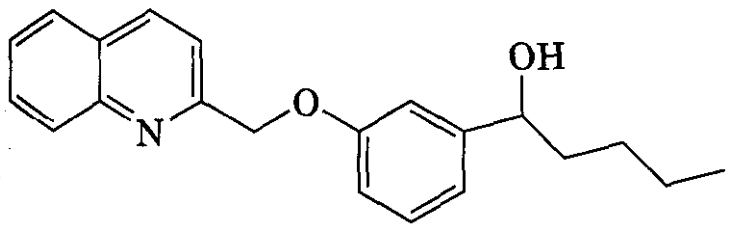
series of indole-3-alkanoic acids related to indomethacin, which possessed the dual pharmacological properties of TXA_2 receptor antagonism and leukotriene biosynthesis inhibition in intact cells.

These properties could be separated and MK0886 had no effect on isolated, purified 5-LO, although in intact leukocyte preparations it inhibited LTs at low nM IC_{50} values (3–5 nM) (167). Thus, a novel mode of action of leukotriene inhibition had been discovered, which was then exploited to find new inhibitors. Indeed, it further helped the understanding of the mechanism of 5-LO activation.

As discussed earlier, FLAP is associated with a cellular membrane; initially, this was believed to be the plasma membrane, but work at Merck Frosst and Michigan (168, 169) showed that localization is cell and activation state dependent. In the neutrophil, inactive cytoplasmic 5-LO translocates to membranes on activation by Ca^{2+} and associates with FLAP, as demonstrated in an elegant series of experiments by two laboratories (170). The nature of this association was initially thought to consist of 5-LO possibly docking onto the membrane-bound FLAP (171), although it has been proposed that FLAP presents AA to 5-LO in a conformation favorable for reaction (172). Whatever the true mechanism, it is clear from transfection experiments that the association of 5-LO and FLAP is essential for intact cell LT biosynthesis (173). The FLAP inhibitors, like MK0886, inhibit complex formation, preventing the complete translocation of 5-LO, and block LT biosynthesis.

MK0886 was shown to have a high binding affinity for FLAP (IC_{50} = 23 n*M*) (174). Clinical studies with MK0886 in atopic asthmatics showed good improvement of early (58%) and late (44%)asthmatic responses in pulmonary function to an allergen challenge, but only showed modest inhibition of *ex vivo* ionophore-stimulated whole blood LTB_4 biosynthesis and urinary LTE, excretion (175).

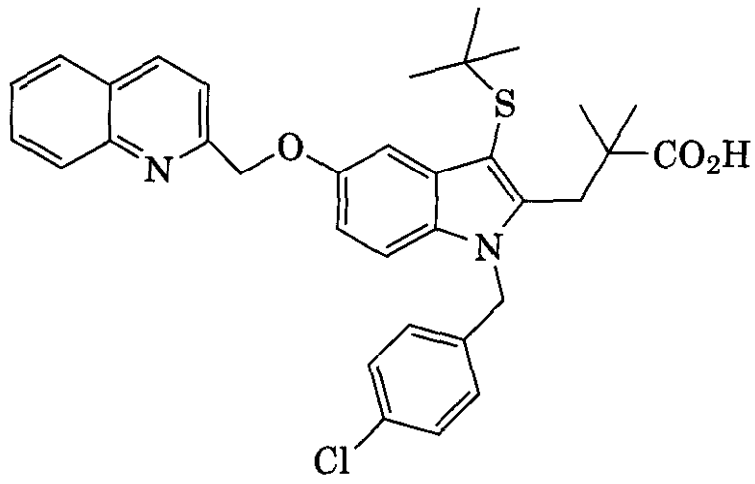
A key observation that would impact the design of new FLAP inhibitors was that an older leukotriene inhibitor, Rev-5901 (**15**),



(15) Rev-5901

which contains a quinolinylmethyloxy substituent, was an inhibitor of this newly discovered protein. The quinoline moiety is a prominent binding motif of many FLAP inhibitors. Two FLAP inhibitors developed from these leads are L-674,636 (16) and MK0591 (17).

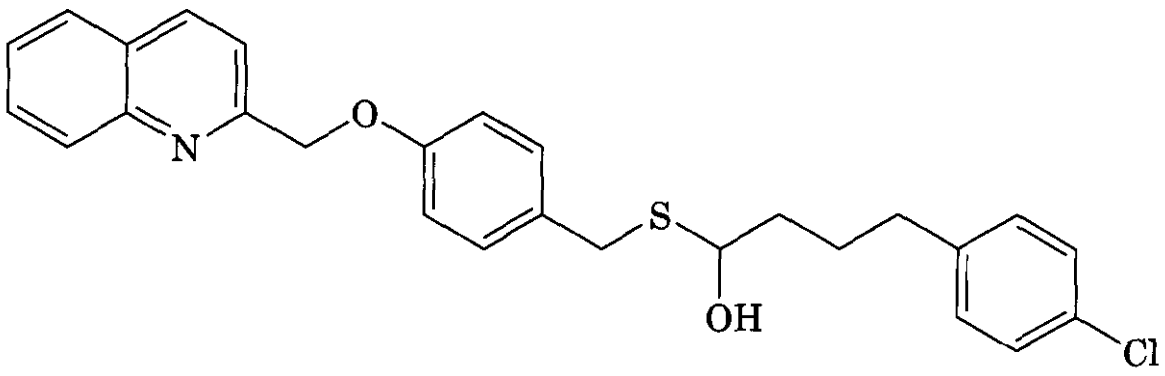
Clinical studies with MK0591 were initially encouraging. Allergen-challenged atopic asthmatic patients were treated p.o. with 3 × 250 mg of MK0591 (24, 12, and 1.5 h before allergen), leading to reduced bronchoconstriction in early (79%) and late (39%) asthmatic responses. Furthermore, in contrast to MK0886, LT biosynthesis *ex vivo* in stimulated whole blood from these patients was totally inhibited, as were urinary LTE_4 levels (87%), for 24 h after allergen challenge (176). The study was extended to several hundred moderate and severe asthmatics (177, 178) and orally dosed MK0591 over 4–6 weeks caused improvements in pulmonary function tests including FEV, and morning and evening peak expiratory flow. Similarly, rescue β-agonist use was diminished. Adverse effects were no different from placebo. However, the compound was not taken into chronic asthma studies.



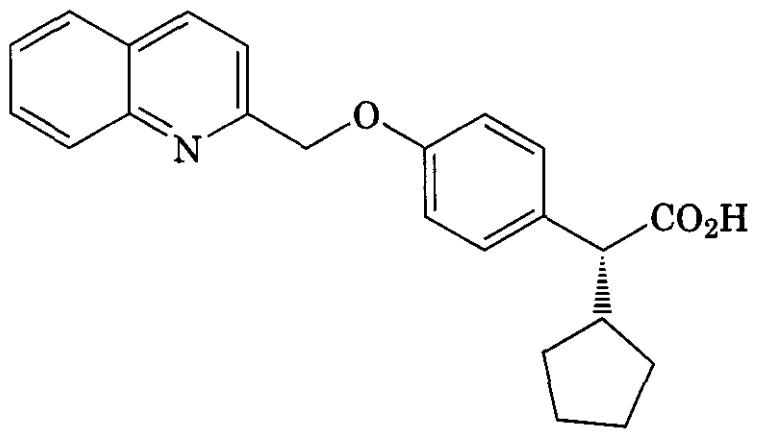
(17) MK0591

Also using the Rev-5901 lead, a series of FLAP inhibitors were developed in which the hydroxyl group of Rev-5901had been replaced by a carboxyl and the n-pentyl alkyl chain had been cyclized to an α-cycloalky substituent. From this series BAY X1005 (18)was selected for clinical development.

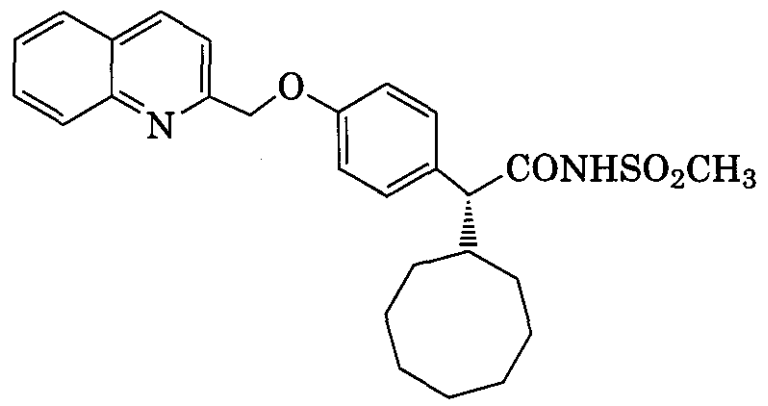
The cyclopentyl substituent was also expanded to cyclohexyl and cycloheptyl. The cycloalkyl substituent provides an important lipophilic interaction, which makes a significant contribution to the affinity. Similar substituents in MK0591 that may correspond with this key lipohilic interaction include the t-butylthiol group or the 4-chlorobenzyl substituent. Stereoselective effects were observed, the R enantiomers displaying a greater affinity for human FLAP. Modification of cyclopentyl to a cycloheptyl alkyl substituent and modification of the acid to a



(16) L-674,636



(18) BAY X1005

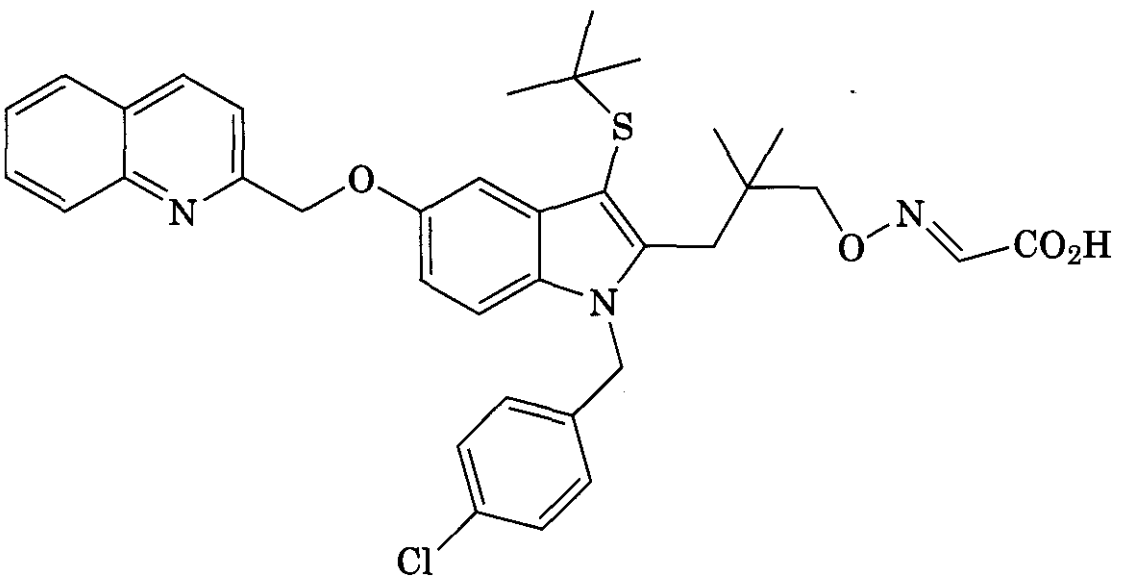


(19) BAY Y1015

methanesulfonylamide provided the backup clinical candidate, BAY Y1015 (19).An interesting modification that has been applied to MK0591 had been the insertion of an oxime moiety **(179)** to connect the terminal carboxylate to the indole skeleton.

Analog **(20)(180)** was similar in preclinical leukotriene inhibition but somewhat superior in monkey pharmacokinetics to MK0591 (R. Bell, unpublished observations, **1994).** Reported SAR trends for structures related to (**20**) and MK0591 are similar; thiazoyl and pyridine were found to be bioisosteric to quinoline in both series **(181, 182).** This oxime can be inserted with two orientations: the oxyimino derivative, shown where the carboxylate is attached to the carbon terminus of the carbon nitrogen double bond; or the oxime ether orientation, where the acid is attached to the oxime as an *O*-alkylated-α-acetic acid oxime ether (179). The oxime moiety provides a structural unit that can present the carboxylate in different spatial orientations for binding. In addition the heteroatoms of the oxime can possibly increase affinity through hydrogen-bonding interactions. Another potential advantage of the oxime may be in improving pharmacokinetic parameters through solvation of the heteroatoms of the oxime; this solvation can affect solubility and absorption.

The oxyimino orientation proved to be superior. This modification has also been applied to the Bayer series as shown (**21**). The **S** isomer (183) was found to be the most potent with the oxime modification **(184). A** drawback of the **BAY X1005** and related candidates such as **(21)** are issues of synthetic complexity and resulting cost of goods associated with a resolution or asymmetric synthesis. The key elements of **BAY X1005,** the quniolylmethoxy, the cycloalkyl, and the acid functionality, are attached to a single carbon center. This stereogenic center could be eliminated if a second quinolylmethyloxyphenyl substituent could replace the lipophilic cycloalkyl substituent but maintain the critical lipophilic interaction



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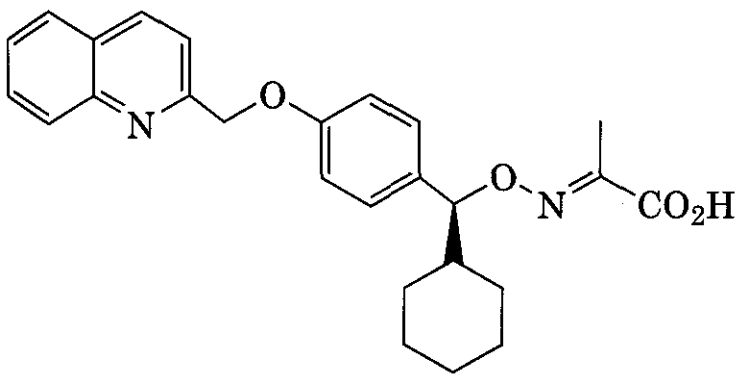


(21)

with the protein. This hypothesis was verified by the symmetrical analog (23).

Development of this symmetrical core led to analogs with and without the oxime linker (184). Analogs were found with superior human neutrophil potency and in *vivo* efficacy inhibiting LT formation in the rat.

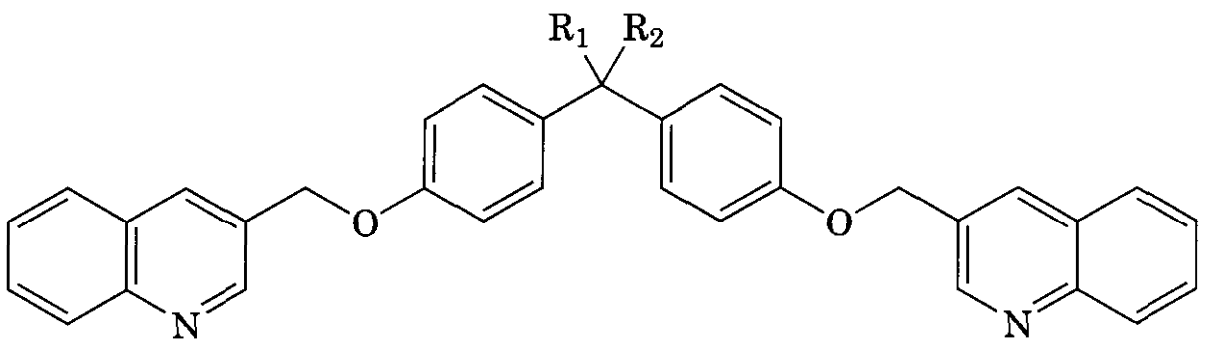
This series culminated in the identification of ABT-080 (**24**). This compound can be readily synthesized by simple bisalkylation of commercially inexpensive 4,4-bis(hydroxyphenyl)valeric acid, also known as diphenolic acid. Of the FLAP compounds synthesized at Abbott, ABT-080 had superior activity in inhibiting rodent leukotriene formation, pulmonary responses in antigen-challenged guinea pigs, and pharmacokinetics in cynomolgus monkey (184, R. L. Bell et al., in preparation). This compound (ABT-080, VML530) has progressed to Phase I trials.

5.3 Inhibition of LTA, Hydrolase

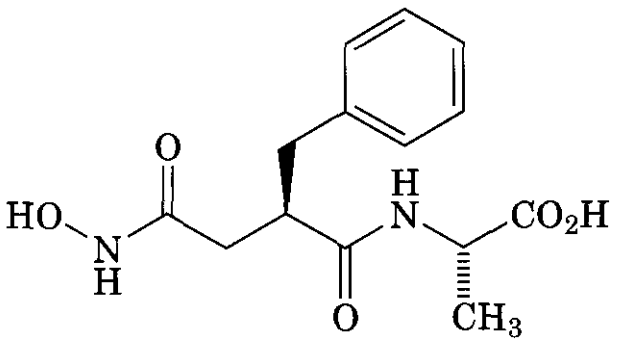
Progress on probing the mechanism of action of leukotriene A_4 hydrolase [EC 3.3.2.6.1 is elegantly summarized in a recent review by Haeggstrom and Wetterholm (185). This enzyme is a 70-kDa zinc-containing monomeric enzyme (186), which catalyzes the vinylogous hydrolysis of LTA_4 to LTB_4. The zinc ion is essential for its hydrolase activity and this enzyme also exhibits aminopeptidase activity. Indeed, it appears that the peptidase and epoxide hydrolase activities occur at the same active site (187). Exploitation of the aminopeptidase function of LTA_4 hydrolase allowed Samuelsson et al. (188–190) to produce a series of tight-binding inhibitors that also inhibit its epoxide hydrolase activity. Elaboration of this type of inhibitor could deliver highly potent and selective anti-inflammatory drugs.

Further work has been published on developing a series of compounds based on the β-mercaptoamino derivative, of which the R enantiomer is two orders of magnitude more potent than the *S* isomer (191). Acetylation of the thiol or its conversion to thio ethers or a hydroxy group results in substantial loss in binding, thus suggesting that selectivity for the free thiol arises from the formation of a highly favorable zinc-sulfide interaction in the enzyme-inhibitor complex.

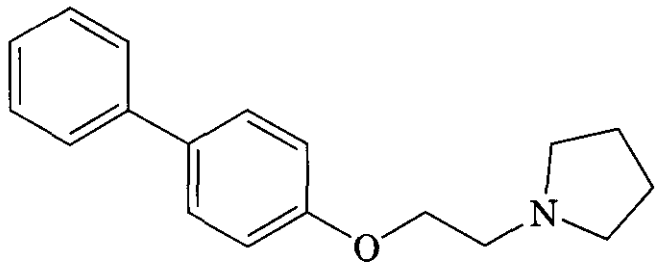
The research on inhibitors of LTA_4 hydrolase has recently been reviewed (192). Extensive research at Searle provided for the only LTA_4 hydrolase inhibitor taken to clinical trials thus far. Early inhibitors described by that group were based on a zinc-binding hydroxamate lead, kelatorphan (25) (193). Unfortunately, reversed hydroxamate compounds with superior in *vivo* pharmacokinetics were considerably less potent. This was also true of carboxylic acids. Another series described by the Searle group used SC-22716 (26) as a non-zinc-chelating phenoxyethyl amino lead. This compound had excellent potency against the



	R1	R2
(23)	H	$ON{=}CHCO_2H$
(24)	CH_3	$CH_2CH_2CO_2H$

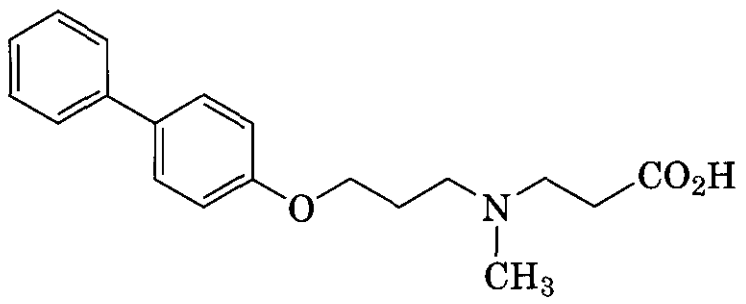


(25) kelatorphan



(26) SC-22716

enzyme and in human whole blood LTB_4 inhibition, but was poorly active in *vivo* (192). Further SAR work around this series led to SC-57416A (27)(194). This compound was potent



(27) SC-57461A

in rodent models of LTB_4 formation and had excellent pharmacokinetics in several species (195). In a preclinical efficacy trial in cotton-topped tamarins this compound also had excellent activity in this animal model of human colitis (196). However, "accumulation of a long-lived metabolite and additional toxicity issues precluded the compound from further development" (197).

At this time there are no known LTA_4 hydrolase inhibitors in development. Recent studies in LTA_4 hydrolase-deficient animals, however, continue to affirm the potential for safe, effective inhibitors of this class (198).

5.4 LTC_4 Synthase

LTC_4 synthase is an 18-kDa integral membrane protein that catalyzes the conjugation of reduced glutathione (GSH) with the epoxide LTA_4 to form LTC_4, the intracellular parent of the cysteinyl leukotrienes. The purification and characterization of LTC_4 synthase has been hindered because of its instability in the semipurified state and the lack of an abundant source of this enzyme. In 1992 Nicholson (199) was able to specifically label this 18-kDa polypeptide in a human monocytic leukemia cell line by use of photoaffinity probes and showed it to be distinct from glutathione *S*-transferase. Most recently, molecular cloning of the gene for human LTC_4 synthase indicates that this enzyme shows significant homology to FLAP (Section 5.2) (200, 201). Details of the biochemistry of this enzyme have been recently reviewed (201, 202).

Perhaps the most important information leading to inhibitors comes from the fact that LTC_4 synthase has significant homology to FLAP (200). An overall homology of 31% is seen for these two proteins with 44% in the N-terminal two-thirds of the proteins. FLAP lacks the amino acids thought to be used in catalysis by LTC_4 synthase. Early studies indicated that FLAP inhibitors also inhibit LTC_4 synthase, albeit in the μM range (200, 201). Surprisingly, little medicinal chemistry has come forth in recent years describing new, more potent inhibitors.

6 AGENTS ANTAGONIZING LEUKOTRIENES

6.1 Peptide-Leukotriene Antagonists

6.1.1 Introduction. There have been thousands of compounds prepared as CysLT antagonists, of which perhaps two dozen have made it to Phase I safety studies in humans. In this section, we focus on a few key compounds, especially those that have advanced to clinical studies. For more detail, the reader is referred to several excellent reviews by Shaw (203), Salmon (204), and Brooks (205).

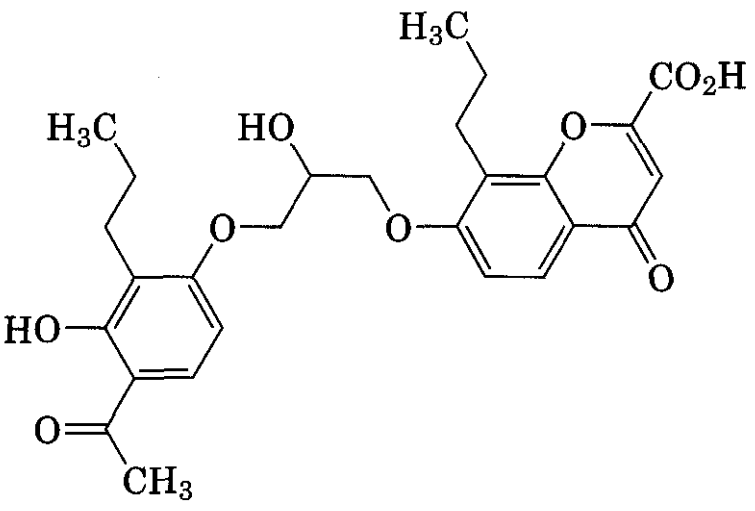
Testing for leukotriene antagonism as for LT inhibition has required many biochemical, pharmacological, and clinical assays. Because

of the variety of assays, only a few that are critical for understanding this section are described. Early researchers tended to measure inhibition of the contraction of a tissue sample (primarily guinea pig trachea or ileum) induced by a standardized unit of "SRS-A" that had been isolated and (partially) purified from a biological source. Later, after synthetic (pure) leukotrienes became available, similar assays putting them into service became widely used (e.g., determination of the percentage inhibition of the 8 n*M* LTD_4-induced contraction of guinea pig tracheal spirals at a specific drug concentration). For compounds warranting greater effort, either IC_{50} values or the calculated pK_B (negative log molar dissociation constants) values for inhibition of the LTD_4- and LTE_4-induced contraction of specific tissues (e.g., guinea pig tracheal or ileal strips, or human bronchus) were determined. In some cases the pA_2 values (the affinity of an antagonist drug as generally determined from the mean of equally effective agonist doselresponse ratios in the absence or presence of a fixed concentration of antagonist) against LTD_4 or LTE_4 were calculated. For reasons that are not clear, most antagonists tend to profile as being more potent against LTD_4 on guinea pig ileum than on guinea pig trachea. For comparison purposes, the IC_{50}, pK_B, and pA_2 values in this section were determined on guinea pig trachea, unless otherwise indicated.

Later, LTD_4-binding assays measuring displacement of [^{3}H]LTD_4 from guinea pig lung membranes were developed. Subsequently, a binding assay using the human receptor in U937 cell membranes has been used by some groups. Study of the binding assay revealed that guinea pig tracheal receptors could be subdivided into high and low affinity classes, with LTE_4 preferentially acting at the high affinity site (206). Comparison of the functional results with several compounds showed that the receptor in human bronchi most closely resembled the guinea pig LTE_4 receptor (207).

In the absence of a structure or sequence for the CysLTl receptor, the starting points for developing antagonists were limited. The choices were either a novel lead obtained by screening of a compound collection or variation of one of the two known ligands, FPL-55712 or the leukotrienes.

FPL-55712 (28) was developed by chemists at Fisons Pharmaceuticals working from a

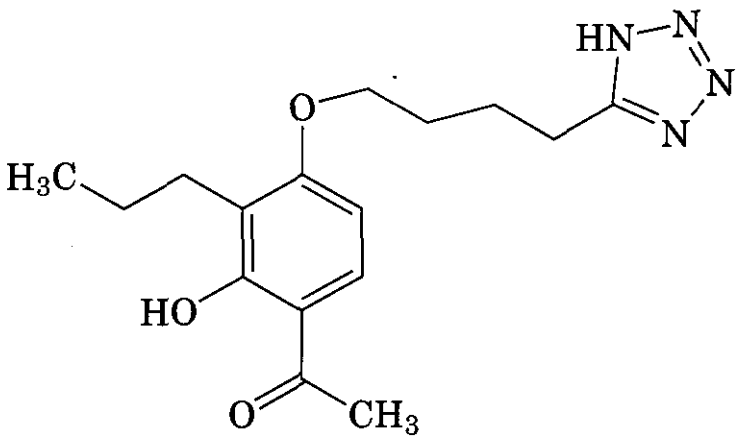


(28) FPL 55712

lead that they had discovered by broad screening of a set of antiallergic compounds against an SRS-A-based assay. This was accomplished before the structure of SRS-A had been assigned as a mixture of the cysteinyl leukotrienes. FPL-55712 served not only as a starting point for other chemists' efforts to develop specific CysLTl antagonists but also helped in the biological isolation and structure elucidation of the LTs.

6.1.2 FPL-55712-Derived Compounds

6.1.2.1 LY171883 (Tomelukast). The most significant of the early CysLTl antagonists that progressed to clinical evaluation was LY171883 (29, tomelukast). Using exploration of the SAR around FPL-55712, the group at Eli Lilly & Co. first simplified the carboxy-



(29) tomelukast (LY 171883)

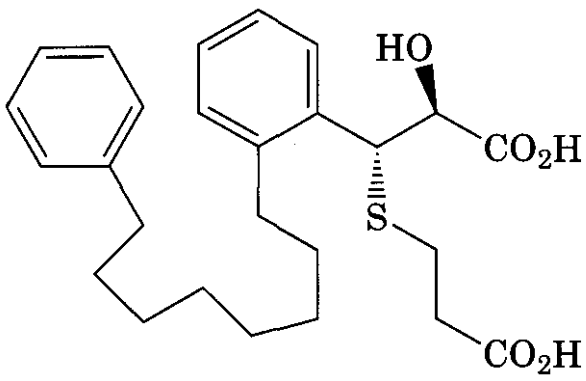
substituted chromanone, resulting in an analog containing an aliphatic acid chain. They then made the key discovery that replacement of the terminal carboxylic acid group with the bioisosteric tetrazole functionality afforded a significantly improved profile both in *vitro* and in *vivo* (208). Optimization of the chain between the tetrazole group and the hydroxyacetophenone group yielded LY171883.

In anesthetized guinea pigs LY171883 3–30 mg/kg p.o. given 2 h before challenge dose-dependently inhibited the increase in pulmonary resistance induced by i.v. LTD_4 (209). In conscious guinea pigs, aerosolized LY171883 was able to reverse the bronchoconstriction induced by either LTD_4 or LTE_4. Clinical trials with LY171883 were pursued to a Phase II trial in chronic asthma that, although showing small significant effects on lung function, was not deemed potent enough to proceed. Merck Frosst also worked in this area but did not find clinical candidates (2).

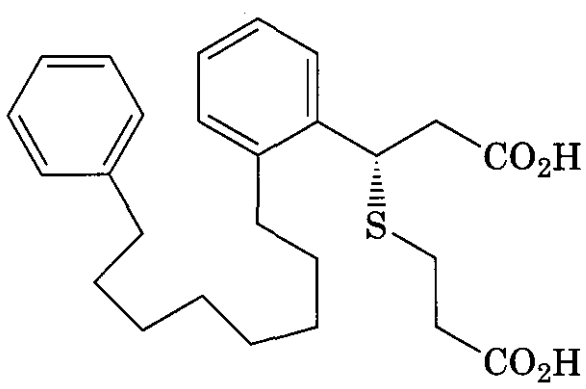
6.1.3 Leukotriene Analog Antagonists

6.1.3.1 SKF 104353 (Pobilukast) and SKF 106203. SKF 104353 (**30**) (210) and SKF 106203 (31)(211) were both developed by the SK&F group through the sequential modification of the structure of LTD_4 and were the first CysLT1 antagonist clinical candidates to be so derived (212).

Although SKF 104353 is about 10-fold more potent in *vitro* than SKF 106203, it has very poor oral bioavailabilitysuch that its clinical development was limited to aerosol formulations. In contrast, SKF 106203 had much better oral bioavailability and entered early clinical studies as an oral formulation. SKF 104353 is a potent, competitive inhibitor of $[^3H]LTD_4$, binding to both human and guinea pig lung membranes with K_i values of 12 and 5 n*M*, respectively (213). In functional assays it inhibited the actions of LTD_4 on human bronchus ($pA_2 = 8.2$) and guinea pig trachea ($pA_2 = 8.6$) (214). Similarly, in functional assays on these two tissues, using LTE_4 as the agonist, it afforded pK_β values of 8.9 and 8.2, respectively. SmithKline had some early clinical success with SKF 104353 but not sufficient to go to pivotal trials (205).

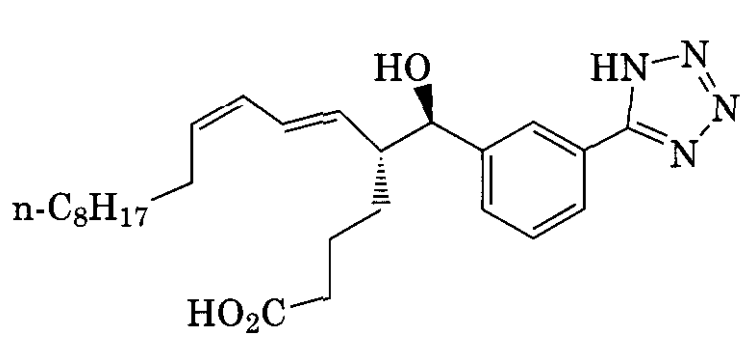


(30) SKF 104353 (pobilukast)



(31) SKF 106203

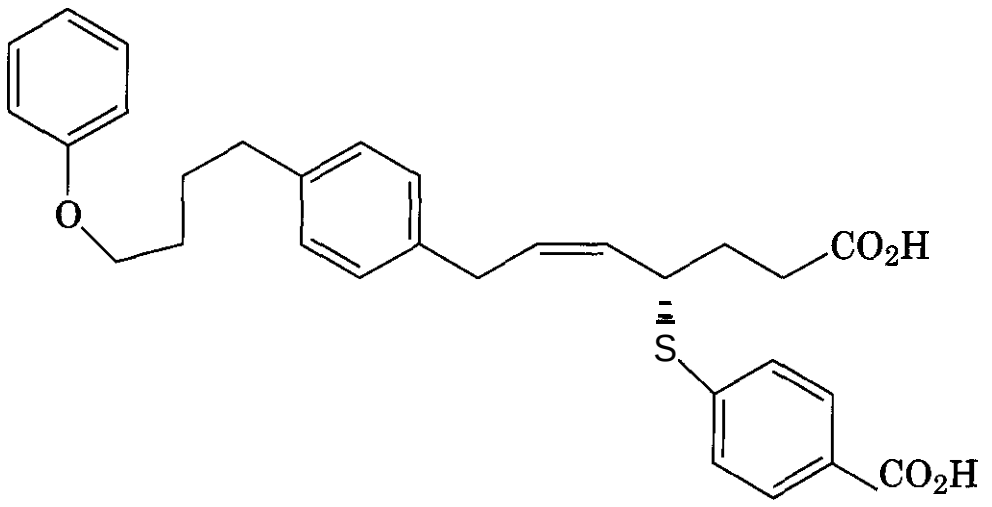
6.1.3.2 LY170680 (Sulukast). Concurrent with the efforts on FPL-55712 analogs at Eli Lilly's U.S. labs, a second team at their UK labs developed LY170680 (**32**), starting from



(32) LY 170680 (sulukast)

the structure of the natural agonists (215). This compound was also dropped from development after some antigen-challenge trials where the drug was given as an aerosol.

6.1.3.3 Bayer x7195. The sequence of steps taken by the Bayer group in going from leukotriene D_4 to Bayer x7195 (33)has been described in two articles (216,217). This interesting candidate incorporates several of the discoveries in LT analogs published earlier by other laboratories. For example, optimization of activity, particularly oral activity, required both shortening the linking group to the C_1-carboxylic acid and removal of the hydroxyl substituent, both precedented by the efforts at

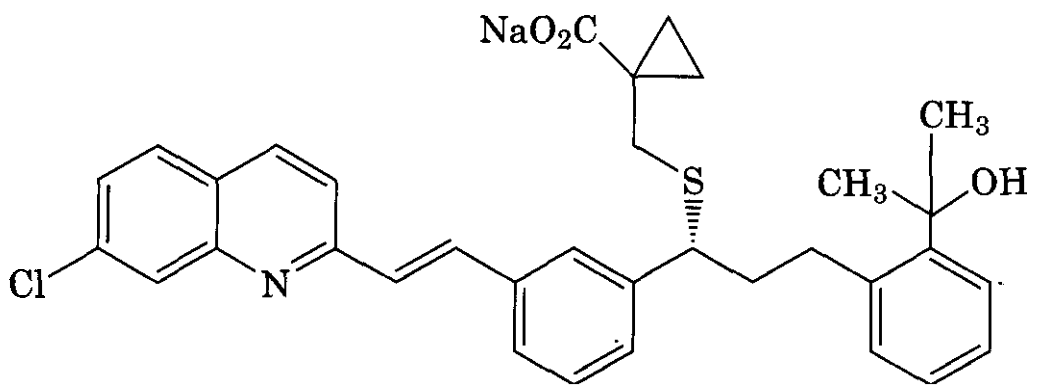


(33) Bayer x7195

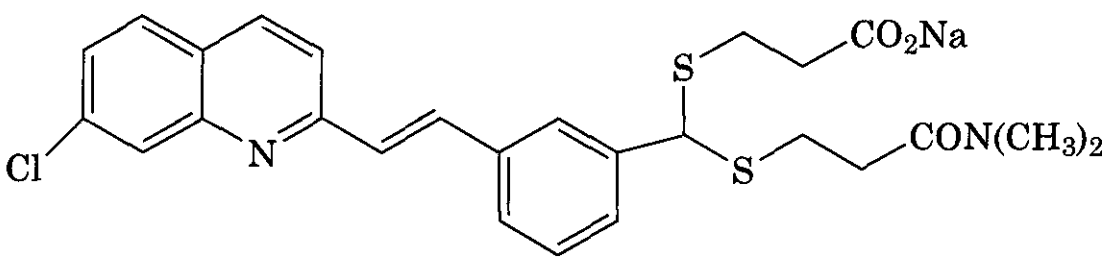
SK&F laboratories, that led to SKF 104353 and SKF 106203. Use of thepara-alkoxy-substituted homo-cinnamyl replacement for the triene backbone was also first described by the Zeneca team (218).

Bayer x7195 inhibits the binding of [^{3}H]LTD_4 to guinea pig lung membranes with a pK_i of 7.8 (219). In functional assays it had pK_β values of 8.4 and 8.2 against LTD_4-induced contraction in guinea pig trachea and human bronchi, respectively. *In* vitro, about 1 μM Bayer x7195 was also effective at blocking the antigen-induced contractions of trachea from sensitized guinea pigs and the anti-IgE response of human bronchi. In the anesthetized guinea pig, orally administered Bayer x7195 blocked LTD_4-induced bronchoconstriction (ID_{35} = 3 mg/kg) for at least 8 h. Topically administered Bayer x7195 was also effective in the guinea pig model, whether given by aerosol (ID_{35} ~ 200 ng) or as a powder (ID_{35} ~ 30 μg). Again, although the compound gave significant improvement in FEV, in Phase II trials in chronic asthma, the compound was not pursued to pivotal trials.

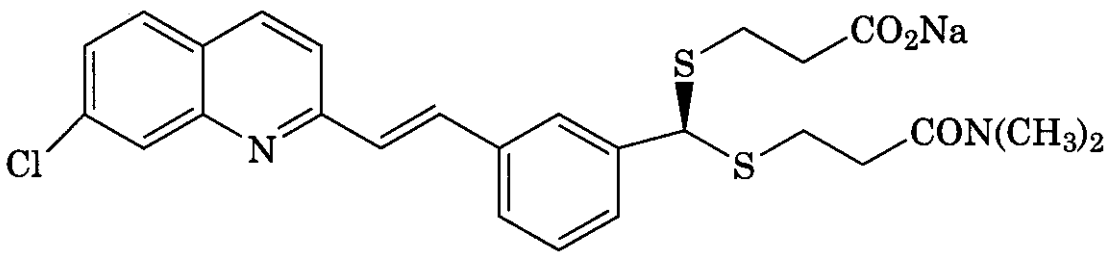
6.1.3.4 MK-0476 (Singulair, Montelukast Sodium, 34) and Its Precursors. Because the series of compounds exemplified by MK-0571, MK-0679, and MK-0476 (34–36) contain mimetics for the three regions of LTD, (lipid, acid, peptide) they may be viewed as leukotriene analog antagonists. However, unlike the



(34) montelukast sodium (MK-0476, Singular)

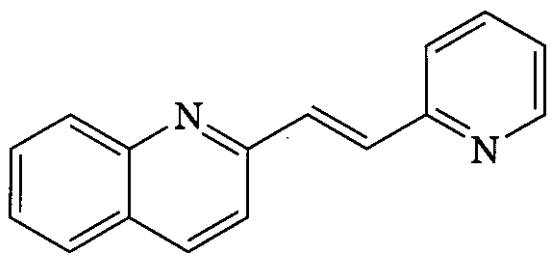


(35) MK-0571



(36) MK-0679 (verlukast)

other antagonists in this section, the starting point for these compounds was not the structure of the leukotrienes. Instead it was a styryl quinoline-containing compound (37) that was



(37)

found, by broad screening, to be a weak LTD_4 antagonist. This lead appears related to a series of quinoline-containing LTD_4 antagonists (220), derived from REV 5901, that are no longer in development and that are not covered in this review.

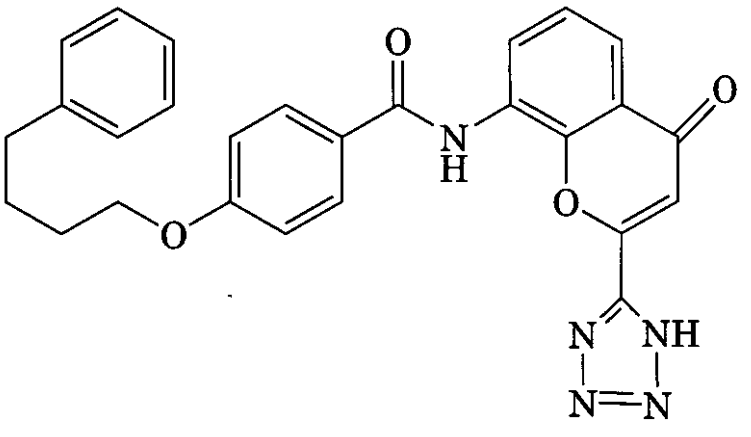
The chemists at Merck hypothesized that compound (37) was mimicking the conjugated olefin (lipid) region of LTD_4 and chose to add mimetics for the acid and peptide regions through the use of a dicarboxy-dithioacetal derivative first pioneered by the SK&F group. This change afforded a compound that displayed a tremendous increase in *in vitro* potency. However, the dicarboxy group appeared to be detrimental to oral efficacy. Replacement of one of the carboxyl groups with an *N,N*-dimethylamide improved oral efficacy and yielded MK-0571 (35) (221), which entered clinical development. This racemic compound was found to be a potent inducer of liver peroxisomal enzymes and was replaced in the clinic by its (*R*)-enantiomer MK-0679 (verlukast, **36)** that had similar LT antagonist activity but an improved safety profile.

In extended studies this compound also induced (limited) liver function abnormalities in animals and in the clinic. An extensive effort by the Merck group to find a compound in this series that was devoid of peroxisomal enzyme induction led to the discovery of MK-0476 (34, montelukast, Singulair) (222).

Montelukast is significantly more potent than either MK-571 or MK-0679 *in vivo*. An i.v. dose of 10 μg/ kg MK-0476 produced a 70-fold and a greater than 100-fold shift in the dose-response curves to i.v. LTD_4 in the anesthetized guinea pig, at 5 or 15 min pretreatment times, respectively. The i.v. $ED_{\_}$ at inhibiting the LTD_4-induced bronchoconstriction was estimated at 0.001 $\mu g/kg$. Montelukast had a long functional half-life after i.v. administration in the guinea pig. As discussed below, montelukast has been successfully tested in human asthma and approved for human use.

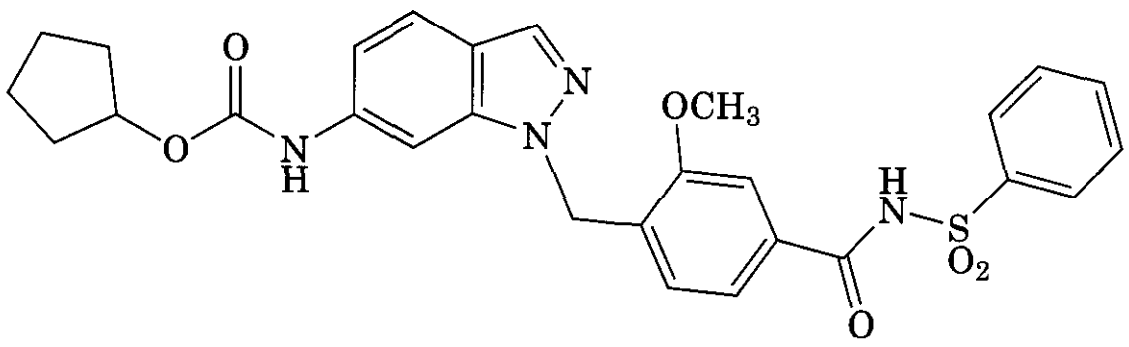
6.1.4 Peptide-Leukotriene Antagonists of Diversified Structure

6.1.4.1 ONO 1078 (Pranlukast). ONO 1078 (pranlukast, 38) is not a member of either the



(38) ONO RS-411, ONO 1078 (pranlukast)

FPL-55712- or the leukotriene D_4-derived sets of antagonists. It was developed from a weak lead, compound (19) (IC_{50} = 14 μM versus LTD, on guinea pig ileum). Replacement of the benzoic acid group with a chromanone carboxylic acid (similar to that found in FPL-55712) resulted in an analog that dis-



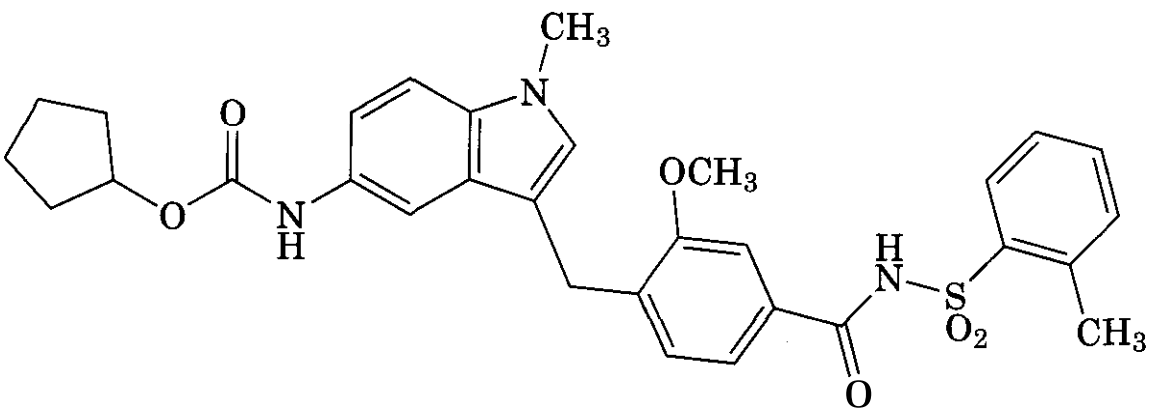
(39) ICI 198,615

played a 150-fold increase in potency *in uitro* (IC_{50} = 100 n*M*) and modest levels of *in vivo* activity (ID_{50} = 500 μg/kg in the guinea pig model). Similar to the discovery that led to LY171883, replacement of the carboxylic acid with a tetrazole yielded increases in both *in vitro* and *in vivo* potency (not illustrated). Optimization of the lipophilic alkyl tail then yielded ONO RS-411, the hemihydrate form of which has been clinically developed as ONO 1078 (pranlukast).

ONO 1078 inhibited the binding of [^{3}H]LTD_4, on guinea pig lung membranes with a K_i value of 46 n*M* (223). *In uitro*, it had pK_β values of 10.40 and 7.5 against LTD_4 on guinea pig ileum and trachea, respectively (233). It also inhibited the LTC_4-induced contraction of guinea pig ileum, with an IC_{50} value of 0.044 n*M* (224). *In vivo*, it was effective (ID_{50} = 1.0 μg/kg) against LTD_4-induced bronchoconstriction in guinea pig when administered i.v. 2 min before LTD_4 (225). ONO 1078, also effective against LTD, (5 ng), induced increases in vascular permeability in guinea pig skin with activity at 0.8 mg/kg p.o. and 0.1 mg/kg i.v. (225, 226). Antigen-induced bronchoconstriction in guinea pig was inhibited by ONO 1078 at a dose of 30 mg/kg p.o. (224).

6.1.4.2 ICI 198,615 and ICI 204,219 (Accolate, Zafirlukast). Several articles have reviewed the discovery of Zenaca's series of indole-based CysLT1 antagonists exemplified by ICI 198,615 **(39)** and ICI 204,219 (40, Accolate, zafirlukast) (227–229). These compounds were developed from a novel hybridization between a leukotriene analog and a hydroxy-acetophenone series of antagonists (230,231).

The first member of this series, ICI 198,615 **(39)** showed remarkable *in uitro* potency and selectivity as a CysLTl antagonist when it was disclosed. It inhibited the binding of [^{3}H]LTD_4 on guinea pig lung membranes with a pK_i value of 9.6 (232). On guinea gig trachea it had pK_β and pA_2 values against LTD_4 of 9.7 and 9.3 and against LTE_4 of 10.3 and 10.1, respectively. ICI 198,615 was also a potent antagonist of LTD, in human bronchi with a pK_a value of 9.2. It also showed *in vivo* antagonist activity in guinea pig in both the classic anesthetized pulmonary mechanics model and in a new conscious labored abdominal breathing model (233). Pharmacokinetic studies in dog and rat, however, showed poor oral bioavailability (<l%). ICI 198,615 was not developed further. Later studies that have shown ICI 198,615 (i.m.) reduces antigen-induced airway hyperresponsiveness in monkeys have con-



(40) zafirlukast (ICI **204,219, Accolate)**

firmed its importance as a standard and indicated yet another mode of action for such antagonists (234).

This team introduced ICI 204,219 (40) as its first broadly studied CysLTl antagonist clinical candidate (235–237). In vitro, this compound has an excellent profile that is similar to that shown by ICI 198,615. In human lung membranes it inhibited the binding of [^{3}H]ICI 198,615 with a K_i value of 3.70 ± 2.90 nM (n = 5). The pK_β values for inhibition of the LTD_4- and LTE_4-induced contraction of guinea pig tracheal strips were concentration independent and were, respectively, 9.52 ± 0.12 and 9.67 ± 0.30, at 3 nM ICI 204,219. The distinguishing characteristic of ICI 204,219 is its excellent in *vivo* profile. It had excellent bioavailability in rat (68%) and dog (67%). The overall profile of ICI 204,219 was such that its oral formulation was chosen for extensive clinical evaluation as Accolate (zafirlukast).

6.2 CysLTl Antagonist Clinical Studies

Inhalation of peptide leukotrienes has been shown to induce a reproducible, robust, well-tolerated bronchoconstriction in humans. Because of this, most clinical studies of CysLTl antagonists have begun with an assessment of their in vivo potency through measurement of their ability to inhibit LT-induced bronchoconstriction. Because these studies can be done over a broad time range, they can also be used to determine human pharmacodynamic values. The most frequent measure was the shift in the LT-induced dose-response curve that is produced by a specified dose of a drug at a particular pretreatment time. Friedman (238) has reviewed the pulmonary-oriented clinical results obtained with most of the early peptide leukotriene antagonists and lipoxygenase inhibitors.

LY171883 was the first CysLTl antagonist to be evaluated in broad clinical trials (239). It was eventually withdrawn from clinical evaluation as a result of both long-term toxicity seen in mice and induction of peroxisomal liver enzyme in rats (240,241). In spite of this, it was an especially important compound because it produced encouraging clinical results that appeared to confirm the hypothesis linking the cysteinyl leukotrienes to disease and thus acted as a strong spur to the development of other CysLTl antagonists.

Aerosolized SKF 104353, 100 and 800 μg, shifted LTD_4-induced bronchoconstriction by about 10-fold in normal and asthmatic individuals, respectively, when administered 2–3.5 h before challenge (242). The same dose protected against exercise-induced asthma (243). SKF 106203 was examined in normal volunteers and a 200 mg p.o. dose was effective in reducing LTD_4-induced bronchoconstriction, with the maximal effect being observed 8 h after dosing (244).

ONON (pranlukast, ONO-1078, ONO-RS-411 hemihydrate) was approved in Japan in 1995 and is the first CysLTl antagonist to be commercially available. However, it has not yet been approved in other countries.

The Merck group has entered the greatest number of CysLTl antagonists into clinical studies. Extensive trials were performed with MK-0571 (L-660,711), its single enantiomer MK-0679, and with MK-0476. This compound montelukast (Singulair) has been approved for clinical use in the United States and internationally for the treatment of asthma in adults and children (245).

Montelukast underwent extensive clinical testing. The studies used to support the clinical use of this agent have been reviewed in several venues (256–258). A daily oral dose of Singulair controlled asthma symptoms in 3- and 9-month extension trials. As with Zyflo the use of Singulair also allowed steroid tapering in moderate to severe asthmatics.

Zafirlukast formulated as Accolate was the first leukotriene modulator approved in the United States. This compound has also been extensively reviewed (256–258). Given 40 mg bid it has been shown to be clinically effective in the treatment of asthma.

Since the registration of Accolate, Zyflo, and Singulair (Table 5.2), most clinical studies have centered on the effects of these agents on inflammatory processes. The clinical trials that formed the basis for their approval demonstrated that these agents are safe and effective, and subsequent studies have also borne this out (251,252). These agents are especially effective in aspirin-sensitive asthma and exercise-induced asthma. They are also effective in treating the upper airway responses to IgE-

mediated immunological medial nasal congestion, although none of the agents has regulatory approval for the nasal indication.

Yanase and David-Bajar have reported that Singulair has a modest but significant effect in treating atopic dermatitis (253). Asero (254) has reported that in NSAID-induced uticaria that Singulair may also have an effect. Accolate has also been reported to have an effect in atopic dermatitis (255). In addition, Zyflo has shown efficacy in significantly improving the symptoms in a small open label study in atopic dermatitis (256). There have been, however, no large clinical trials to date to define the potential activity of these agents in the treatment of atopic dermatitis.

The major side effect that has been reported for the CysLTl antagonists is Churg-Strauss syndrome (257). A recent review of the literature suggests that Churg-Strauss is related to an underlying eosinophilic disorder in patients in whom corticosteriods use was being reduced, and thus not directly associated with the antagonists (257,258). Thus far, this syndrome has not been reported with Zyflo.

7 LEUKOTRIENE B_4 ANTAGONISTS

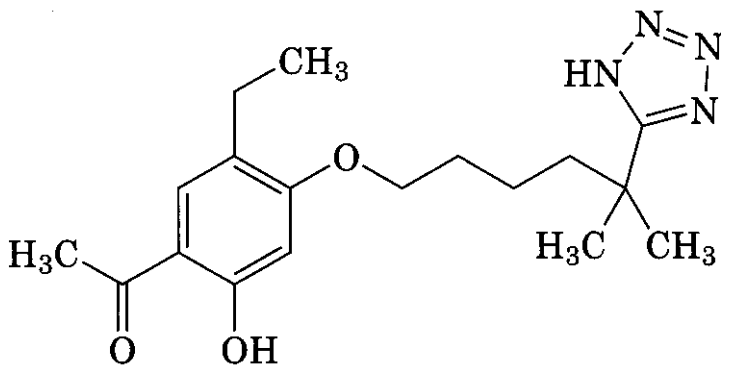
7.1 Introduction

Although the structure of LTB_4 was discovered at about the same time as the cysteinyl leukotrienes, the pace of development of LTB_4 antagonists has lagged significantly behind that of CysLTl receptor antagonists. This delay may be attributed to the fact that, unlike with the CysLTs (SRS-A), there was not, at first, a clear recognition of which pathophysiologies to associate with LTB_4. Also, as limited as the starting points for drug development were in the SRS-A area, they were even more limited in the area of LTB_4 antagonists. Most research teams began their efforts with the structure of LTB_4. Other teams started with known LTD_4 antagonists and hoped that, because LTB, and LTD_4 were biosynthetically related, some of these molecules would display weak LTB_4 antagonist activity. Several previous reviews on the development of leukotriene B_4 antagonists have been published (259–261) and, although clinical data on LTB_4 antagonists are much more limited than with the LTD_4 antagonists, this section focuses on the development of those compounds that have advanced into clinical studies.

The in vitro and in vivo activities of LTB, antagonists have been evaluated by use of several different assays. Binding assays have been developed that measure the inhibition of binding of [^{3}H]LTB_4 to guinea pig lung membrane (262)and to isolated human neutrophils (263). Functional activity has been assayed through inhibition of the LTB_4-induced contraction of guinea pig lung parenchyma (262) and through the inhibition of the LTB4-induced up-regulation of CD11b/CD18 expression in human neutrophils. In vivo, in guinea pigs or cynomolgus monkeys, inhibition of LTB_4-induced neutropenia and interdermal neutrophil migration have been examined.

7.2 LTB, Antagonists Related to HAP-Type LTD_4 Antagonists

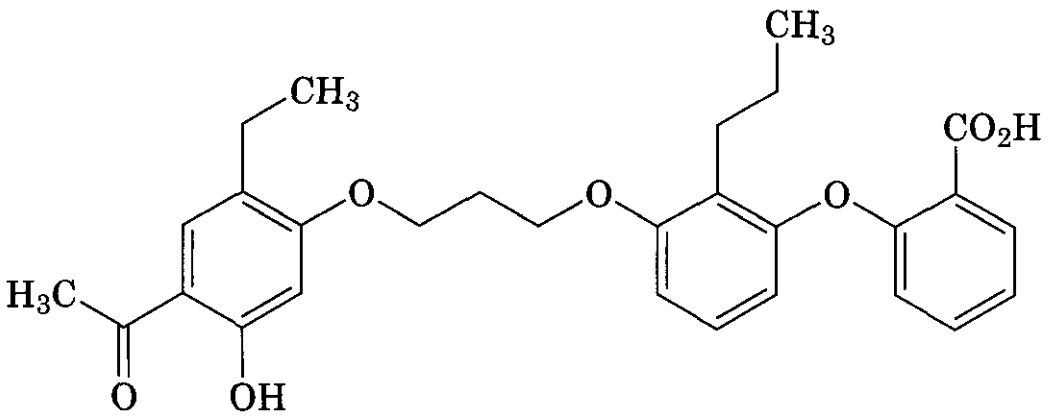
A team at Eli Lilly discovered that moving the alkyl chain from the hydroxyacetophenone (HAP) 3-position, as found in FPL-55712 (**28**), resulted in selective LTB, antagonists. For example, LY255283 (41) inhibited LTB_4 binding



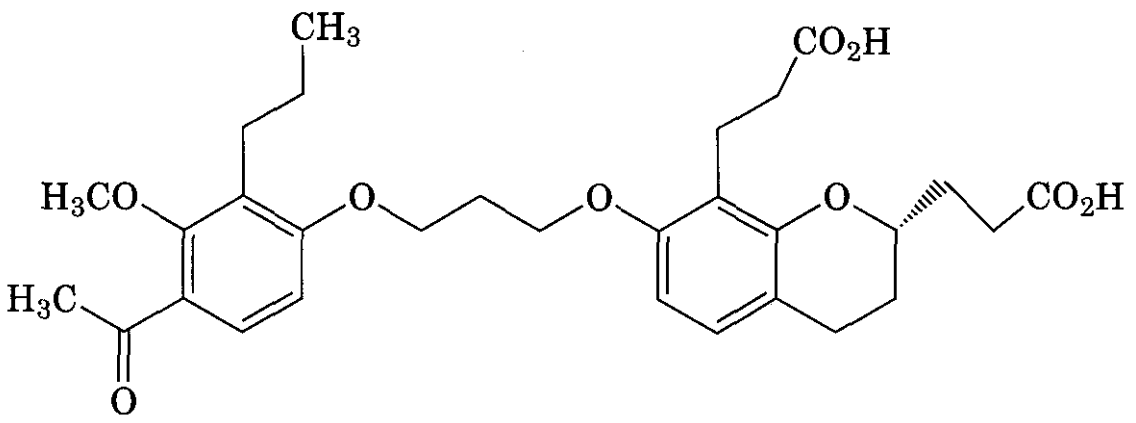
(41) LY 255283

to neutrophils with an IC_{50} value of 87 n*M* (264). Further work in this class yielded LY293111 (**42**), which was active in blocking LTB_4-induced bronchoconstriction in vivo (265). A publication on LY293111 has appeared describing its pharmacology in vivo (266). Unfortunately, the compound was not effective in clinical trials in asthmatics.

The Searle group apparently started with FPL-5512 analogs and arrived at SC 53228 (**43**), which antagonized LTB_4 binding with an



(42) LY293111

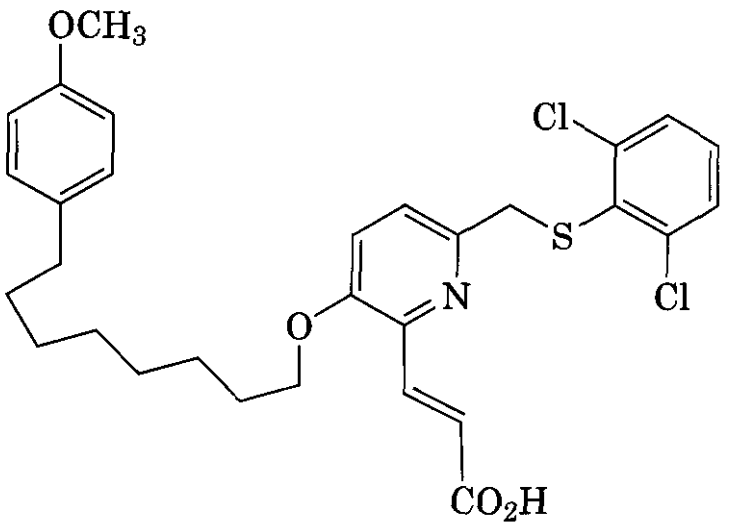


(43) SC 53228

IC_{50} value of 1.3 n*M*. The compound was also active in blocking PMA-induced ear edema (<2.5 mg/kg).

7.3 LTB_4 Antagonists Derived from the Structure of LTB_4

A number of agents have been reported based on the structure of LTB_4. The early pharmacology was previously reviewed (2). The compounds that have advanced furthest are ONO-4057 (**46**) and two compounds from SmithKline Beecham, SB 201993 (44) and SB 209247 (**45**). Early ONO compounds used a variety of groups to replace the triene in LTB_4 **(267).** The diacid ONO-4057 (**46**) had an IC_{50} in binding assays of 15 n*M* and was active in the guinea pig in LTB_4-induced neutropenia. SB209247 was also orally active in rodents (268) and was taken to clinical trials (2). CGS-25019c (**47**), also taken to phase I trials, was unique compared to earlier discussed compounds in that it had an amidine group functionality instead of an acidic group (269). More recently, a series of prodrugs of an amidine group were described by Boeringer Ingelheim (270). The lead compound from this series,

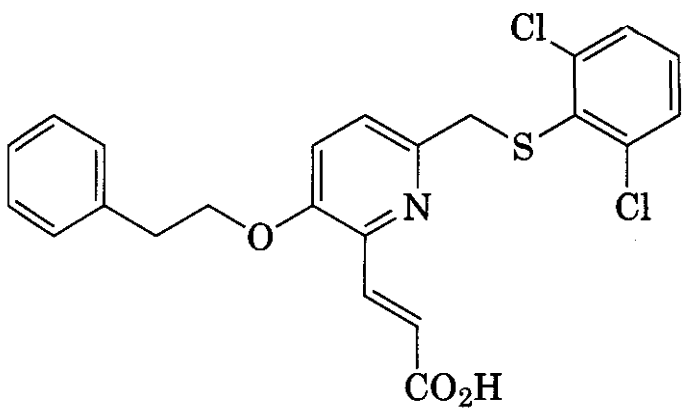


(44) SB 201993

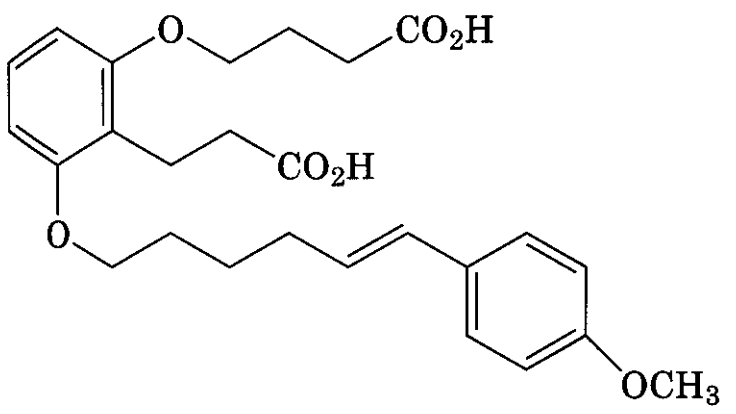
BIIL 284 (48) was longer acting in monkeys than either CGS-25019c or LY 293111.

7.4 Clinical Trials with LTB_4 Antagonists

Clinical success has not yet been achieved with LTB_4 antagonists. It is unclear whether this is because LTB_4 is only one of many leukocyte chemoattractants in inflammation or whether a significant portion of the biology of LTB_4 is directed by binding to BLTR2. Several compounds have been in phase I trials (2) but no



(45) SB 209247



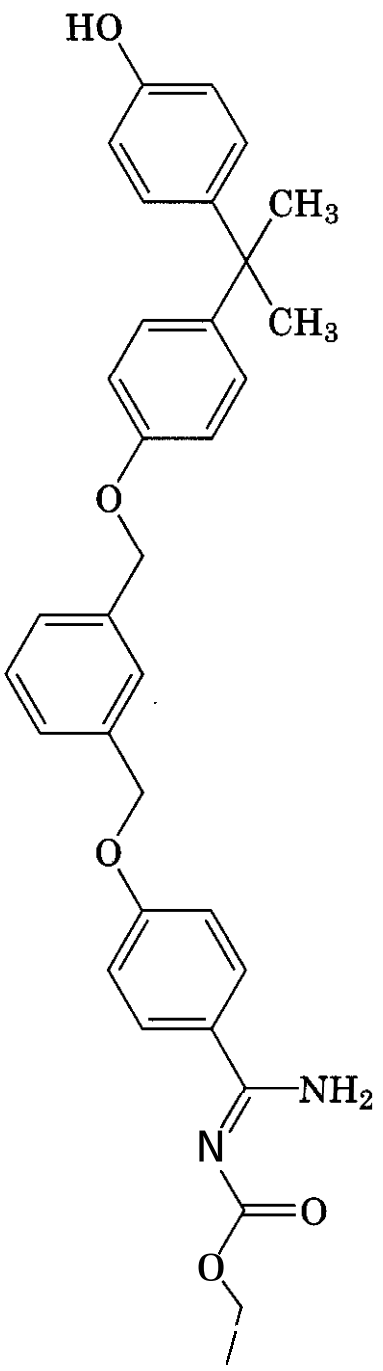
(46) ONO-4057

substantial success has been reported and no compounds have been registered. Attempts in asthma and psoriasis have been deemed marginal or unsuccessful (271–272).

8 BIOCHEMISTRY AND STRUCTURAL BIOLOCY OF CYCLOOXYCENASES

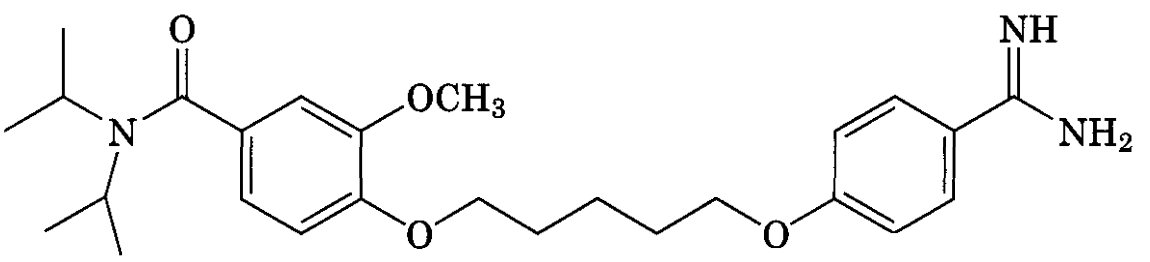
8.1 Prostaglandin Discovery and the Connection to NSAIDs

Prostaglandins are the earliest of the eicosanoids to be discovered. Originally described by von Euler in the late 1940s as the active principal components in semen that induced uterine contractility (273), the structures of the most important of these (PGE_2, PGF_{α}, PGD_2) were elucidated in the 1960s by Bergstrom, Hamberg, Samuelsson, and others



(48) BIIL 284

(Fig. 5.3) (274). The multiple activities of these substances were the topic of intense research in the 1960s and 1970s, where roles in reproductive, cardiovascular, pulmonary and renal physiology, and pathophysiology (pain, inflammation, cancer) were identified. The discovery of thromboxane (275) and prostacyclin (276) came later and expanded the role of the eicosanoids as powerful substances in human physiology.



(47) CGS 25019C

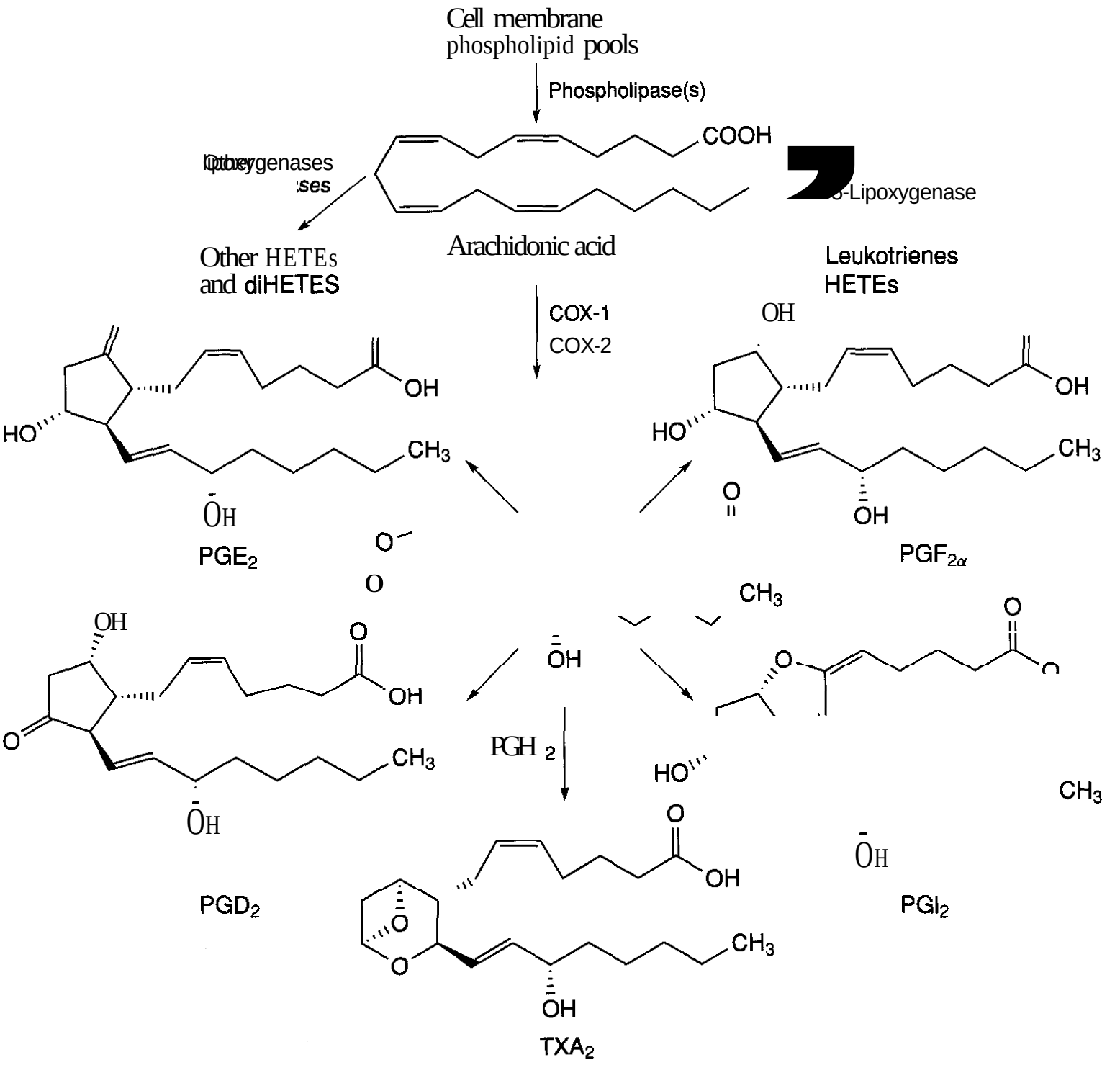


Figure 5.3. Cyclooxygenase pathway.

In a parallel fashion, albeit starting much earlier, the discovery of **nonsteroidal** anti-inflammatory drugs (NSAIDs) were derived from early **folk** medicine use of natural substances, the active agent of which was ultimately discovered to be aspirin in the 1800s. Once the negative gastric side effects of aspirin were established around 1940, a number of synthetic agents were developed **with** the goal of achieving safer drugs. These drugs were eventually classified as NSAIDs, in contrast to steroids, the other major class of anti-inflammatories then available.

Assignment of NSAIDs as cyclooxygenase inhibitors and thus prostaglandin inhibitors, was made in the early 1970s by Vane and colleagues using classical tissue strip pharmacology (277). This discovery was elegantly followed by **Majerus** et **d.,** who identified the amino acid site of the covalent **acylation** of platelet cyclooxygenase by aspirin (278).

8.2 Cyclooxygenase 1

Cyclooxygenase 1 (COX-1) is the common name used to describe the protein prostaglandin H **synthase** 1 (**PGHS-1**), which performs both **peroxidase** and cyclooxygenase reactions to make PGH, the precursor to prostaglandins E, D, **F2$_\alpha$ prostacyclin,** and **thromboxane A,** (see Fig. **5.3).** Originally described as an

enzymatic activity in seminal vesicles, it was purified in 1977 by two groups (279). The active enzyme is a homo-dimer with subunits of 74,000 Da. As described earlier (2), the dimer containing two noncovalent heme moieties initially interacts with endogenous peroxide where a two-electron reaction occurs. A one-electron translocation provides the tryosyl radical required for interaction with arachidonic acid. When the cyclooxygenase site is occupied by arachidonic acid, the 13 pro-S hydrogen is extracted to yield an arachidonyl radical. The fatty acid radical then reacts with molecular oxygen to form the 11-hydroxyl radical, which subsequently cyclizes a C_{11} to C, endoperoxide moiety, interacts with a second molecule of O_2, and ultimately produces PGG_2. As described in more detail (280) the tyrosyl radical can cycle through the peroxidase site if no arachidonate is available. Although reasonably well understood and accepted, there are still controversies in the PGH mechanism (281). Subsequent to purification efforts, cyclooxygenase 1 was cloned first from sheep (282) and then from other species. Expression of COX-1 occurs in most if not all mammalian tissues and cells in culture (283, 284). The crystal structure of ovine COX-1 was solved in 1994 and allowed rigorous interpretation of numerous biochemical and site-directed mutagenesis work (285). Important aspects of the crystal structure are discussed below in comparison to COX-2.

8.3 Cyclooxygenase 2

As described above, the first described cyclooxygenase was purified and characterized in the late 1970s. This work provided the molecular framework for connecting the biochemistry of the enzyme studies predominantly in seminal vesicle preparations and in the platelet, with the biology that had expanded to include all major organ systems and most cell types. In the 1980s, however, several groups began to obtain data inconsistent with the characteristics of the cyclooxygenase in platelets and seminal vesicles. In particular, hormonal control of prostaglandins in cells such as fibroblasts and macrophages (286,287) and *in vivo* (288,289) were inconsistent with a single enzyme. These publications proposed two pools of cyclooxygenase, one static and unresponsive to steroids and the other inducible and inhibited by steroids such as dexamethasone. Some investigators also reported increased enzyme concentrations in response to growth factors by Western blot with antibodies thought to be specific for COX-1 (287).

The discovery of a second cyclooxygenase enzyme initiated an explosion of research. This research showed that COX-2 was distinguished from COX-1 by a slightly larger molecular mass, rarity of mRNA expression, and induction by growth factors and cytokines (280). Further work has yielded information with significant detail in the biochemical, cellular, pharmacological, and structural differences between the two enzymes.

8.4 Structural Features of COX-1 and COX-2 Enzymes

A large body of structural biology information on the two cyclooxygenases, COX-1 and COX-2 (PGHS-1 and PGHS-2) now exists. Overall, the data indicate a remarkable degree of similarity between the enzymes. They differ in length by only 11 amino acids, with COX-2 being slightly larger. The differences in sequence are predominantly in the membrane-binding domain. The overall structure of the human and mouse COX-2 is superimposable on the ovine COX-1 structure (280). Both enzymes are dimers with three structural domains: an N-terminal EGF domain of approximately 50 amino acids, a membrane-binding domain of about 50 amino acids, and a large C-terminal globular domain that contains the catalytic domain. The dimer interacts with the membrane in a novel way, associating with the inner leaflet but not penetrating the entire membrane, as supposed before the crystal structure of COX-1 was elucidated (285).

The nature of the active site. how substrate is bound, and in particular how inhibitors bind in the two active sites have been extensively studied (280, 290). As COX-2 was discovered, the availability of the COX-1 crystal structure allowed for models, particularly site-directed mutagenesis, to explore differences in the two active sites. Early modeling studies indicated clearly that the first layer of amino acids in the active site were largely conserved, with the exception of valine^{509} (291). A few differences also occur at the next "layer" of amino acids

surrounding the active site. Good evidence for the importance of the valine residue comes from mutation of that residue causing changes in the selectivity of agents (292)and from crystal structures with SC-558 (51c), where it was found that this inhibitor "wedges" the sulfonamide group into a hydrophobic side pocket that is inaccessible in COX-1 (292). Movement of the valine and insertion of sulfonamide or methyl sulfone moieties common to many selective COX-2 agents may be critical for the selectivity seen for these agents. Structures with inhibitors and using nitroxide-labeled enzyme show clearly that the active site is modified by inhibitor and substrate binding (293). Indeed, structural flexibility may be important for catalysis (293). Recently, the crystal structures of COX-2 with arachidonic acid and PGG, (294) in the active site have been determined. In particular, the structure with PGG_2 is supportive of earlier biochemical and modeling data, illustrating again the similarities between the two enzymes.

8.5 Biosynthesis of Prostaglandins

Prostaglandins are made by nearly every organ system, tissue, and cell in the body. Under normal conditions this is predominantly driven by COX-1. This enzyme is broadly expressed and is constitutively active (290). Under homeostatic conditions, prostaglandin formation is controlled by substrate availability and perhaps peroxide tone (295). Most cells and tissues tightly regulate free fatty acid concentrations to be very low. The chief enzymes responsible for this are the fatty acid transacylases, which rapidly reacylate free fatty acid into either phospholipids or triglycerides. Agents that stimulate production of prostaglandins from COX-1 have primarily been agents that cause a calcium spike inside cells. In contrast to COX-1, expression of COX-2 is normally limited in the body to specific cells in kidney, brain, and pancreas (290). In general, COX-2 must be induced for substantial concentrations of prostaglandins to occur (290). A plethora of inducers have been reported and fall into several classes including cytokines, growth factors, and hormones (296–298). COX-2-dependent prostaglandin production thus occurs over hours rather than minutes, in contrast to COX-1-driven eicosanoid production that requires only seconds or a few minutes. Additionally the expression of COX-2 enzyme is usually the rate-limiting step for COX-2-driven eicosanoid formation, not substrate release.

Several groups have explored the molecular details of how COX-2 expression is induced. It appears that agents that induce COX-2 expression do so by both inducing COX-2 mRNA production as well as stabilizing the mRNA. Recent work by Song et al. indicates that COX-2 expression is normally silenced through a hypermethylation mechanism (299). Induction of COX-2 mRNA has been reported to occur through IL-1 mediation, ceramide-dependent MAP kinases, p38 MAP kinase, and IκB kinases (296–298,300). Recently, the PEA3 family of transcription factors as well as NFκB p65 have been shown to increase COX-2 mRNA levels (301). PPAR γ has been shown to suppress LPS induction of COX-2 in macrophages (302). In addition, p53 has been shown to be a transcriptional inhibitor, explaining the expression of COX-2 in tumor cells that in many cases lose p53 (303). Associated with COX-2 expression in tumors is the report that k Ras, a protein associated with tumors, increases the stability of COX-2 mRNA (304). The anti-inflammatory activities of salicylate and corticosteroids are at least partly attributable to suppression of COX-2 expression by suppression of transcription and mRNA destabilization, respectively (288).

In several cellular systems, higher levels of prostaglandin production have been associated with COX-2 expression. In some cells (e.g. WISH) expression of both enzymes yields prostaglandin production from only COX-2, as shown by specific inhibitors (Hulkower and Bell, unpublished observations, 1996; 305). In addition COX-1 appears to prefer exogenous arachidonic acid, whereas with endogenous substrate COX-2 metabolism is predominant (298). These phenomena have three possible explanations: *(1)* specific linkage of each enzyme with a specific phospholipase, (2)linkage of COX-2 to an inducible PGE, synthase, and **(3)** regulation by peroxide tone.

A number of enzymes have been linked to arachidonic acid release and subsequent prostaglandin formation. Types II, V, and IV PLA_2s have been linked to prostaglandin re-

lease as well as phospholipase Cldigyceride lipase and phospholipase D (306). The enzyme responsible for release of substrate may be stimulus and cell type dependent. In the mast cell early phases of AA release are dependent on a phospholipase different from that in the secondary phase (307). A number of workers have suggested specific phospholipase activation coupled to either COX-2 or COX-1 (308,309).

Very recently, two laboratories have made progress in identifying the enzymes responsible for PGE_2 production from PGH_2. Two enzymes have been identified. The first is a cytosolic, constitutively expressed enzyme and appears linked to COX-1 (308,310). The other enzyme is microsomal in location, is inducible, and appears linked with COX-2 PGE_2 production (310–313). The importance of the inducible PGE_2 synthase in control of the relative contribution of COX-1 versus COX-2 PGE_2 production remains to be thoroughly understood but has significant potential, perhaps even as a new drug target.

Finally, a third molecular mechanism for the difference between the two COX enzymes' turnover and activation rate has been proposed. Elegant kinetic studies show clearly that the two enzymes differ in the peroxide reaction kinetics of stabilization of the active cyclooxygenase species. The interpretation of these studies is that a lower level of peroxide is required for COX-2 turnover than that for COX-1. Practically, this means that under some cellular conditions COX-1 is silent, whereas COX-2 produces prostaglandins in significant amounts (291).

A final aspect of cyclooxygenase biosynthesis of prostaglandins is the cellular location of the enzymes. A number of investigators (280, 290) have described a preference for COX-2 to localize to nuclear membranes compared to COX-1, although this is cell and stimulus dependent. Recent publications have also described COX-2 location in lipid bodies and in caveoli (314).

8.6 Actions of Cyclooxygenase-Derived Eicosanoids

8.6.1 Homeostatic Effects of Prostaglandins.

A large number of organ systems are influenced by prostaglandins. These include cardiovascular, gastric, kidney, and reproductive organ systems. Recent research, taking advantage of the availability of selective COX-2 inhibitors and reagents differentiating the expression of the two enzymes, has begun to detail the relative contribution of the two enzymes in these organs and their function.

The clearest function is in the gastrointestinal (GI) tract, where prostaglandins clearly play a protective role, as evidenced by the superior safety of COX-!&selective agents and the protective effects of misoprostal on the GI lining. The primary drawback of traditional nonselective agents is in the gastric system, where reduction of protective prostaglandins causes ulceration, bleeding, and occasionally death.

Cardiovascular effects of prostaglandins are more complex. The coagulation system is clearly modulated by platelet-derived thromboxanes, which have procoagulation effects and the anticoagulative effects of endothelial cell-derived prostacyclin. Thromboxanes are clearly COX-1 derived because platelets do not express COX-2. The source of endothelial cell prostacyclin production is less clear with both enzymes expressed and mixed opinions on the relative contribution of the two enzymes. Recent results with Vioxx, a selective COX-2 inhibitor, imply a modest effect on myocardial infarction rate in patients taking the compound but do not define whether this is caused by a change in ratio simply by a lack of platelet effect or that inhibition of endothelial cell prostacyclin also skews the pro- and anticoagulant ratio.

Prostaglandins regulate renin-angiotensin secretion and thus glomerular filtration rate and sodium homeostasis. These effects appear to be COX-2 driven (315). The kidney is a rare organ, one that expresses COX-2 under nonpathological situations. Expression in the loop of Henle apparently drives prostaglandin formation in the kidney and the subsequent physiological responses. Thus a selective agent would likely have similar negative effects on kidney function as those of the nonselective NSAIDs. This is apparently the case with both Vioxx and Celebrex (315).

Prostaglandins play important roles in each step of mammalian reproduction. These prostaglandins appear to be COX-2 derived, as

delineated by COX-2 null mice (316). Thus the reproductive effects associated with NSAIDs would be expected to occur with COX-2-selective agents as well.

8.6.2 Pathophysiological Effects of Prostaglandins. Nonselective COX inhibitors, the NSAIDs, have long been used as anti-inflammatory, antipyretic, and analgesic agents. Studies in animals and in humans show clearly that selective COX-2 inhibitors such as rofecoxib and celecoxib are as effective in these areas as earlier nonselective agents. These results strongly indicate that the majority of the prostaglandins causing inflammation, pain, and fever are COX-2 derived.

Older nonselective agents and more recently new selective COX-2 inhibitors have been shown to have anticancer effects in animal models and some human experience (317). These studies imply effects of prostaglandins on several stages of cancer progression. Early studies have shown prostaglandins to be important as modulators of cell proliferation (318) and, not unexpectedly, COX-2 inhibitors have shown pro-apoptotic effects (319). More recently, expression of COX-2 has been shown to be cell cycle dependent in human fibroblasts (320) and associated with G1 delay in intestinal epithelial cells (321). Even earlier in cancer cell progression than tumor cell growth, COX-2 inhibitors block carcinogenesis in animal models (322). Finally, COX-2-selective agents block later stages of tumor growth such as angiogenesis (323). Most significantly, it was shown recently that overexpression of COX-2 in mice was sufficient to cause tumorigenicity (324).

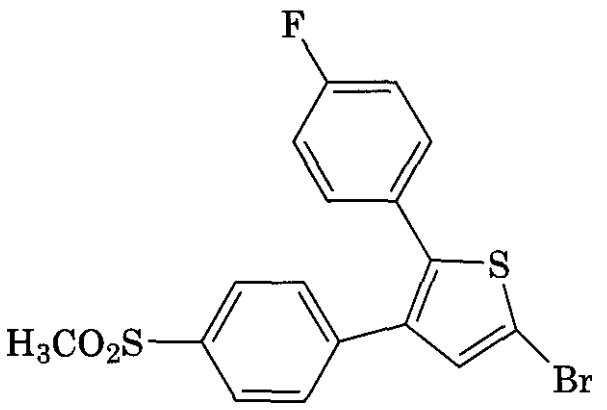
9 SELECTIVE COX-2 INHIBITORS

9.1 Agents Inhibiting COX-2

The hypothesis that a selective COX-2 inhibitor would be safer and therefore provide an attractive alternative to NSAIDs has been validated by the clinical successes of Celebrex and Vioxx. Both of these agents are from the largest or most studied class of COX-2 inhibitors, although other classes have been identified and each has characteristic kinetic and thermodynamic parameters describing a mechanism for cyclooxygenase inhibition. Several reviews have addressed various aspects of the COX-2 story (428), a very recent review (386) and two minireviews or summaries (398,399) are cited within the following discussion on this section.

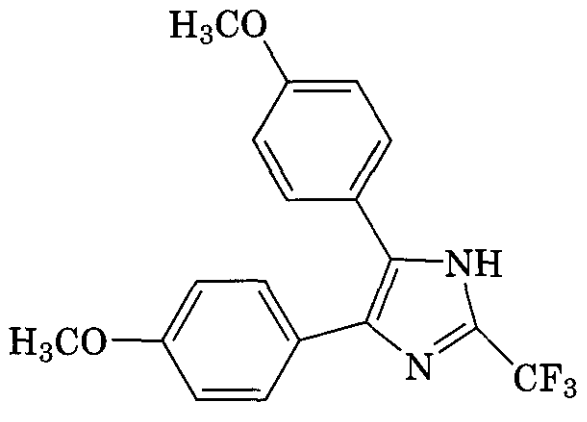
9.2 Structure-Activity Relationships

9.2.1 4-Sulfonylphenyl COX-2 Class. The most investigated area of selective COX-2 inhibitors is the 4-sulfonylphenyl super genus or family, to which both Celebrex (celecoxib) and Vioxx (rofecoxib) belong (325, 326). Often referred to as the "tricyclic," "diaryl," or "*cis*-stilbene" class, the lead structures for this general class were a series of known diaryl anti-inflammatory agents. The most referenced lead structure appears to be DuP 697 (49) (327).



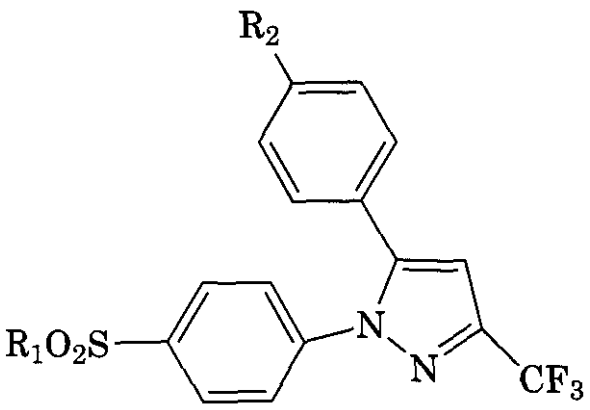
(49) DuP **697**

The development of this compound was in part driven by the observation of less gastrointestinal irritation in animal models. A structural representative of this early class of anti-inflammatory agents, predating DuP 697, is illustrated by flumizole (50) (328). After the discovery of the inducible COX isoform, analogs having a methanesulfonylphenyl moiety



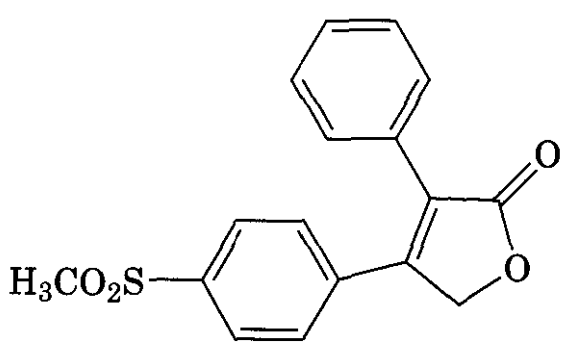
(50) Flumizole

as one of the aryl rings were quickly segregated as a subclass having high COX-2 selectivities. The 4-methylsulfone or 4-sulfonamide-substituted phenyl ring is the hallmark of this class of selective COX-2 inhibitors. Subsequent crystallographicstudies of the protein with inhibitors have provided the rationale for this structural requirement (285, 329–331). Initial studies showing that mouse and human enzyme proteins were quite similar were followed by an X-ray structure of SC588 (**51c**) cocrystalized with mouse COX-2 (329). These studies revealed an extra side pocket created by an isoleucine/valine[523] switch and that this new "nook" apparently is engaged in binding of the methylsulfone moiety and provides an opportunity for COX-2 selectivity. This site is the most prominent difference in the COX-1 vs. COX-2 active-site comparisons. This difference provides a key design element of increased steric bulk to augment COX-2 selectivity. SAR studies reported for celecoxib (**51a**) and rofecoxib (52) illustrate many general characteristics of this class.



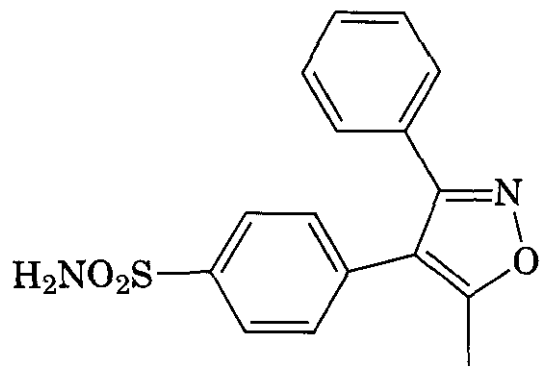
(51) Searle Series

(51a)	$R_1 = NH_3$, $R_2 = CH_3$	Celecoxib
(51b)	$R_1 = CH_3$, $R_2 = Cl$	SC-263
(51c)	$R_1 = NH_3$, $R_2 = Br$	SC-588
(51d)	$R_1 = NH_3$, $R_2 = F$	SC-58451
(51e)	$R_1 = CH_3$, $R_2 = F$	SC-58125



(52) Rofecoxib (MK 966)

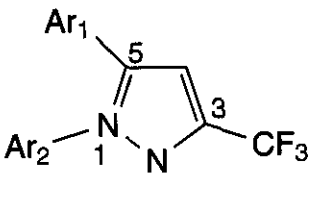
The common pharmacophore shared by these two structures and this class consists of two vicinal substituents being attached to adjacent sp^2 atoms of a five-membered heterocycle or carbocycle. One of the two must be a 4-sulfonylphenyl moiety and the other is often a second substituted phenyl ring. This concept has been applied very successfully to many heterocycles and confirmed the generality of this scaffold or template model for this class. Analogs using the 1,5-diarylpyrazole (51, 57) of celecoxib (**51a**) as a scaffold as well as related 3,4-diarylpyrazoles (332), 1,2-diarylpyrroles (**63,** 64, Table 5.1), 1,2-diarylimidazoles (59,Table 5.1) (334), 4,5-diarylimidazoles (58) (335), 4,5-diarylthiazoles (**60**) (336, 337), 4,5-diaryloxazoles (332, 338), and 3, 4-diarylisoxazoles (valdecoxib,SC-65872, Bextra, 53)have



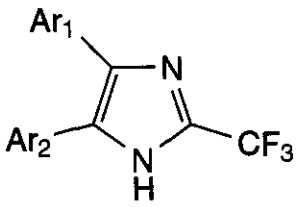
(53) Valdecoxib SC-65872

been thoroughly studied (339,340). In addition to the 2(5*H*)-furanone scaffold of rofecoxib (**52**), related cyclopentenone (341), 3(2*H*) furanone (342), and 1,5-pyrrolin-2-ones (343) have also been reported as closely related series based on carbonyl-containing heterocyclic templates or scaffolds. Other five-membered heterocycles have been developed, including3,4-diarylthiazolin-2-one (344), 4,5-diaryloxazolones (345,3461, diarylthiadiazole (347), and diaryltriazole (348). Other core scaffolds or templates have also been developed from modified five-membered heterocycles that have been fused to a second cyclic ring (349351). Many examples of these heteroaryl-bicyclic templates have been reported, including FR-228352 (**54**), reported to be in phase I testing (340). Six-membered heterocyclic scaffolds have also been shown to be effective and include pyridine (352) [etoricoxib, (55) (353)], pyridazinone (354), and pyranone (355).

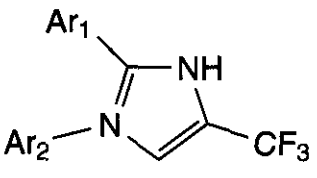
Table 5.1 COX Activity/Selectivity of Selected Inhibitors



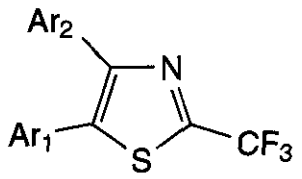
57 a, b



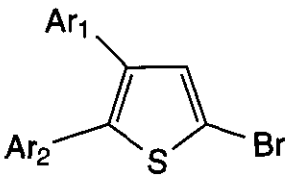
58 a, b



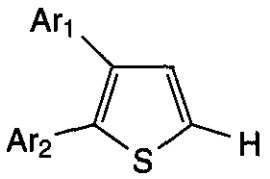
59 a, b



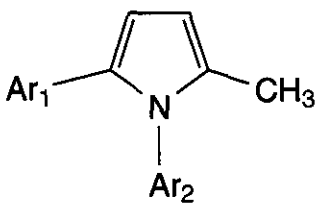
60 a, b



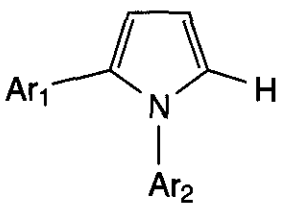
61 a, b



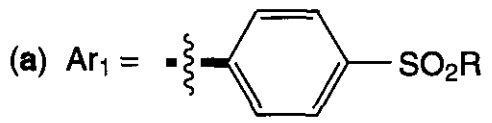
62 a, b



63 a, b



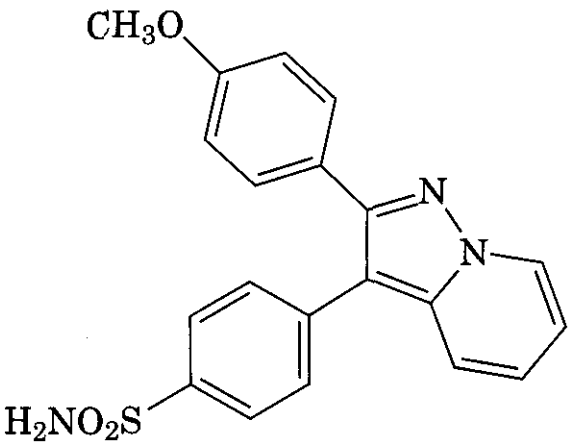
64 a, b



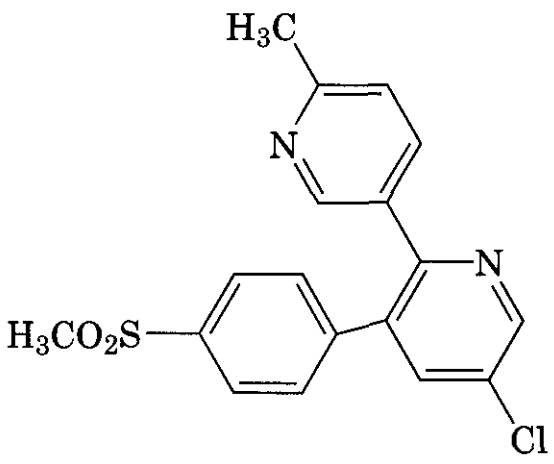
Heterocyclic Template	COX-1 (μM)	COX-2 (μM)	R	Reference
(**57a**)	25.5	0.41	NH_2	325
(**57b**)	0.08	0.01	NH_2	325
(**58a,b**)	>100	0.19	CH_3	335
(**59a**)	>100	5.85	CH_3	334
(**59b**)	36	0.1	CH_3	335
(**60a**)	>100	0.023	CH_3	337
(**60b**)	1.07	0.006	CH_3	337
(**61a**) (DuP 697)	0.6	0.005	CH_3	364
(**61b**)	1.1	0.02	CH_3	364
(**62a**)	>100	0.25	CH_3	364
(**62b**)	>50	4.3	CH_3	364
(**63a**)	>100	0.06	CH_3	333
(**63b**)	>100	>100	CH_3	333
(**64a**)	>100	0.51	CH_3	333
(**64b**)	>100	10.2	CH_3	333

Five- and six-membered carbocycles have also been incorporated as efficient scaffolds. Examples using phenyl (known as terphenyls) (356, 357), cyclopentene (356, 358–362), cyclopentadiene (359), cyclobutenone (363), and naphthyl (356) cores or scaffolds have been described. The cyclopentene series was optimized to a high degree, producing many very potent and selective COX-2 inhibitors with good *in vivo* activity (e.g., 56, SC-57666).

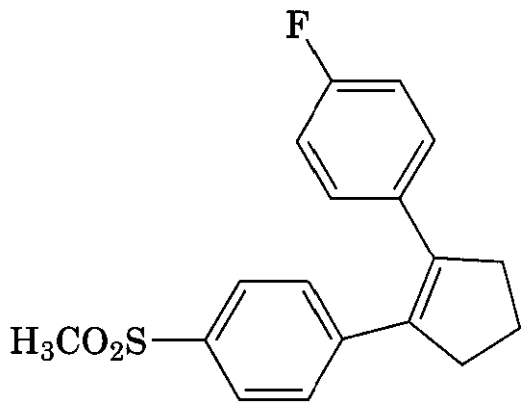
Other structural elements on the scaffold may be relevant in relation to the regiochemistry of the two key adjacent substituents on the template. Minor changes in positioning the two aryl rings can result in dramatic differences in selectivity between two very similar isomers on the same template. Table 5.1 shows data from several analogs where the relative positions of the two key substituents have been interchanged on a given template. In the 3-trifluoromethylpyrazole template, or celecoxib series, the 4-sulfonylpheny moiety must be attached in a 1,3 relationship relative to the 3-trifluoromethyl substituent of the pyrazole, as in both (**57a**) and (**57b**) (Fig. 5.4). Both of these isomers are potent COX-2 inhib-



(54) FR-228352



(55) etoricoxib (MK-0663)



(56) SC-57666

itors. One isomer (**57a**) has superior selectivity. The isomer with the 4-sulfonylphenylring in the 5-position and F-phenyl in the **1-position** (**57b**) had higher affinity for COX-2 but was less selective because of a dramatic simultaneous increase in COX-1 potency.

The **4,5-diaryl-3-trifluoromethylimdazole scaffold** produces a selective COX-2 inhibitor (**58**); these isomers have the same 1,3 relationship of the 4-methanesulfonylphenyl and the 3- CF, substituents. Scaffold symmetry eliminates further analysis, given that the isomers with respect to the imidazole NH cannot be resolved. The 1,2–4-trifuoromethylimidazole (**59a**, 59b; Fig. 5.4) shows a somewhat different result in terms of selectivity relative to the **1,5-pyrazole** of the celecoxib series (**37a**, **37b**). The isomer (**59b**), which is more potent at both isoforms, turns out to be the more selective because of an increase in affinity at COX-2. When applied to the **4,5-thiazole** (**60a**, **60b**) scaffold, the **4-sulfonylphenyl** moiety adjacent to the nitrogen of the heterocycle, (**60a**), is much more selective than the other (**60b**) (Fig. 5.4) isomer by virtue of a much weaker COX-1 activity and little change in the COX-2 potency. Reported SAR for the **thiophene** series reveals a much smaller difference in potency and overall COX-2 selectivity between isomers (**61a** and **61b**). This template

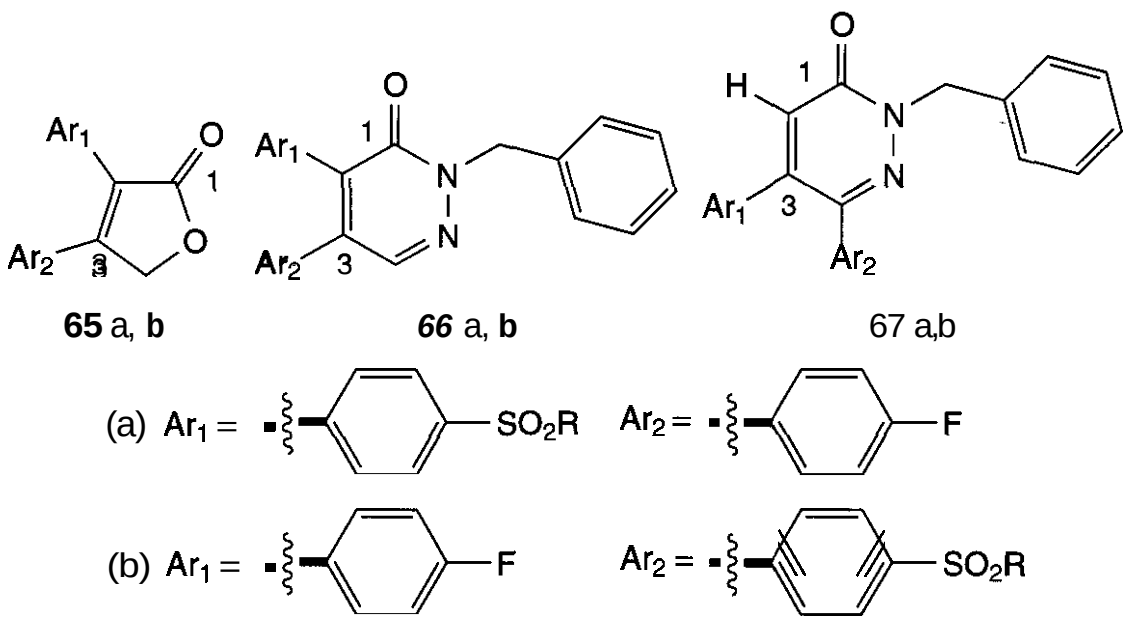


Figure 5.4. **Carbonyl** containing heterocyclic templates.

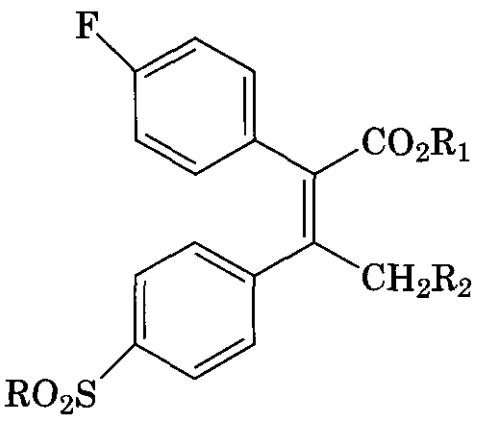
has the sulfur heteroatom as well as the 2-bromo substituent as structures introducing template asymmetry, but shows little preference with respect to the relative 4,5 position of the 4-methylsulfonephenyl moiety. For comparison, in the thiophene template without the 2-bromo substituent (**62a, 62b**) an approximately 2 log shift to the right in COX-1 potency is observed but only about a 10-fold shift in COX-2 potency for the "a" isomer (**62a**), which has the most "useful" selectivity of the 4-thiophene compounds shown. Of the two possible isomers of the 5-methyl pyrrole scaffold, the "a" isomer (**63a**) has good COX-2 selectivity, with the "**b**" isomer (**63b**) being one of the few analogs completely inactive at COX-2. The unsubstituted pyrrole (**64a, 64b**) exhibits a similar trend, with the a isomer having superior activity at COX-2 and therefore being a selective agent, although the relative increases are dramatically different from what was observed in the methyl-substituted template (63).

A related phenomenon is the similar requirement of a 1,3 relationship of the 4-sulfonylphenyl ring and a structural feature of the scaffold. In the rofecoxib series, the relative position of carbonyl of the furanone is critical.

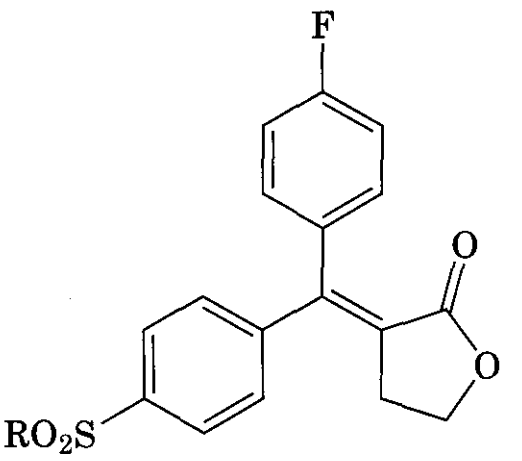
The analog (**65a**) (Fig. 5.4), where the relative position of the 4-sulfonylphenyl ring and the carbonyl are 1,2, is inactive at both isoforms; the reverse orientation, (**65b**), is of course the highly selective rofecoxib template. Similar to the rofecoxib series, the 4,5-disubstituted pyridazinones (365) show a required 1,3 relationship of the sulfonylphenyl moiety and the template carbonyl. Of the possible four isomers, only two analogs (**66b** and **67a**) (Fig. 5.4) are potent selective COX-2 inhibitors.

Broader variations on the scaffold model have been reported in the patent literature. A prodrug of the rofecoxib furanone template has been reported (366). The cis-stilbene-like structure (68) can undergo metabolic oxidation to produce the selective 2,3-diary furanone. A variation on the 1,2 vicinal substitution is the reported analog (**69**), with geminal positioning of the two aryl rings off the exocyclic olefin of the butyrolactone template (367, 368).

The rationale for these related phenomena, involving the critical relative positions of the



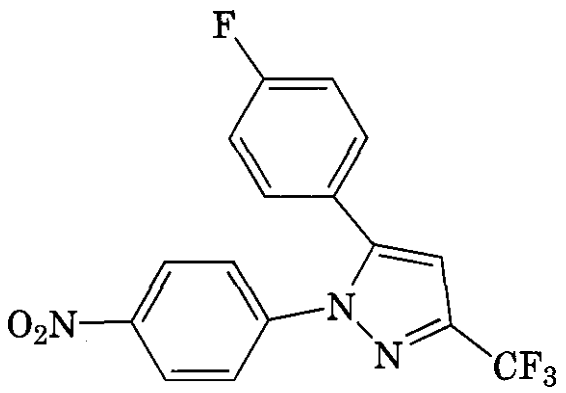
(68)



(69)

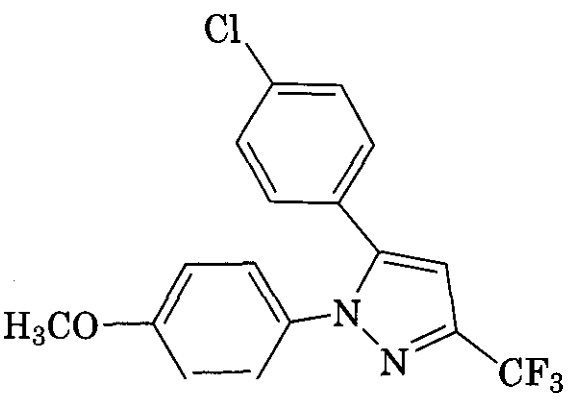
two key aromatic substituents on the scaffold, is currently not well developed. Although X-ray structures and modeling techniques have provided valuable gross features of the enzyme, to date a large number of X-ray structures with bound ligands have not been available. The scaffold model does seem valid, based on the wide range of heterocycles that have been used and, with a few exceptions (see 65a and the other non-1,3-carbonyl analogs 66a and **67b**), most produce potent COX-2 inhibitors with less potent COX-1 activity.

One of the only successful modifications for the methylsulfone moiety has been the unsubstituted sulfonamide. Both sulfonamide and sulfone analogs can also be quite potent against COX-1, however. The extra isoleucine/valine[523] space is limited, even in COX-2, and the SAR for this substituent is very restrictive. For example, the N-methyl sulfonamide in the celecoxib series was inactive (325). Reversal of the N and SO, atoms gave an inactive compound. The introduction of other strong electron-drawing group replacements for sulfonyl like nitro or $COCF_3$ lead to inactive compounds at COX-2. The 4-nitro analog (70) did



(70)

have some COX-1 activity (IC_{50} COX-1, 1.75 μM) (325). Only a few highly COX-1-selective inhibitors have been reported, which are often found as part of SAR analysis of sulfonyl replacements. Examples of very selective COX-1 analogs from the pyrazole (71) (325) and thiophene (72) (364) series have been identified.



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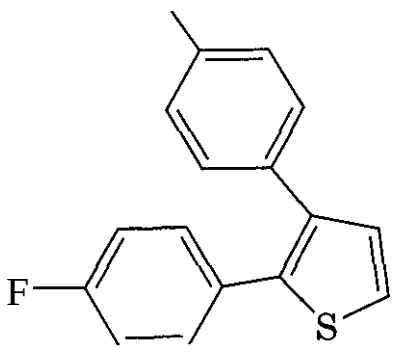
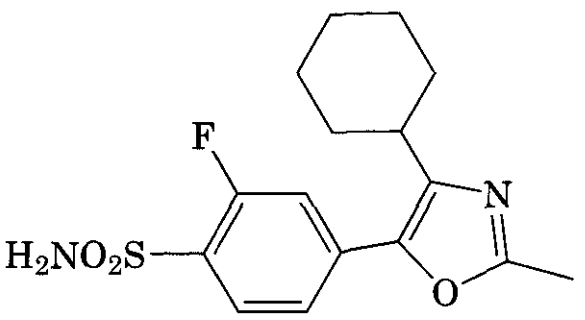


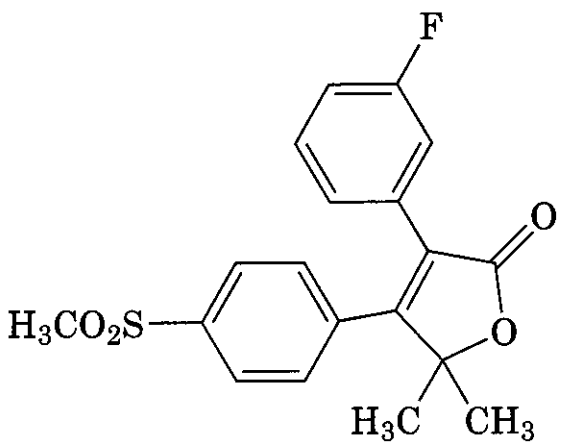
(72)

A significant empirical trend has been identified by comparison of direct sulfonamide and sulfone analogs. The sulfonamide analog is generally more potent but this usually applies to both isoforms. In many cases the COX-1 potency is often disproportionately increased, leading to a decreased selectivity ratio (332, 359–361,364). The sulfonamide can have superior bioavailability and physicochemical properties that are manifested in greater in *vivo* efficacy in the anti-inflammatory models. Celecoxib has incorporated the sulfonamide as a balance of selectivity and in vivo efficacy. The JTE-522 compound (73) introduced a fluorine *ortho* to the sulfonamide and reported the compound with this modification regained COX-2 selectivity more characteristic of the methylsulfone analog (369, 370).



(73) JTE 522

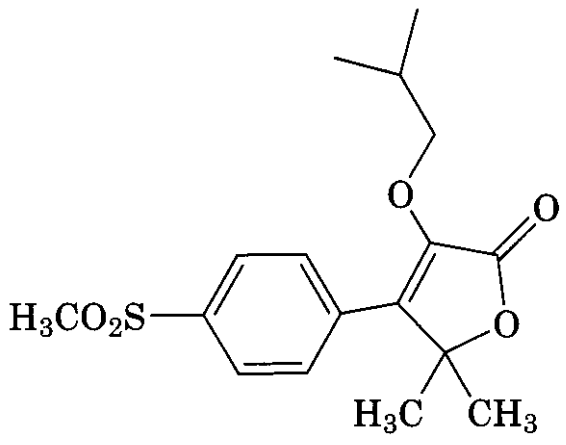
In contrast to the restrictive nature of allowed modifications for the 4-sulfonylphenyl substituent, a wide variety of structures can be incorporated to replace the second phenyl ring. 4-Halogen-substituted phenyl is the most used reference substituent for this position. The early Searle candidate, SC-263 (**51b**) with a 4-Cl substituent was found to have an unacceptably long half-life, which can also be characteristic of this class. In celecoxib (**51a**), introduction of the para-methyl group on this ring, as a site for metabolic oxidation, reduced the rat plasma half-life from 221 h for SC-263 (**51b**) to 3.5 h for celecoxib (**51a**) (325). Clinical trials with DuP 697 also identified its extremely long half-life of 292 h as a development issue (371). In general halogen and small alkyl groups are well tolerated on this ring; stronger electron-donating groups, such as methoxy, are tolerated in the 4-position as well. Other halogen effects on this ring are noted in the rofecoxib derivative DFU (**74**) [the name DFU appears to be an acronym related to the 5,5-dimethy-2(5*H*)-furanone scaffold] (372, 373). Improved selectivity can in part be attributed to the introduction of the *ortho* fluorine atom into the non-sulfone-bearing phenyl ring. Dihalogen substitution also frequently gave compounds with a 1000-fold in vitro selectivity (325). SAR studies indicate



(74) DFU

many heterocycles to be bioisosteric, with phenyl for many templates (337, 352, 374). The traditional, older diaryl classification or terminology to describe this class has been supplanted by demonstration that the non-4-sulfonylphenyl vicinal substituent can be a number of lipophilic as well as polar nonphenyl substituents. JTE-522 (73) was one of the first widely referenced analogs with a cyclohexyl ring in place of substituted phenyl (369).

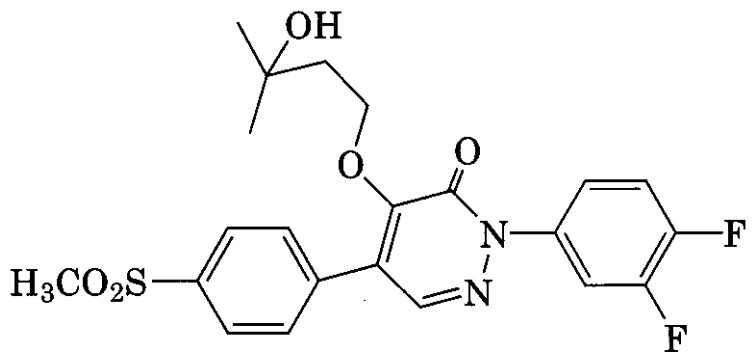
DFP (75) (DFP is also a possible acronym indicating propyloxy substitution on the di-



(75) DFP

methyl-furanone scaffold), a potent analog of DFU, introduced methylpropoxy subtitution for phenyl and maintained COX-2 selectivity and potency (375). In addition to alkoxy, thioalkoxy and aminopyridyl substitution adjacent to the sulfonylphenyl ring using a pyridine ring central scaffold were reported to produce potent COX-2 inhibitors (376). The presence of these polar side chains was proposed to improve oral bioavailability through improved absorption. Arylpyridazinones have also been shown to tolerate 3-methylbutoxy and 2-methylpropoxy substituents adjacent to the phenylsulfonyl substituent (354). Even more polar substituents, such as alkoxyhydroxy substitution, have been reported to maintain good COX-2 selectivity in the pyridine scaffold series (376).

The Abbott compound ABT-963 (76)has a 3-hydroxy-3-methylbutyloxysubstituent adja-



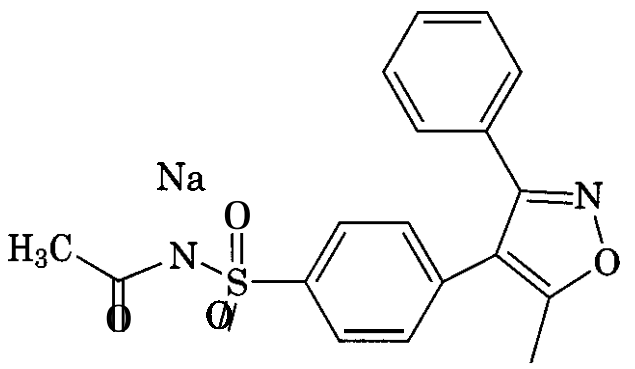
(76) ABT-963

cent to the 4-methanesulfonylphenyl moiety and shows improved selectivity as well as exhibiting superior solubility, protein binding and pharmacokinetics (365). Additional ternplate or scaffold substituents, beyond the well-studied 4-methanesufonylphenyl and its 1,2 vicinal partner, are limited and are more specific to the template or scaffold. In the celecoxib series the 3- CF, pyrazole substituent appears optimized, although a wide variety of substituents were tolerated (325). Several substituents, including hydroxymethyl, cyanomethyl, benzyloxymethyl, 4-methoxyphenyl, and 5-chloro-2-thienyl in the 3-position, were shown to maintain potency and selectivity comparable to those of the CF, analog. The hydroxymethyl-substituted analog was active, similar to a reported hydroxymethyl metabolite of valdecoxib that was isolated and reported to have a potent selective COX-2 activity as well as oral anti-inflammatory activity (339). Some minimal size or specific electronic nature of the 3-pyrazole substituent appears to be important, given that the 3-methyl analog of celecoxib displays very weak COX-2 activity and the 3-unsubstituted analog is completely inactive (>100 μM at COX-2). This example illustrates one of the weaknesses of this heteroaryl template (347) general strategy.

A small change in the template can render it a poor scaffold. The 1,3 relationship of the

3-trifluoromethyl substituent and the 4-methanesulfonylpheny substituent on the pyrazole ring is an example of an optimized template. In an attempt to survey a large number of five-membered templates to find a replacement for the thiophene template, the unsubstituted 4,5-diaryloxazole was discarded for lack of potency (347). The 4,5-diaryl-2-methyl oxazole is a template that produces selective analogs and is found in JTE-522 and, like the 1,5-diarylpyazole scaffold of celecoxib, requires an additional small substituent for maximun activity. A series of 1-arylpyrrolin-2-ones (343) employs an additional aryl substituent on the scaffold similar to the pyridazinones (354).

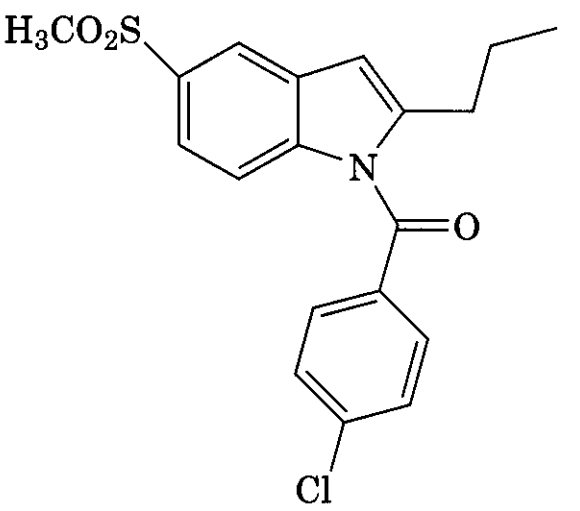
This class of COX-2 inhibitors is generally lipophilic and lacks water solubility; the formulation of a parental or i.v. administrable COX-2 inhibitor has been addressed by Pharmacia in development of a water-soluble prodrug of valdecoxib (53), known as parecoxib sodium (77) (340, 377).



(77) Parecoxib Sodium

This compound is derived from acylation of the sulfonamide moiety of valdecoxib, followed by treatment with sodium hydroxide. The acidic acylsulfonamide forms a water-soluble sodium salt that is stable for administration but cleanly delivers valedecoxib once in plasma.

Related methanesulfonylheteroaryl inhibitors (78 and 79) from Chugai are reported in the patent literature (378). These compounds may be interacting in a manner similar to that of the methanesulfonylphenyl inhibitors, although they do not fit the general model because there is no substituent adjacent to the sulfonyl group on the aromatic scaffold. The direct attachment of the methanesulfonyl group to a nonphenyl ring appears to be novel. Both cores reported are indole derived and



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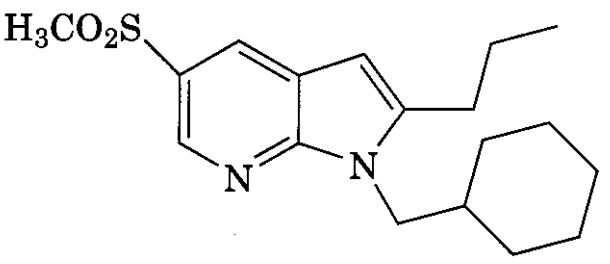
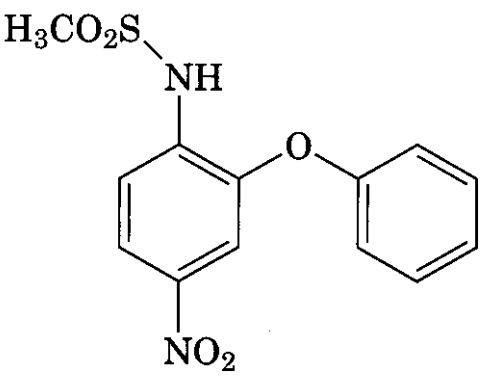


(79)

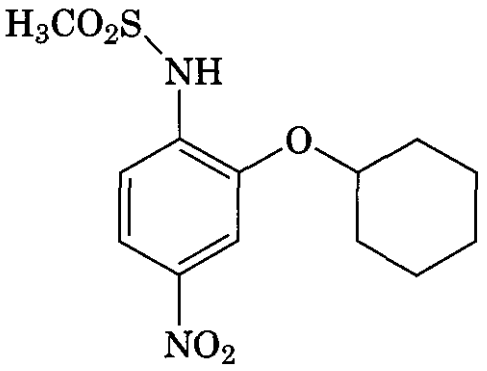
this compound (79) is reported to be 33-fold selective for COX-2.

9.2.2 *N*-Arylsulfonamides. Lead structures for this class of selective COX-2 agents were also compounds developed as anti-inflammatory agents before the discovery of the COX-2 isoform. Nimesulide (R-805, 80) was identified

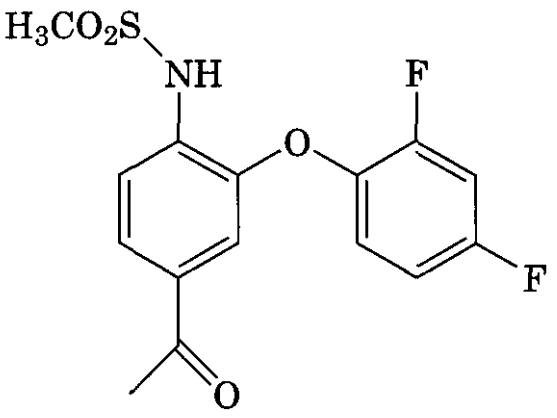


(80) nimesulide **(R-805)**

as an NSAID anti-inflammatory drug with weak prostaglandin synthetase activity (COX-1) but potent in *vivo* in the carrageenan-induced edema model (379). An analog of nimesulide, from Taisho, NS-398 (81) was shown to be a selective COX-2 inhibitor (380). This class has a characteristic acidic proton by virtue of the N-arylsulfonamide or methanesulfonanilide. A related analog, FK-3311 (83), incorporated a

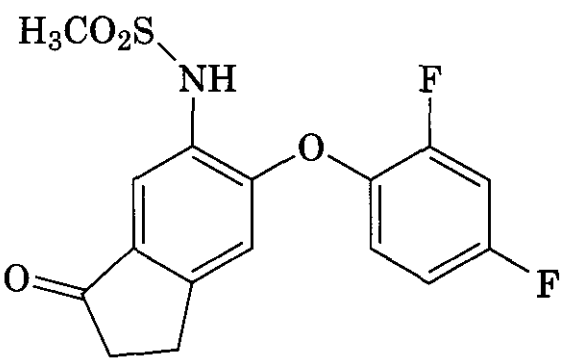


(81) NS-398



(83) FK 3311

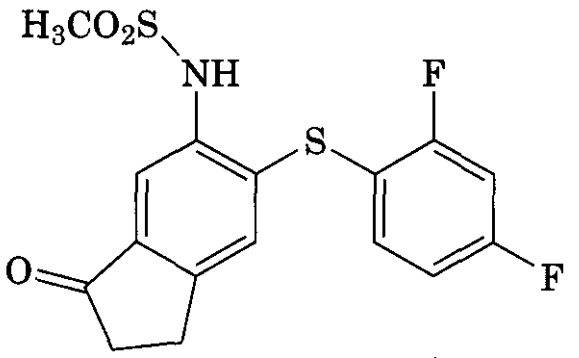
methyl ketone as an alternative to the nitro electron-withdrawing group and introduced 2,4-difluoro substitution into the phenoxy ether ring (381). This series was expanded to show that thiophenoxy ethers were as potent as the oxygen analogs. Flosulide (GP-28238, 84) incorporates a difluorophenoxy substitu-



(84) Flosulide (CGP 28238)

ent *ortho* to the sulfonanalide and introduces a modified electron-withdrawing keto group, as part of a conformational restricted fused cyclopentanone (382).

Flosulide was shown also to be a selective COX-2 inhibitor, less potent than NS 398 but with better pharmacokinetic properties (383). Flosulide was used as a lead for development of L-745,337 (85), which is the direct thio-

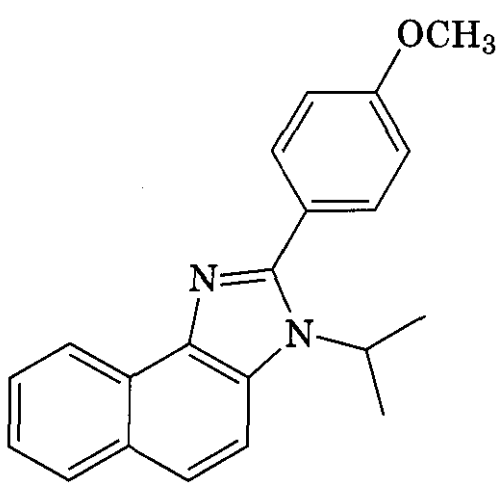


(85) L-745,337

ether congener of Flosulide. L-745,337 is more active than Flosulide in the rat paw edema *in vivo* assay (383,384). The SAR for this series is reported to be very limited with cyclohexyl, thiazole, and to a lesser extent pyridyl being some of the very few substituents that can replace the phenoxy ring (385). This restrictive SAR may also explain the lack of further reported development of this series.

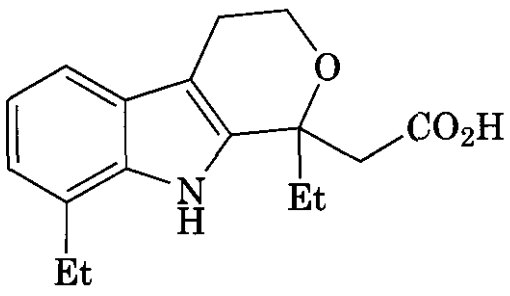
9.3 Selective NSAIDs and NSAIDs Modified for Improved COX-2 Selectivity

Given the homology between the two cyclooxygenases, the reexamination of known anti-inflammatory agents, with particular interest in reported PGHS inhibitors, is an area of research regenerated by the identification of the inducible COX-2 isoform. Some of the most selective NSAIDs, such as diclofenac, have COX-1/COX-2 selectivities in about the threefold range (386). However, tomexiprole (86) was reported to have 30-fold COX-2 selec-



(86) tornoxiprole

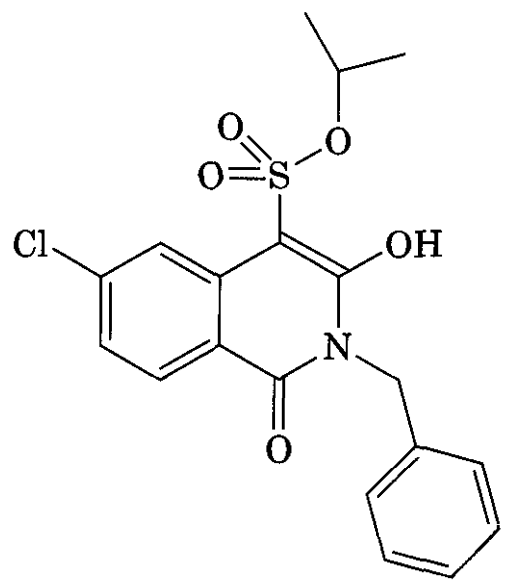
tivity (387). Surprisingly, etodolac (87) has been reported to be as selective as the rofecoxib derivative DFU (COX-1/COX-2, 1000×)



(87) etodolac

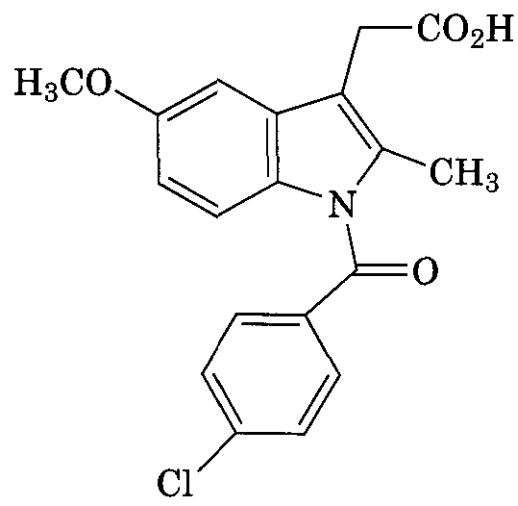
(372, 388). There is COX-2 patent activity related to the etodolac skeleton (389).

Other NSAIDs with weak COX-2 selectivity, like meloxicam, have been modified, with considerable structural reorganization, to yield structures like (**88**) with enhanced selectivity (390).



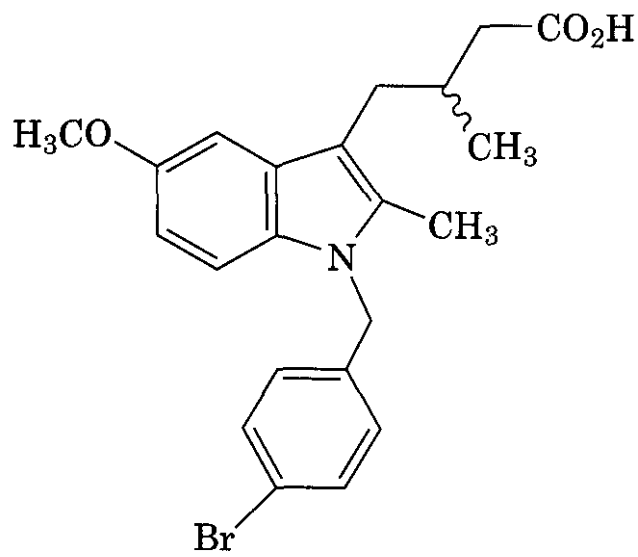
(88)

The model of the COX-2 protein as having a larger binding site has been used for rational design of superior COX-2 selectivity into existing NSAIDs. In addition to modeling studies, supporting evidence has been the observation that after acetylation by aspirin, COX-2 still produces large amounts of 15-HETE after addition of AA (391). This activity was not observed with COX-1, which is inactivated by aspirin acetylation. The rationale that follows implies that the COX-2 pocket of opening is large enough, even after acylation of key residues, to allow AA access, whereas AA does not have steric access to the smaller COX-1 pocket after acetylation. Two types of modifications of indomethacin (89) have been shown to increase COX-2 selectivity. Modifications to the *N*-benzoyl moiety, including trichloro substi-



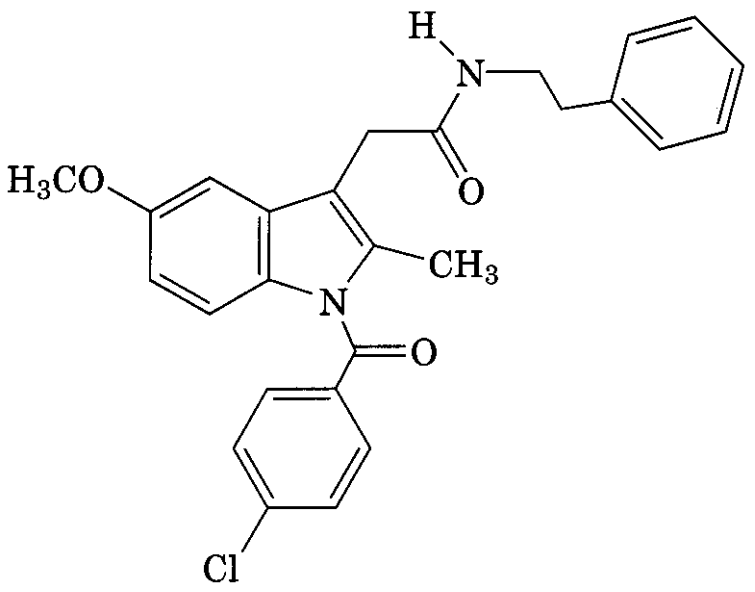
(89) indomethacin

tution or conversion to 4-bromobenzyl, have improved selectivity (391). A second, more general modification has been the homologation or extension of the carboxyl functionality in combination with alkyl branching, producing selective inhibitors like L-761,000 (90) (392).



(90) L-761,000

The alkyl branching on the carbon framework extending the acid primarily blocks sites of oxidative metabolism. Amides and esters formed using bulky or substituted alcohols and amines have also produced indomethacin derivatives like (**91**), with improved selectivity through structural extension of the carboxylate domain (393). Incorporation of the carboxylate of indomethacin into the latent carbonyl of a thiazole ring (92) as a way to extend the structural framework and take advantage of the larger binding cavity of COX-2 is a related approach (394). The carboxyl functionality of zomepirac has been extended as a sulfonamide (**93**), with improved COX-2 selectivity



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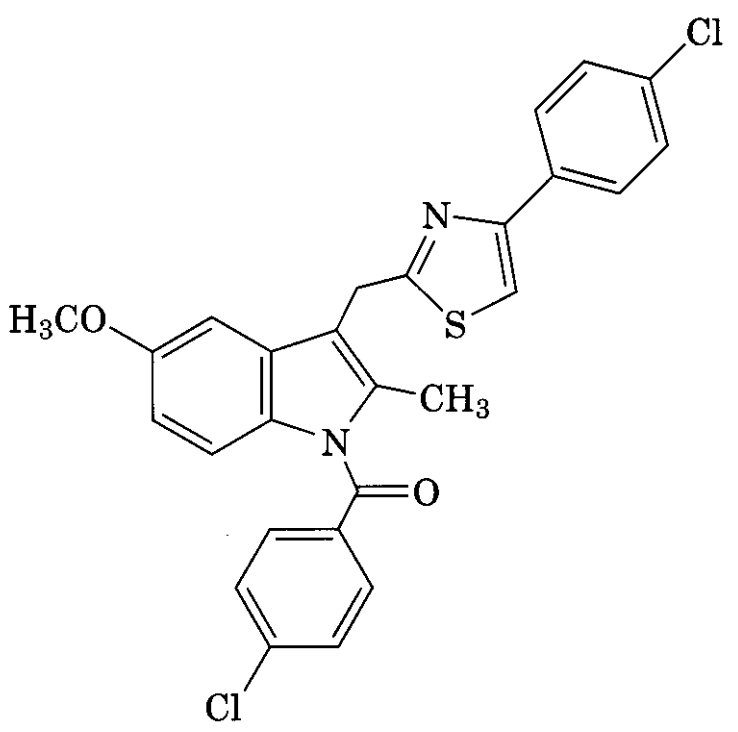
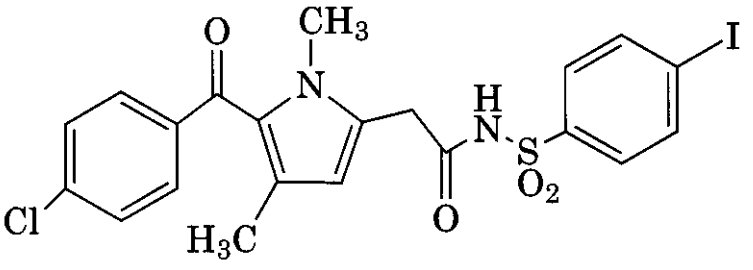


(92)

(386). Flurbiprofen can be converted into a more COX-2-selective agent (94) by addition of steric bulk in the form of two ethoxy groups to the terminal phenyl ring (395). No clinical data have been reported with modified NSAIDs thus far.

Selective compounds have been designed de *novo*; the most selective analogs reported



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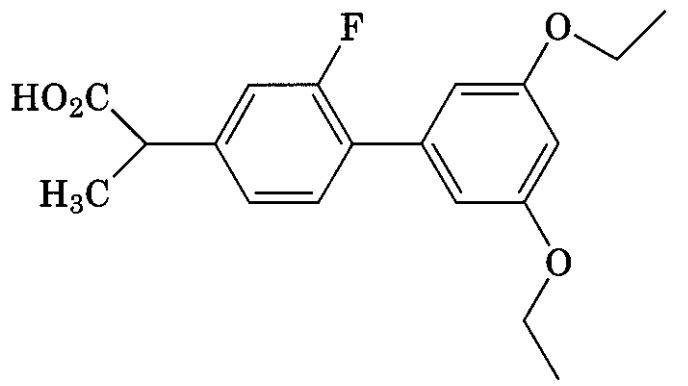

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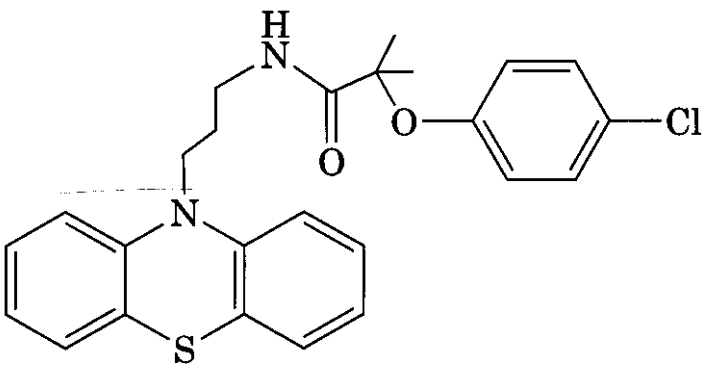
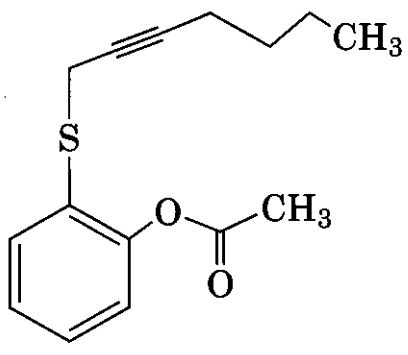


(95)

were also extended bulky amides like (95) and presumably correlate to a difference in binding pockets (396). Modification of COX-1 binding ligands for greater COX-2 selectivity has been successfully achieved and has been supported by structural information about the enzyme.

9.4 Irreversible Inhibitors

Aspirin is known to covalently acetylate the Ser^{530} residue in COX-1 and is the only known NSAID to irreversibly inactivate COX-1 by this mechanism. Acetylation by aspirin of the COX-2 enzyme did not lead to inactivation but did modify the product formed to 15-HPETE (391). The acetoxyheptynyl sulfide (**96**), has



(96)

been developed as a selective COX-2 inhibitor, which similar to aspirin covalently acetylates Ser^{516} in COX-2 (397).

Unlike aspirin, this compound is reported to selectively acetylate the COX-2 serine residue and selectively inactivate COX-2. The bulky heptynyl side-chain was optimized for COX-2 selectivity in this SAR study and the selective inhibition reported must be related to the differences in active site or binding pocket size, similar to the strategy for creating many other selective inhibitors from modifications of COX-1 inhibitors.

9.5 Biochemical Assays, Selectivities, and Potencies

One of the challenges associated with evaluating COX-1/COX-2 selectivity is trying to define or identify accurate affinities or dissociation constants for compound comparison. Inhibitory concentration data against purified protein obtained from baculovirus or CHO cells transfected with COX-1 or COX-2 have provided a majority of the information with regard to relative potency of individual agents. A complicating factor is that inhibitory activity exhibits a characteristic time-dependent phenomenon with many classes of COX-2 inhibitors; only under the simplest kinetic conditions, do K_m values give good approximations of equilibrium dissociation constants that are needed to facilitate traditional SAR studies. In addition, the stable cell lines expressing the two enzymes must be stimulated with exogenous AA, quite unlike the cellular situation where AA is released in smaller concentrations internally. Typical values reported for highly selective compounds against broken cell or transfected CHO cells are micromolar COX-1 potencies and low nanomolar potencies for COX-2 activity, giving approximately 1000-fold selectivity ratios.

Celecoxib has IC_{50} values of 40 nM and 15 μM for COX-2 and COX-1, respectively, against the recombinant human enzyme assay (325). Rofecoxib has IC_{50} values of 20 nM and >15 μM for COX-2 and COX-1, respectively, using protein from the CHO cell assay (326). Some of the nonselective reference compounds are quite potent in COX-2; IC_{50} values reported for indomethacin range from 30 to 900 nM for COX-2 in the broken cell assays (380, 326) and 10 to 50 nM for COX-2 in the engineered CHO cell (383, 392). DuP 697 is a potent COX-2 inhibitor in the CHO cell, with an IC_{50} value of 2–10 nM (383, 363). It is also COX-2 selective, having a weaker corresponding COX-1 IC_{50} (59–100 nM). The most potent COX-2 IC_{50} values are single-digit nanomolar and many of the cyclopentene derivatives approach this maximal activity (360, 362). The furanone derivatives appear to have a slightly weaker potency against COX-2 but are also much weaker against COX-1, resulting in similar selectivity ratios.

Later reported data indicate most companies switching to human whole blood (HWBL) assays for better indications of the effects of protein binding and other pharmacokinetic parameters considered relevant in correlating in *vivo* potencies and selectivity. Most of the reported compounds, including celecoxib and rofecoxib, are much weaker in the HWBL assays, with potencies on the order of 0.5–1 @. Merck reports an IC_{50} value for rofecoxib in the human COX-2 HWBL assay of 0.5 μM, and in the corresponding COX-1 HWBL assay, an IC_{50} value of 19 μM (38-fold selectivity) (326, 353). Comparison values for celecoxib are reported to be an IC_{50} of COX-2 HWBL assay of 1.0 μM and the corresponding COX-1 HWBL assay, an IC_{50} value of 6.3 μM (–sixfold selectivity). In this same comparison the JTE-522 compound was found to have HWBL potencies of approximately 33 and 100 μM for COX-2 and COX-1, respectively, for an approximate only threefold selectivity. More recent agents such as etoricoxib (Arcoxia), valdecoxib (Bextra), and ABT-963 have greater reported selectivity in the human blood assays compared to that of rofecoxib and celecoxib.

One of the most important mechanistic characteristics of COX-2 inhibition exhibited by many COX-2-selective inhibitors is the observed time-dependent inhibition of COX-2 but not of COX-1. A slow, noncovalent, pseudo-irreversable conformational change in the protein occurs after binding of the inhibitor, but this is only observed in binding with the COX-2 enzyme (353,398,399). As a result, a much lower concentration of inhibitor is required for effective inactivation of the COX-2 enzyme after a longer incubation, even for a

compound with equal dissociation constants at the two isoforms. This time-dependent inhibition of prostaglandin biosynthesis by certain NSAIDs was examined by Lands and his analysis showed that 0.3 μM flurbiprofen (an NSAID that shows this time-dependent phenomenon associated with a second conformational change after binding) could over a short time period compete with 50 μM ibuprofen (a freely reversible NSAID), even though they both had similar dissociation constants (400). In effect, this boosts the apparent selectivity for COX-2 of any inhibitor that can induce this COX-2-selective and time-dependent conformational change. This appears to be an immense advantage to this type of inhibitor and would argue that a lower selectivity could be tolerated, as long as the COX-1 activities were relatively high. Additional detailed models on the complex kinetics of COX-2 binding have been proposed (401).

9.6 *In* Vivo Models and Corresponding Efficacy

Several models of inflammation and analgesia have been used to characterize the in *vivo* pharmacology of COX-2-selective inhibitors. In addition to efficacy in inflammation and pain models, models of gastric irritation have been used to define superior safety indices for these agents. ^{51}Cr excretion in the rat has been used most often for a gastric safety model, with selective COX-2 inhibitors showing no leakage from possible intestinal lesions at doses often starting around 100 mg/kg (358). For acute inflammation the rat carrageenan paw edema model is most often used. $ED_{..}$ values for celecoxib and rofecoxib in this model are 7.1 and 1.5 mg/kg, respectively (325, 326). Indomethacin (375, 326) and DuP 679 (327, 363) are very potent in this assay, with $ED_{..}$ values around 1 mg/kg. Rofecoxib and related furanones seem slightly more potent in this assay than the celecoxib pyrazole or related templates, including the cyclopentene series. Flosulide was reported to have one of the lowest $ED_{..}$ values reported for this assay, with an $ED_{..}$ value of less than 1 mg/kg (0.6 mg/kg) (383).

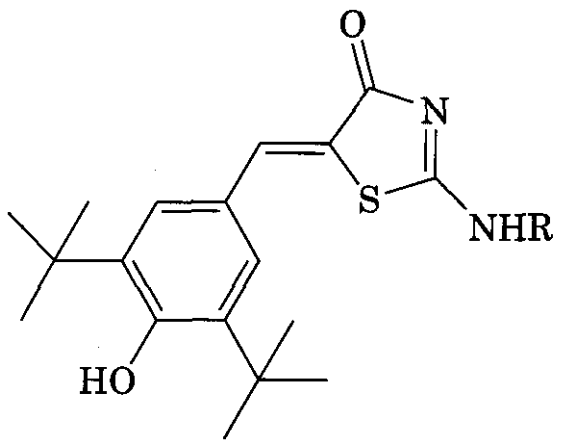
In models of chronic inflammation, for which the rat adjuvant arthritis assay is frequently used, these selective COX-2 inhibitors are very potent. $ED_{..}$ values of 0.1 to 1 mg/kg are achieved with analogs from many different series; representative efficacies are as follows: celecoxib, $ED_{..}$ 0.37 mg/kg (325); rofecoxib, $ED_{..}$ 0.15 mg/kg (326); DuP 697, $ED_{..}$ 0.18 mg/kg (379); and indomethacin, $ED_{..}$ 0.11 mg/kg (325). COX-2 inhibitors have also shown excellent analgesic activity. Hyperanalgesic paw models, either yeast-induced (Randall-Selitto) or carrageenan-induced (Hargraves), are most commonly reported as a measure of analgesic activity. Another model that is often reported is the rat air pouch inflammation model. This model is reported to measure anti-inflammatory events at the site of inflammation. The cyclooxygenase inhibitors are also very potent in this model, often giving 90% and greater inhibition at 2 mg/kg doses (R. Harris and R. Bell, personal communication, 1996).

9.7 Conclusion

Since the discovery of the COX-2 isoenzyme, a significant number of chemical agents have been synthesized with the goal of finding a selective inhibitor. As just described, defining what is meant by "selective COX-2 inhibitor" in *vivo* has been difficult. This is not surprising, given the similarities of the active sites for COX-1 and COX-2, complexities of the enzyme reaction, the possibility for metabolism of compounds, and so forth. As described in greater detail in section 11, the search, although difficult, has been successful. Clinical examination of Celebrex and Vioxx have shown a clear safety advantage for the compounds, with no loss of efficacy.

10 AGENTS INHIBITING COX-2 AND 5-LIPOXYGENASE

Agents that can inhibit more than one inflammatory mediator may be advantageous. Compounds that inhibit both COX-2 and 5-LO may have an efficacy advantage over current agents in the treatment of rheumatoid arthritis. Parke-Davis has reported CI-1004, darbufelone (98), and PD 138387 (97) as dual 5-LO/COX-2 inhibitors (402). Darbufelone was reported to be in clinical trials and has recently been described in detail (402). PD-

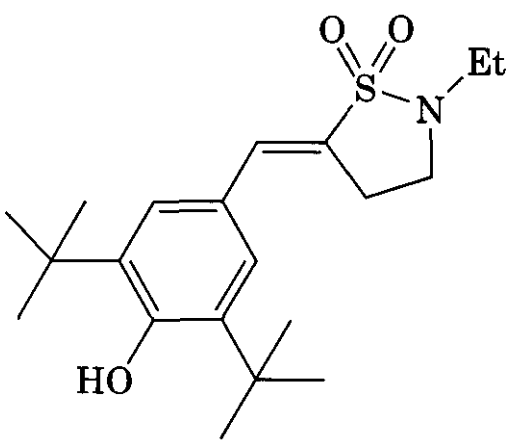


(97) R = OCH_3, **PD 138387**
(98) **R = H, (CI-1004)**

138387 was developed by SAR studies and exhibited superior selectivity to the earlier compound CI-1004.

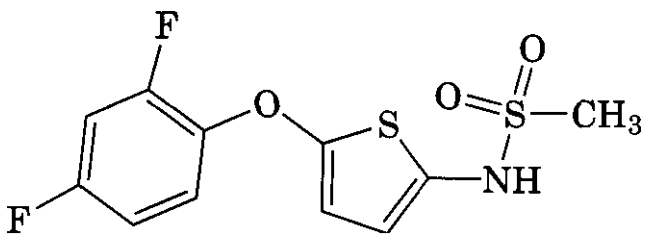
PD-138387 (**97**) was reported to have an IC_{50} value of 170 n*M* against COX-2 and >10 μM for the corresponding COX-1 potency.

PD-138837 was reported to be active in the rat CPE assay, with a modest oral ED_{40} value of 15.7 mg/kg. Analgesic activity was measured using acetic acid writhing in mice and gave an ED_{40} value of 0.1 mg/kg but also indicates COX-1 activity (Harris and Bell, unpublished observations, 1997). Several structurally related compounds have been reported that incorporated a sulfonamide into the heterocyclic moiety, for example, (99) (S-2474),



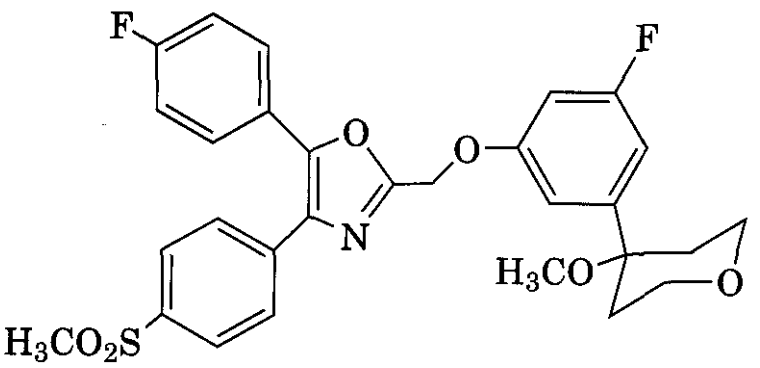
(99) S-2474

which has COX-2 selectivity of 2500-fold over COX-1 (COX-2, IC_{50} = 0.11 μM; COX-1, IC_{50} = 27 μM) (403). S-2474 has oral *in vivo* efficacy in the CPE model, with an ED_{30} value of 3.5 mg/kg and in the rat adjuvant arthritis model, an ED_{40} value of 0.76 mg/kg. Another series of reported dual COX-2/5-LO inhibitors are a series of phenoxy-substituted sulfonamido thiophene derivatives (100) (404). The phenoxythiophene template has been used as template for a series of N-hydroxyurea-based 5-LO inhibitors (131); the inclusion of the sulfonamide moiety results in compounds with dual inhibition at 5-LO and COX-2. In *vitro* activities of a preferred compound (100) were reported as IC_{50} values of 1.97 μM for COX-2 and 0.73 μM for 5-LO. Other compounds have been specifically designed by combining pharmacophores from both COX-2 inhibitors and 5-LO inhibitors.



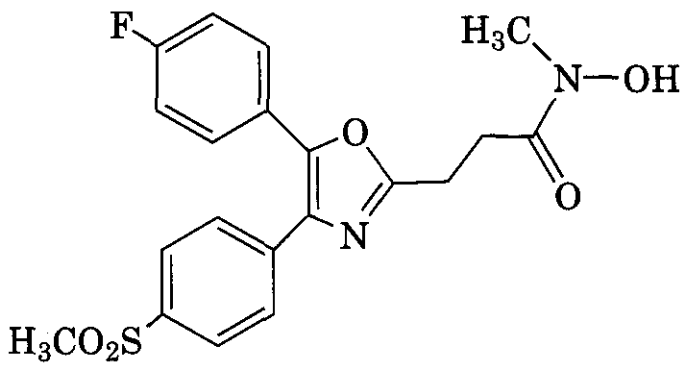
(100)

The patent report of a combined inhibitor (101) (405) reveals the familiar oxazole (369,



(101)

340) COX-2 inhibitor attached to the Zeneca methoxyphenylpyran, which was a very potent binding element for a series of 5-LO inhibitors (155). In a similar manner, the oxazole COX-2 inhibitor has been linked to a hydroxamic acid, which is also known to be a potent iron ligand and primary pharmacophoric moiety for many potent 5-LO inhibitors, to give dual inhibitors like analog (102) (406). The pyran-containing compounds (101) exhibited potent and selective COX-2 inhibition (COX-2, IC_{50} < 0.1 μM; COX-1, IC_{50} > 100 μM) and 5-LO inhibition (IC_{50} = 0.02 μM). This compound exhibited oral in *vivo* activity of 15% inhibition at 10 mg/kg in the rat CPE model.



(102)

Thus far no clinical results with dual 5-LO/COX-2 inhibitors have been reported.

11 CLINICAL EFFICACY AND SAFETY OF SELECTIVE COX-2 INHIBITORS

Celebrex (celecoxib) and Vioxx (rofecoxib) have progressed to registration and have established the utility of this class of drugs in treating the pain associated with surgery, osteoarthritis, rheumatoid arthritis, and muscle pain.

Celebrex was the first COX-2 inhibitor to be approved by the FDA (December 31,1998) and Vioxx was approved somewhat later (May 22, 1999). More recently, Bextra (valdecoxib) was approved (November 16, 2001) (Table 5.2).

The clinical trials, which were conducted by Searle/Pharmacia to define the activity of Celebrex, were extensive and involved nearly 16,000 subjects. Merck also examined a large number of patients to define the activity of Vioxx.

The first study by Hubbard et al. (407) showed that Celebrex was effective in third molar extraction, with significant difference from placebo but somewhat less effective than ibuprofen. In another study, Celebrex at either 100 or 400 mg was compared to aspirin, and in this study all treatments were equally efficacious and were significantly different from placebo.

Malmstrom et al. (408) compared Celebrex, Vioxx, and ibuprofen in an acute osteoarthritis study and the treatments were rank-ordered as placebo, Celebrex, ibuprofen, and Vioxx.

In a study examining the potential activity of Celebrex in orthopedic surgery it was found that, after a single dose, Celebrex was equal to hydrocodone/paracetamol, but with multiple doses, Celebrex was superior (409).

11.1 Osteoarthritis

The efficacy of the COX-2 inhibitors in osteoarthritis was expected, given that classical NSAIDs were effective in treating the pain associated with this degenerative disease. In a study examining the effects of Celebrex in osteoarthritis of the hip, Geis et al. (410) showed that 50, 100, and 200 mg doses were effective in treating the pain in hip osteoarthritis and the two higher doses were about equal to a 500 mg dose of naproxen. In a larger study examining the effects of Celebrex in osteoarthritis of the knee (again where 50,100, and 200 mg doses of Celebrex were compared to a 500 mg dose of naproxen), significant improvements were seen in the standardized scores in all treatment groups; and at both the 100 and 200 mg doses the scores for Celebrex were better than those seen with naproxen (411, 412). In similar studies Merck also established the efficacy of Vioxx. Vioxx at doses of 12.5 and 25

Table 5.2 Approved Eicosanoid Drugs

USAN	Trade Name	Mechanism	Date Approved[a]
Leukotriene			
Zafirlukast	Accolate	CYSLT antagonist	Sept. 1996
Zileuton	Zyflo	5-LO inhibitor	Dec. 1996
Montelukast	Singulair	CYSLT antagonist	Feb. 1998
COX-2			
Celecoxib	Celebrex	COX-2 selective	Dec. 1998
Rofecoxib	Vioxx	COX-2 selective	May 1999
Valdecoxib	Bextra	COX-2 selective	Nov. 2001
Etoricoxib	Arcoxia	COX-2 selective	Submitted 2002

[a]FDA approval.

mg resulted in improvement in both hip and knee osteoarthritis in studies that lasted from 6 to 86 weeks of duration (413,414).

Vioxx (50 mg) provided pain relief equal to that of either 550 mg naproxen or 400 mg ibuprofen (415, 416). In a multiple-dose study, Vioxx produced significant pain relief after orthopedic surgery (416).

The clinical results with both Celebrex and Vioxx suggest that inhibition of COX-2 is an effective treatment for the pain that is associated with surgery, osteoarthritis, and rheumatoid arthritis.

Although most of the evidence suggests that COX-2 is expressed only when induced by cytokines or growth factors, there are potential roles this enzyme may play in normal physiology such as in uterine contraction, renal medulla, and in both brain and gut mucosa. Extensive clinical trials with both Celebrex and Vioxx were conducted to determine the safety of these compounds. Celebrex was examined in 3- and 6-month trials and it was found that the incidence of ulcers was the same as that of placebo and significantly less than that seen for either naproxen or diclofenac (410). Vioxx was examined in 1516 patients using endoscopy, in either the 25 or the 50 mg dose, and was compared to ibuprofen 2400 mg. A significantly lower percentage of ulcers were seen in the Vioxx-treated patients than seen in the ibuprofen-treated individuals (417). In another study, in which Vioxx was given at 250 mg (10–20 times the clinical dose) for 7 days, it was well tolerated, with gastric injuries no worse than those of the placebo, and was less than the injuries seen with either 2400 mg of ibuprofen or 2500 mg of aspirin (418,411).

COX-2 is expressed in the renal medulla and therefore there was concern that specific COX-2 inhibitors could cause untoward renal effects. However, renal effects of the compounds have been reported to be mainly related to fluid retention in salt-depleted patients (420).

11.2 COX-2 and Cancer

Various studies have demonstrated a protective role of NSAIDs in the prevention of colon cancer (421). However, the potential for gastric damage intrinsic to conventional NSAIDs precluded their use as preventive therapy. Based on these observations, the scientists at Searle/Pharmacia examined the potential role of COX-2-selective inhibitors in first preventing polyps in mice and then in humans (422, 423). Data from the animal studies showed that in rats treated with celecoxib and then given s.c. injections of azoxymethane to induce colon cancers, there was a 93% inhibition of cancer incidence and a 97% reduction in tumor number. Overall, there was a 87% reduction in the tumor burden (423). In the MIN mouse model of familial adenomatous polyposis (FAP), Jacoby et al. (424) showed that celecoxib inhibited the tumor number by 71% and the tumor size by 83%. In chemically induced bladder cancer in mice and rats, celecoxib was effective at reducing the incidence of lesions in both mice and rats.

Howe et al. (425) reviewed the potential of using COX-2 inhibitors for the treatment of breast cancer and suggested that there is good rationale to examine COX-2 inhibition for breast cancer prevention. Limited data are available in animal models of breast cancer; however, Harris et al. (426) showed that celecoxib inhibited the tumor multiplicity by 86% and the incidence by 68%.

Clinically, Celebrex has been shown to be effective in FAP patients. Steinbach et al. (424) showed that twice-daily 400 mg/day of Celebrex reduced the number of polyps in familial adenomatous patients by 28% after 6 months of treatment. There was a similar incidence of adverse events in all of the treatment groups. These data were sufficient for Celebrex to be approved for use in FAP.

Rofecoxib was shown to be effective in chemoprevention of polyps in the APC delta 716 knockout mice by Oshima et al. (428). Rofecoxib was given in food for 8 weeks and then the animals were necropsied and the polyps scored. There was a greater than 57% inhibition of polyps greater than 1 mm in size and up to 100% inhibition of polyps greater than 3 mm. Overall, the data are very interesting and suggestive of potential activity for Vioxx in FAP, although thus far no clinical data have been reported.

12 CONCLUSION AND SUMMARY

The modulation of the synthesis and activity of a number of eicosanoid products has been shown to be of significant importance medically in a multitude of clinical conditions. Nonselective cyclooxygenase inhibitors (NSAIDs) have been broadly used for several decades in the relief of pain and inflammation. The new COX-2-selective agents (Table 5.2) have already proved to be safer and equally efficacious. It is likely that they will continue to replace the older agents. However, the cardiovascular effects of COX-2 agents will surely need to be watched carefully in the future. In addition, the safety profile of these agents will allow for their use in many clinical settings, where the possibility of GI bleeding or platelet effects preclude the use of NSAIDs. These could include cancer prevention, cancer therapy, and both surgical and cancer pain.

Blockade of the effect or synthesis of leukotrienes has proved to be a safe and effective approach to asthma therapy (Table 5.2). Here again, especially for the leukotriene biosynthesis inhibitors, new uses in other pulmonary diseases are likely. Expansion into other allergic diseases is already taking place and will probably continue.

In addition to the already established approaches such as COX-2 inhibitors, 5-LO inhibitors, and CYSLT1 antagonists, several new targets in eicosanoid research are clearly not well explored and have significant potential. These would include inhibitors of LTA_4 hydrolase, LTB_4 antagonists at both receptors, CysLT2 antagonists, inhibitors of inducible PGE, synthase, selective prostaglandin receptor antagonists, lipoxin modulators, and perhaps COX-3 inhibitors (D. Simmers, submitted, 2002). Whatever the final success of any one of these targets, it is clear that modulation of eicosanoid production and action provides clear medical benefit in multiple settings.

13 ABBREVIATIONS

COX	cyclooxygenase
DP	dipeptidase
FLAP	5-LO activating protein
gGTP	gamma glutamyl transpeptidase
HETE	hydroxyeicosatetraenoic acid
HPETE	hydroperoxyeicostetraenoicacid
LO	lipoxygenase
LT	leukotriene
PG	prostaglandin
PLA	phospholipase
TX	thromboxane

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CHAPTER SIX

Agents Acting on Prostanoid Receptors

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1 INTRODUCTION

1.1 Historical Background and Overview of Prostanoid Synthesis

Prostanoids are metabolites of C_{20} fatty acids that act as local hormones in both the periphery and central nervous system (CNS). Classically, they are comprised of the prostaglandins and thromboxanes. However, recently, interest has focused on the isoprostanes, which are formed through the direct oxidation of membrane phospholipids and not through the actions of cyclooxygenase (COX) enzymes. Despite this difference in synthesis, current evidence suggests that they act predominantly through prostanoid receptors (1). Another interesting recent development has been the discovery of the prostamides, which are derivatives of anandamide and are formed through the actions of COX-2 and not COX-1 (2–4).

As a family, the prostanoids are almost ubiquitously distributed, and in general, have a short duration of action. Von Euler first proposed the term 'prostaglandin' in describing the presence of a vasodepressor and smooth muscle stimulating factor in human seminal fluid (5), following the initial observations of Kurzrok and Lieb (6) and Goldblatt (7). However, it was another 20 years before the first prostaglandins, prostaglandin E_1 (PGE_1) and prostaglandin $F_{1\alpha}$ ($PGF_{1\alpha}$), were successfully purified (8). During the next decade it became apparent that the family included more than the original two and these were named alphabetically from PGA, to PGH,. Of these, PGG, and PGH, are unstable intermediates (9) generated during the biosynthesis of this family of hormones. In the mid-1970s, two new prostaglandin-like compounds were discovered: thromboxane A, (TXA_2) (10) and prostacyclin (PGI_2) (11).

The natural prostanoids derived from arachidonic acid are known as prostaglandin D, (PGD_2), prostaglandin E_2 (PGE_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), prostaglandin I, (PGI_2), and thromboxane A, (TXA_2). They exert their physiological and pathophysiological actions through specific high affinity receptors and are responsible for a multitude of actions, some of which have been exploited to produce clinically effective medicines. Prostaglandins are characterized by a cyclopentane ring, with two side-chains, termed α and ω, that are both in the ***trans*** configuration. Also present are a hydroxyl group at C-15 and a Δ^{13} ***trans*** double bond. The structure of PGE, as representative of the entire group is shown in Fig. 6.1. Prostanoids can be biosynthesized from three related fatty acid precursors to give rise to three different series based on the number of double bonds in their side chains. Thus, prostanoids derived from 8,11,14-eicosatrienoic acid (γ-homolinolenic acid) give rise to the 1-series, whereas the 2- and 3-series are derived from

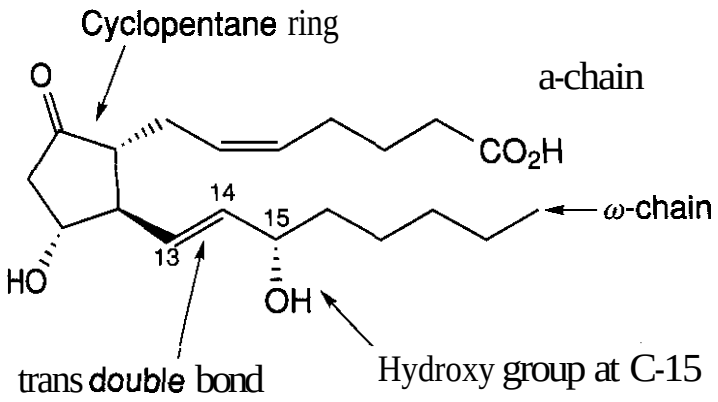


Figure **6.1.** The structure of prostaglandin E_2. Note the presence of the a- and ω-chains, the cyclopentane ring, ***trans*** double bond, and hydroxy group at C-15.

5,8,11,14-eicosatetrienoic acid (arachidonic acid) and 5,8,11,14,17-eicosapentaenoic acid (timodonic acid), respectively. Arachidonic acid is the most abundant precursor in mammals, including humans, making the 2-series by far the most common. However, eicosapentanoic acid (EPA, 20:5ω3) is present in large amounts in marine animals, and consequently in the bodies of those species living largely on a diet of these animals. For example, Greenland Eskimos have much higher levels of EPA and other ***03*** fatty acids in their phospholipids, and this has been linked to the lowered incidence of heart disease in these populations (12).

Prostaglandin H_2 is the common biosynthetic precursor of the natural prostanoids, and is produced as a result of the action of cycloxygenases on free arachidonic acid. The major source of free arachidonic acid is the phospholipids of the cell membrane, from which it is largely liberated by the actions of phospholipases. The activity of COX, also known as prostaglandin H synthase (PGHS), converts arachidonic acid to PGH_2 through a two-step process: first a cyclooxygenase step, that results in the production of the unstable intermediate PGG_2, and second, a peroxidase step that yields PGH,, which is then converted to the individual prostanoids by specific synthases. An overview of this process is shown in Fig. 6.2. The biosynthesis and breakdown of prostanoids is discussed further in Sections 2.1 and ***2.2.***

1.2 Biological and Therapeutic Functions of Prostanoids

The major roles of prostanoids are in the autocrine and paracrine control of physiological processes, and therapeutically they have found use mainly in obstetrics, gastoenterology, ophthalmology, and the treatment of cardiovascular disease. In the reproductive system, prostaglandins, especially of the E and F series, have potent effects on uterine contraction and have been used to stimulate abortion, cervical ripening, and the induction of labor (13). In addition, in many laboratory and farm animals, but not in humans, $PGF_{2\alpha}$ is a potent luteolytic agent and is used for this purpose in veterinary medicine (14). Similarly, prostaglandin receptors exhibit a widespread distribution in the gastrointestinal tract (15), and prostaglandins are generally thought to have cytoprotective effects in the gastric mucosa (16), through the control of gastric secretions, protection of the mucosal barrier, and the regulation of blood flow (17). This assertion has led to the production of a number of PGE analogs for use as potential gastroduodenal protective agents, against, for example, the effects of non-steroidal anti-inflammatory drugs (NSAIDs). Recently, PGF analogs, such as latanoprost, have found use as novel medicines for the treatment of glaucoma (18). In the cardiovascular system, prostanoids, especially prostacyclin, are involved in, for example, the regulation of platelet aggregation. Prostacyclin and its analogs are administered exogenously in the treatment of several forms of cardiovascular disease, where they may cause vasodilatation and also inhibit platelet clumping and aggregation (19). Prostaglandins are also active in the renal system. Indeed, one of the earliest physiological effects attributed to prostaglandins was the effect of PGE, to block the action of vasopressin on renal water permeability (20). Receptors for prostaglandins have also been localized to all parts of the kidney (21). Prostaglandins, particularly of the E and I series, have also been strongly implicated in inflammation and inflammatory pain (22), leading to recent efforts to produce specific receptor antagonists for the treatment of these conditions.

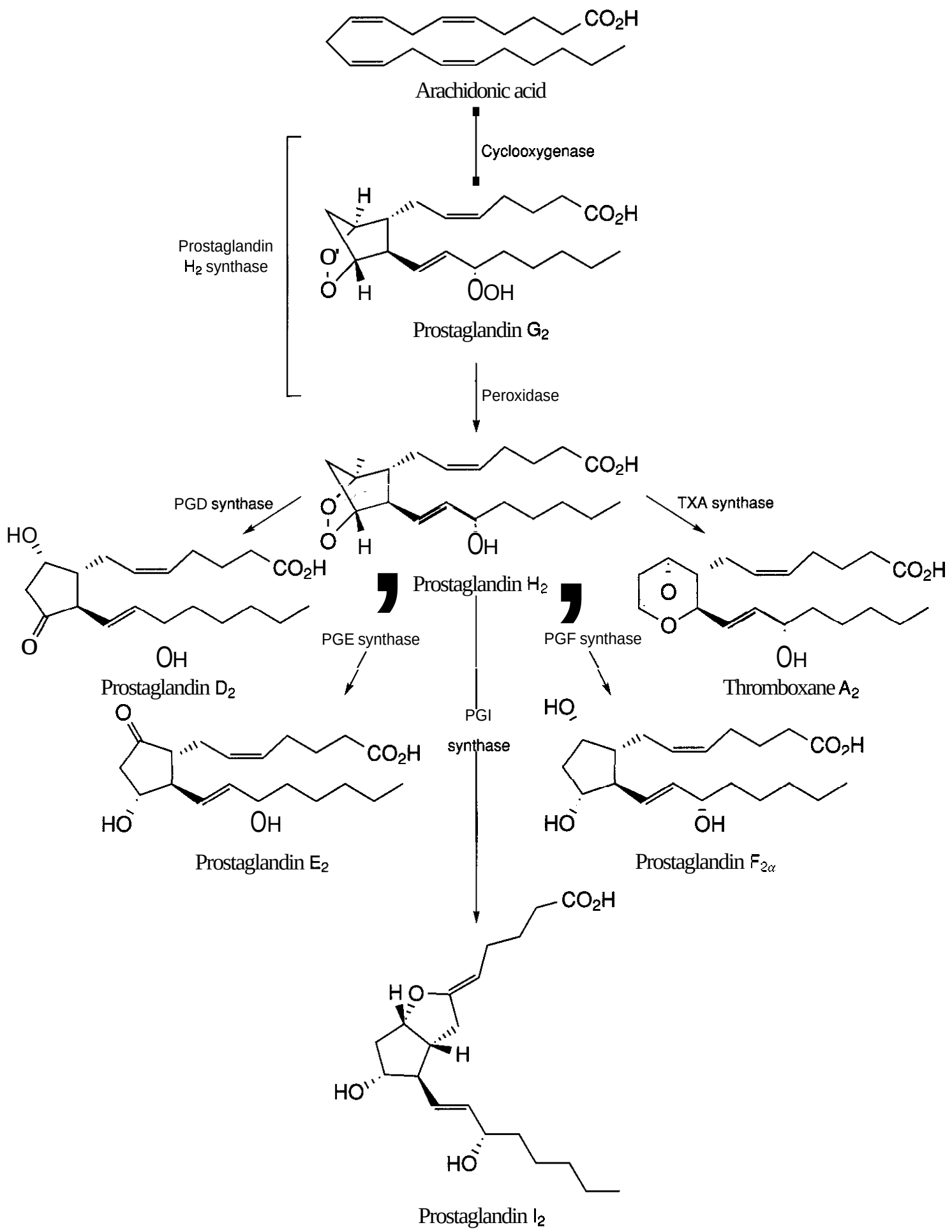


Figure 6.2. The biosynthesis of prostanoids from arachidonic acid. Free arachidonic acid is converted to the unstable intermediates PGG, and PGH, by cyclooxygenase (COX) enzymes. PGH, is then converted to the five primary prostanoids by specific synthases.

1.3 Prostanoid Receptor Classification: A Historical Perspective

Prostanoids, as metabolites of fatty acids, are relatively hydrophobic compounds and it was initially thought that they might act through incorporation into the plasma membrane. However, it has subsequently been shown that prostanoids predominantly exert their biological actions through interactions with specific receptors in cell membranes. In the absence of direct evidence, this conclusion is suggested by a consideration of some of the basic properties and actions of the prostanoids. For example, they are active at low concentrations ($<10^{-9}$ *M*), and thus display high potency, implying the existence of specific binding sites. Furthermore, it is clear that different members of the prostanoid family can evoke different responses in the same system. For example, TXA_2 and PGI_2 exert both aggregatory and non-aggregatory effects on human platelets, respectively, thus suggesting, in platelets at least, the presence of distinct receptor sites for both of these prostanoids.

The first evidence for the existence of more than a single subtype of prostanoid receptor came from the work of Pickles (23), who used both natural and synthetic analogs of E and F series prostaglandins to demonstrate differential effects in several smooth muscle preparations: the rabbit jejunum and human and guinea pig myometrium. These studies led him to conclude that there were at least three types of prostanoid receptors. The presence of multiple receptors was also suggested by the work of Andersen and Ramwell (24), who compared the agonist potency ratios of PGA, PGE, and PGF analogs, and Gardiner and Collier (25), who examined the prostanoid receptor types present in the lung. In 1982, Kennedy and co-workers proposed the first overall classification of prostanoid receptors. In the premolecular biology era, they proposed specific receptors for PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2, and TXA_2, calling them DP, EP, FP, IP, and TP receptors, respectively. At each of these receptors, one of the natural prostanoids was at least one order of magnitude more potent than the other four. Hence, PGD_2 is the most potent natural agonist at the DP receptor, PGE_2 the most potent natural EP receptor agonist and so on. This system was based on functional data obtained with the natural agonists, some synthetic agonists, and a small number of antagonists, but is still valid today (1,26). These initial studies were conducted on isolated smooth muscle preparations that were considered PGE- (e.g. guinea pig ileum), PGF- (e.g. dog and cat iris), or TXA-sensitive (e.g. rat aorta). They compared the agonist potencies of the naturally occurring prostanoids and the TXA_2 mimetic, U46619, in these preparations. Coupled with other studies that suggested receptors for PGD_2 and PGI,, this led to the proposal outlined above that receptors existed for each natural prostanoid. Further studies identified compounds that acted as receptor blockers in some preparations, but not others. Hence, AH19437 was identified as a competitive antagonist in TP receptor containing tissues (26). Whilst SC-19220 was an antagonist in some PGE-sensitive tissues (26), producing rightward shifts in the concentration-effect curves in guinea pig ileum and guinea pig and dog fundus, it had negligible effects in the PGE-sensitive preparations cat trachea or the chick ileum (26). This provided the first evidence for a subdivision of the EP receptors. Further evidence came from the identification and use of the antagonist, AH6809, and the agonists, sulprostone and AY23626, with AH6809 being active in EP_1 receptor-containing preparations, and sulprostone and AY23626 having actions at EP_1 and EP,, and EP_2 and EP, receptors, respectively (27). Subsequently, a fourth EP receptor was identified in piglet saphenous vein (28, 29). The rank order of the naturally occurring prostaglandins at their receptors is shown in Table 6.1.

This chapter will provide an overview of the natural prostanoids and their synthetic and metabolic pathways and then discuss the receptors at which prostanoids exert their actions and the agents that have been generated to aid as either specific receptor agonists or antagonists, some of which have found use in the clinic.

2 NATURAL PROSTANOIDS

2.1 Biosynthesis

Prostanoids are synthesized *de novo* by a wide variety of cells, and synthesis can be induced

Table 6.1 Rank Orders of Potency of the Naturally Occurring Prostanoids for Each of the Five Types of Prostanoid Receptors

Receptor Type	Subtype	Rank Order of Natural PGs[a]
DP	DP_1	D, > E, = F„ = I, = U46619
EP	EP_1	E, > F„ = I, > D_2 > U46619
	EP_2	E_2 > $F_{2\alpha}$ = D_2 = I, > U46619
	EP_3	E, > I, > F„ ≫ D_2 > U46619
	EP_4	E, ≫ I, = $F_{2\alpha}$ > D, > U46619
FP		$F_{2\alpha}$ > D_2 > E_2 = U46619 > I_2
IP	IP_1	I, > D, = E_2 = F„ > U46619
TP		U46619 ≫ D, = E, = $F_{2\alpha}$ = I,

[a] U46619 is a stable TXA_2 mimetic (see Section 4.5) and was used instead of TXA_2. Data taken from Coleman (1).

by a variety of stimuli, including non-prostanoid receptor activation as well as simple mechanical agitation. Prostanoid synthesis requires the presence of free, unesterified arachidonic acid. Under resting conditions, the cytoplasmic levels of this fatty acid are low, necessitating the liberation of this substrate as the first and rate-limiting step in prostanoid biosynthesis. The main source of arachidonic acid is that present in membrane glycophospholipids, esterified in the 2-acyl position in the fatty acyl chains, and this is primarily liberated by the enzyme phospholipase A_2 in a one-step process (30). Arachidonic acid not metabolized by cyclooxygenases or other eicosanoid-producing enzymes (e.g., lipoxygenases) is rapidly re-esterified by acetyltransferases (31).

Arachidonic acid, liberated from membrane phospholipids, can be metabolized to the five primary prostanoids, PGD_2, PGE_2, $PGF_{2\alpha}$, PGI,, and TXA_2. The first two steps in this process are catalyzed by the cyclooxygenase (COX) enzymes and thereafter by specific prostanoid synthases (32). An overview of prostanoid synthesis is illustrated in Fig. 6.2.

2.2 Catabolism

The half-life of most prostaglandins in the circulation is less than 1 min, and this is attributable to the efficiency of the processes that exist to aid in their inactivation. For example, in the lung, around 95% of single doses of either PGE_2, PGE,, or $PGF_{2\alpha}$ are metabolized during the first passage. The enzymes involved in prostanoid metabolism are located intracellularly, thus necessitating the uptake of substrates across the cell membrane (33–36). This is believed to be a carrier-specific mechanism and displays differential selectivity over the prostanoid-catabolizing enzymes. Following uptake, prostanoid metabolism occurs in two stages: first, a rapid inactivation by prostanoid-specific enzymes. This first stage normally results in a loss of biological activity. Second, a slower inactivation process occurs, involving enzymes that are responsible for general fatty acid oxidation. Metabolism occurs in the lung and also the liver and the kidney.

The first important work on prostanoid metabolism was conducted by Anggard and Samuelsson (37), who demonstrated that PGE and PGF compounds are dehydrogenated at carbon-15 to the corresponding 15-keto prostanoid by the nicotinamide adenine dinucleotide + (NAD+)/nicotinamide adenine dinucleotide phosphate (NADP)-linked enzyme, 15-hydroxy-prostaglandin dehydrogenase (PGDH), to form 15-keto-PGE, and 15-keto-$PGF_{2\alpha}$. PGDH has subsequently been shown to have a distribution pattern that includes the lung, kidney, and liver (38). Several compounds can inhibit PGDH, including the substituted phenylazobenzoic acetic acid derivatives, Ph CL28A and Ph CK61A (39). PDGH is not specific to prostaglandins. For example, the NAD+-linked enzyme present in the lung will also metabolize 15-hydroxyeicosatetreonic acid (15-HETE) and other ω6-containing fatty acids (40). These 15-keto metabolites are far less active than the parent compounds, and therefore, these initial transformations represent a functional inactivation.

The second stage of inactivation is catalyzed by a $\Delta^{13,14}$ reductase and involves satu-

ration of the Δ^{13} double bond to form 15-keto, 13,14-dihydro prostaglandins. This reaction can only occur in prostaglandins that have been initially undergone oxidation of the 15-hydroxy group, and the metabolite formed is the major breakdown product found in plasma samples and kidney and lung homogenates. This enzyme uses nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) as a cofactor and can be inhibited by *p*-chloromercuribenzoic acid (41).

Following these steps, β and ω oxidation take place to shorten the carboxyl side-chain and to introduce a further carboxyl group, respectively (42–45). The urinary metabolites detected after prostaglandins are infused in man are generally dinor and tetranor compounds, so the carboxyl-containingside-chain is normally shortened by two to four carbon atoms. This is the result of β-oxidation that occurs mainly in liver mitochondria or peroxisomes. The ω-oxidation that occurs is catalysed by cytochrome P450, and occurs largely in the liver and kidney, and many of the urinary metabolites of prostaglandins are generated through this pathway. Overall, these steps help to make the prostanoids more polar and thus available for excretion by the kidney. Thus, the final metabolites found in the urine are 16-18 carbon atoms and possess carboxyl groups on both the α and ω side-chains.

Prostaglandin D_2 follows the same basic principles outlined above, but in humans at least, a different pattern of catabolism may occur. It has been found that a sizeable proportion of urinary metabolites obtained following injection are recovered as metabolites of PGF compounds (46). For this to occur, the 11-keto group of PGD_2 must be reduced, but it is unclear whether this reduction is of PGD_2 itself, or of one of its subsequent metabolites, for example, those that are present after being oxidized by PGDH.

Prostacyclin, although not taken up by the lungs, is rapidly inactivated by acid-catalysed, non-enzymatic means to the metabolite 6-keto-$PGF_{1\alpha}$. In addition it is also catabolized by PGDH to 6,15-diketo metabolites. 6-keto-$PGF_{1\alpha}$ is biologically inactive and also rapidly metabolised in *vivo*, with the major urinary product being dinor-6-keto-$PGF_{1\alpha}$ (47).

Thromboxane A_2 has a plasma half-life as short as 7 s (48) and spontaneously decomposes non-enzymatically to the functionally inactive TXB_2, which is either excreted unaltered or as one of several other metabolites. For example, TXB_2 can undergo β-oxidation to dinor-TXB_2.

2.3 Physiological Actions of Prostanoids

Prostaglandins have a wide range of actions in normal cell physiology, but also as pathophysiological agents in, for example, inflammation and pain states. The roles of the natural prostanoids are reviewed in this section in relation to studies on receptor distribution, which provide useful information concerning potential pharmacological applications. Recent advances in molecular biology have also allowed the creation of prostanoid receptor "knockout" mice, which have proved valuable in helping to further elucidate the receptor types involved in mediating the various actions of prostanoids.

2.3.1 Prostaglandin D. PGD_2 acts predominantly at DP receptors, although it does have relatively high potency at FP and even TP receptors. DP receptors are probably the least widely distributed of all the prostanoid receptors and often co-exist with other prostanoid receptors, making them difficult to study outside of recombinant systems. However, the availability of potent, selective agonists and antagonists (see Section 4) has proved pivotal in surmounting this problem.

DP receptors are present on vascular smooth muscle, platelets, and in nervous tissue, including the central nervous system (CNS). In the CNS, PGD_2 has been associated with sleep induction, as well as body temperature regulation, hormone release, nociception, and olfactory function. DP receptors have also been localized to smooth muscle in the gut, uterus, and airways. Northern blot and in *situ* hybridization studies have reaffirmed this, with only low levels of expression being detected in human retina and small intestine (49). The physiological actions of PGD_2, mediated through DP receptors, are generally "inhibitory"; that is, smooth muscle relaxation and the inhibition of platelet aggregation. However, "excitatory" actions have also been

reported: for example, in afferent sensory nerves PGD, seems to induce hyperalgesia (50) and can also stimulate the release of neuropeptides (51). PGD, has also been implicated in central roles, such as sleep-induction, in both rats and humans (52). DP receptors have been localized to the leptomeninges (53), and PGD, injected into the arachnoid space below the basal forebrain caused leptomeningeal cellular activation, detected by immunohistochemistry for the immediate early gene, *fos* (54). Furthermore, the brain-specific isoform of PGD synthase has recently been localized in the leptomeninges (55). DP receptors have also been recently detected in the mucous-secreting goblet cells in the rat gastrointestinal tract, suggesting a potentially novel role for PGD, in mucous secretion (56).

DP receptor localization also seems to vary considerably between species. For example, platelet DP receptors seem to be most abundant in humans and not in other animal species (57). PGD, is also the major prostaglandin produced by mast cells following immunological challenge (58). Recent studies using mice deficient in the DP receptor have suggested that PGD, production may be important in some aspects of asthma (59). In a model of asthma, when challenged with ovalbumin, $DP^{-/-}$ mice showed reduced lymphocyte accumulation in the lung compared with wild-type controls, and in addition, failed to develop airway hyperreactivity. These data suggest that, in mice at least, DP receptors may play a role in airway hyperreactivity.

2.3.2 Prostaglandin E, PGE_2 acts predominantly through EP receptors, which are abundantly distributed and are responsible for mediating the multitude of actions of PGE,. These include smooth muscle contraction and relaxation, stimulation and presynaptic inhibition of neurotransmitter release, neuronal sensitization, nociception, inhibition of lipolysis, and gastric acid secretion and modulation (positively and negatively) of water secretion. It was partly because of the sheer diversity of these responses and through the use of what are now known to be subtype specific ligands that it became clear that there was more than one subtype of EP receptor. It is now known that at least four receptor subtypes exist, and these are termed EP_1, EP,, EP,, and EP, receptors (see Section 3).

The first characterized EP receptor subtype, the EP_1 receptor, is probably the least widely distributed of all the EP receptors. It predominantly mediates the smooth muscle contraction actions of PGE, and is present in guinea pig trachea, gastrointestinal tract, bladder, and uterus. This receptor subtype has also been found in other animal species: for example, in the bovine iris sphincter (60), dog gastric fundus (61), and human myometrium (62). These receptors also seem to play a role in inflammatory pain, with EP_1 antagonists currently being investigated as potential antinociceptive agents (63). In *situ* hybridization studies have detected EP_1 receptor transcripts in the kidney, lung, and stomach of the mouse (64, 65). Recently, EP_1 receptor knockout mice have been used to suggest that endogenous EP_1 receptors are important in the mediation of pain perception and also the regulation of blood pressure (66). EP_1 receptor–deficient mice were both healthy and fertile but showed reduced pain sensitivity, similar to that obtained following inhibition of COX with an NSAID. Similar results have also been reported in mice deficient in the IP receptor (67) (see Section 2.3.4), thus suggesting that EP_1 and IP receptors may represent possible novel targets for the treatment of inflammatory pain.

EP, receptors, first recognized because of the lack of effect of EP_1 receptor antagonists in blocking the relaxant effects of PGE,, have a more widespread distribution than the EP, receptor subtype. This receptor subtype also mediates a more diverse array of actions. For example, smooth muscle EP_2 receptors always mediate smooth muscle relaxation [through increases in cyclic adenosine monophosphate (cAMP) through G_s (see Section 3.3)]. Epithelial EP, receptors regulate non-acid secretion, and in mast cells and basophils, they mediate inhibition of mediator release. Furthermore, they may also mediate afferent sensory nerve activation, including neuropeptide release. Northern blotting in the mouse has suggested the presence of EP, receptor mRNA in the ileum, thymus, spleen, heart, and uterus (68). This receptor subtype has also been localized

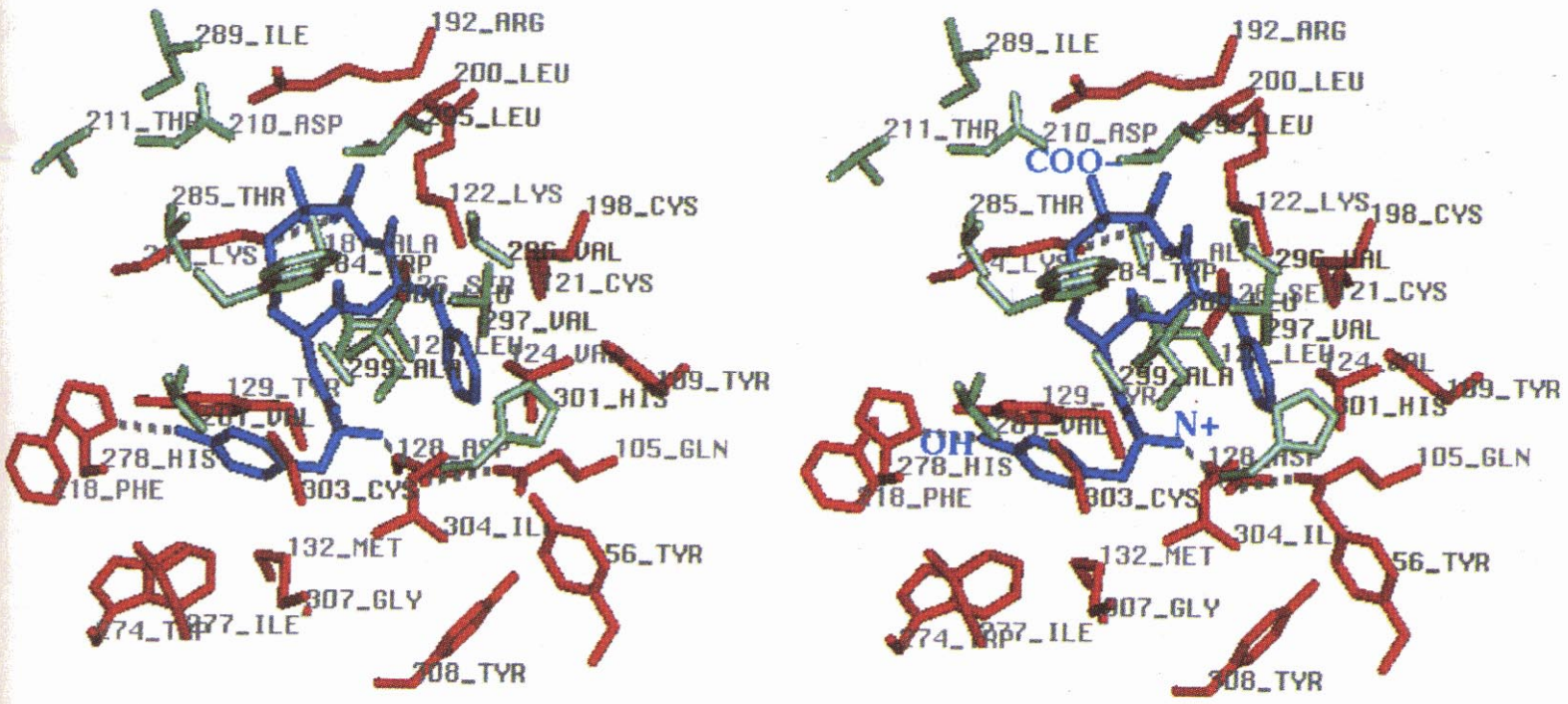


Figure 2.8. JOM-13 (blue) in the 8-opioid receptor binding pocket (stereoview). [Taken from Fig. 2.9 in H. I. Mosberg, *Biopolymers (Peptide* Science), 51, 426 (1999). Reprinted by permission of John Wiley & Sons.]

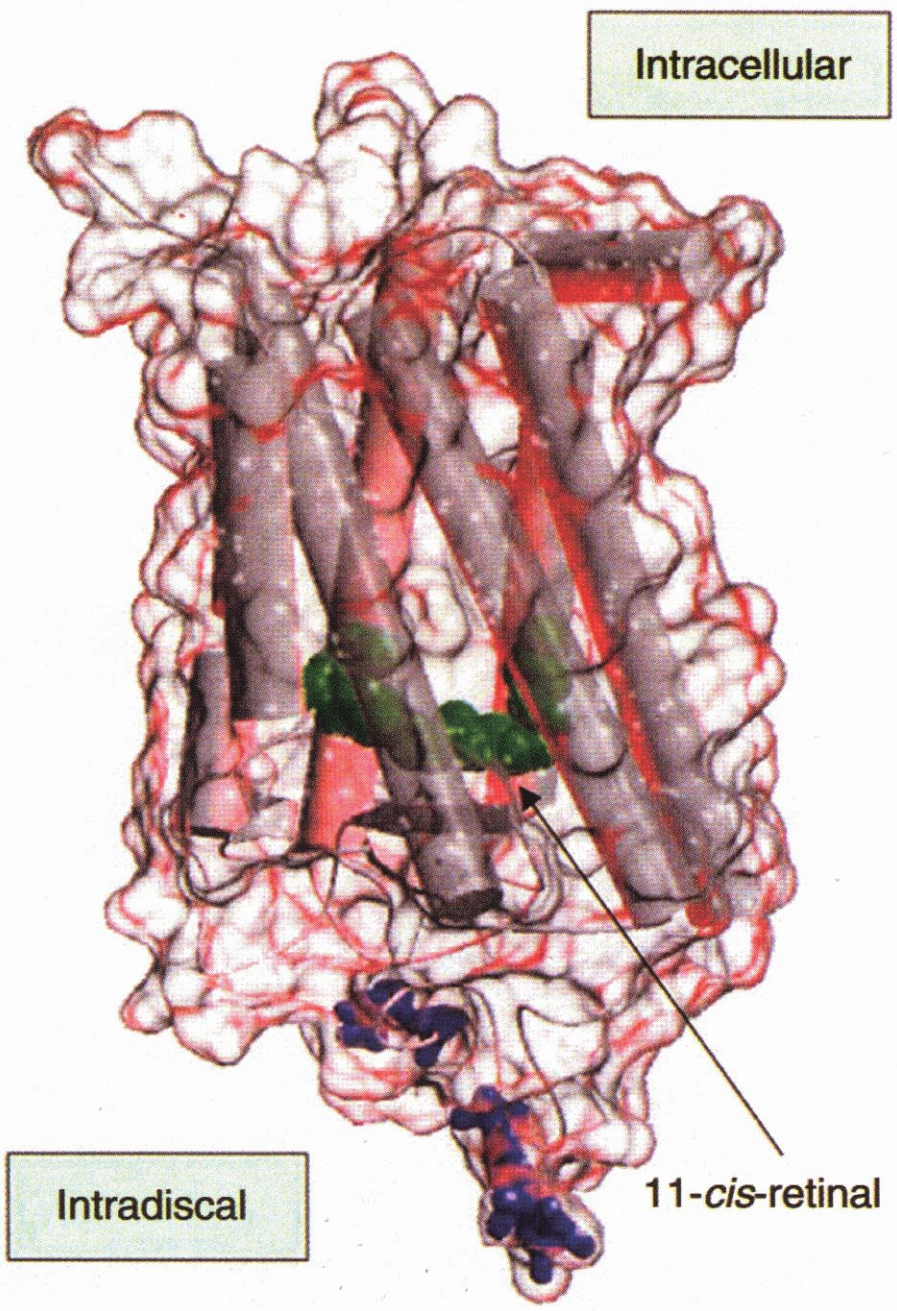


Figure 7.15. **Rhodopsin** crystal **structure. Three-dimensional** structure of rhodopsin based on X-ray crystallography (186). Note that **all-*trans*-retinal** is protected from the **intradiscal** side by multiple structural elements, including several β **strands.** Carbohydrates are in blue, 11-cis-retinal is in green, and helices **are in gray.** Red shadows **are** added for esthetic reasons. This **figure** was generated by C. **Behnke** (University of Washington) and is reprinted with permission from *Prog. Retin.* Eve *Res.,* **20, 469–521** (2001).

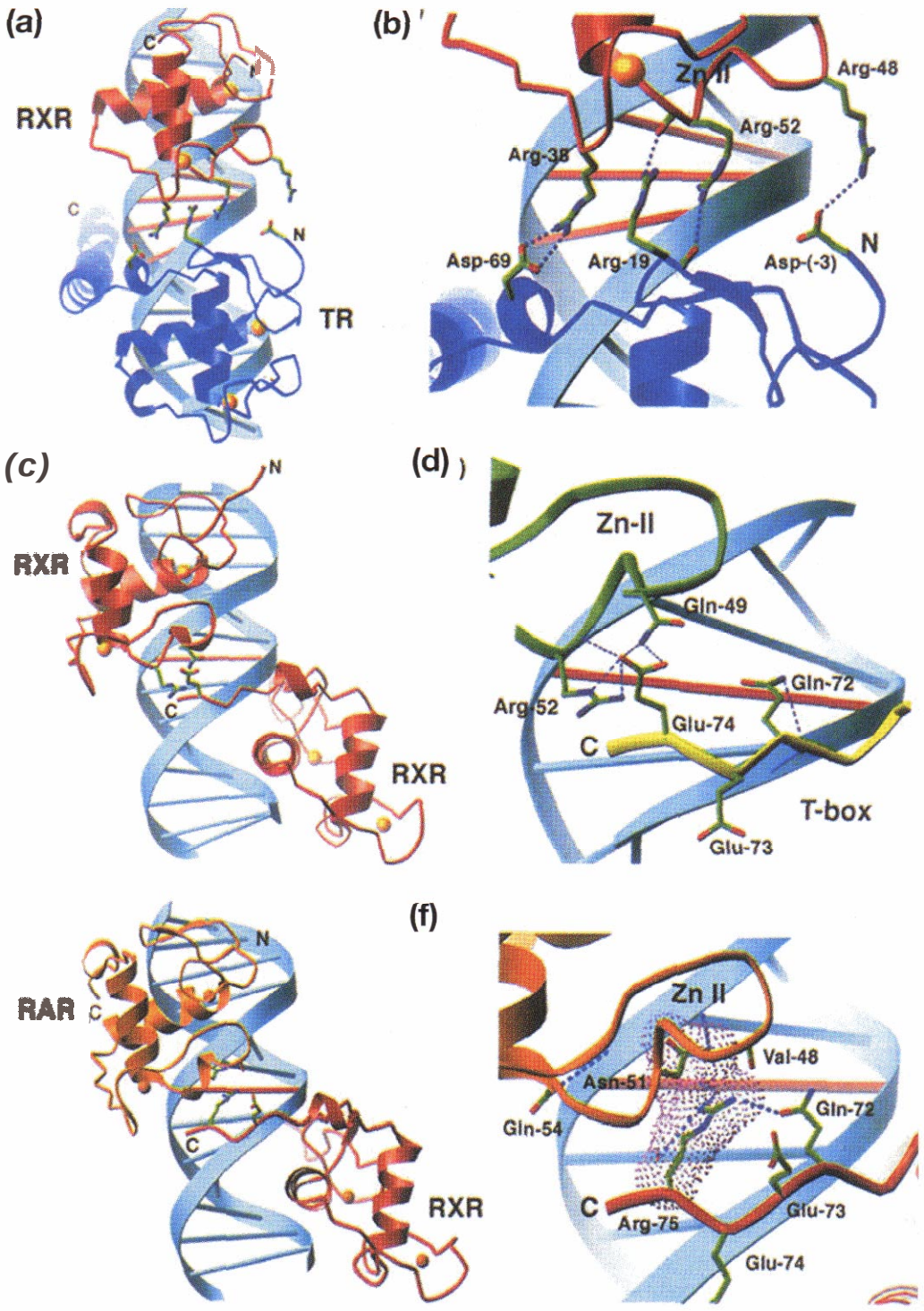


Figure 7.21. Schematic representation of DNA binding domains. Structures of DNA-binding complexes involving RXR and their dimer interfaces. The overall structures are shown on the left, with close-up views of the protein–protein interactions shown on the right. Dotted blue lines indicate hydrogen bonds between proteins or between proteins and the DNA spacing. The dotted surface indicates complementary van der Waals interactions. DNA sequences (cyan) are shown with their 5' ends pointing up, with base pairs belonging to the spacing element of the DRs shown schematically in red. In each case, protein–protein contacts are formed directly over the minor groove of the spacing, with several protein–DNA phosphate contacts stabilizing the assembly. The interacting amino acid side-chains are shown in green, with nitrogen atoms indicated in blue and oxygen atoms indicated in red. (a and b) The RXR-TR DBD heterodimeric complex with DR4; (c and d) the RXR DBD homodimeric complex with DR1, and (e and f) the RXR-RAR DBD heterodimeric complex with DR1. Reprinted with permission from *Curr.* Opin. ***Struct.*** Biol., **11,** 33–38 (2001).

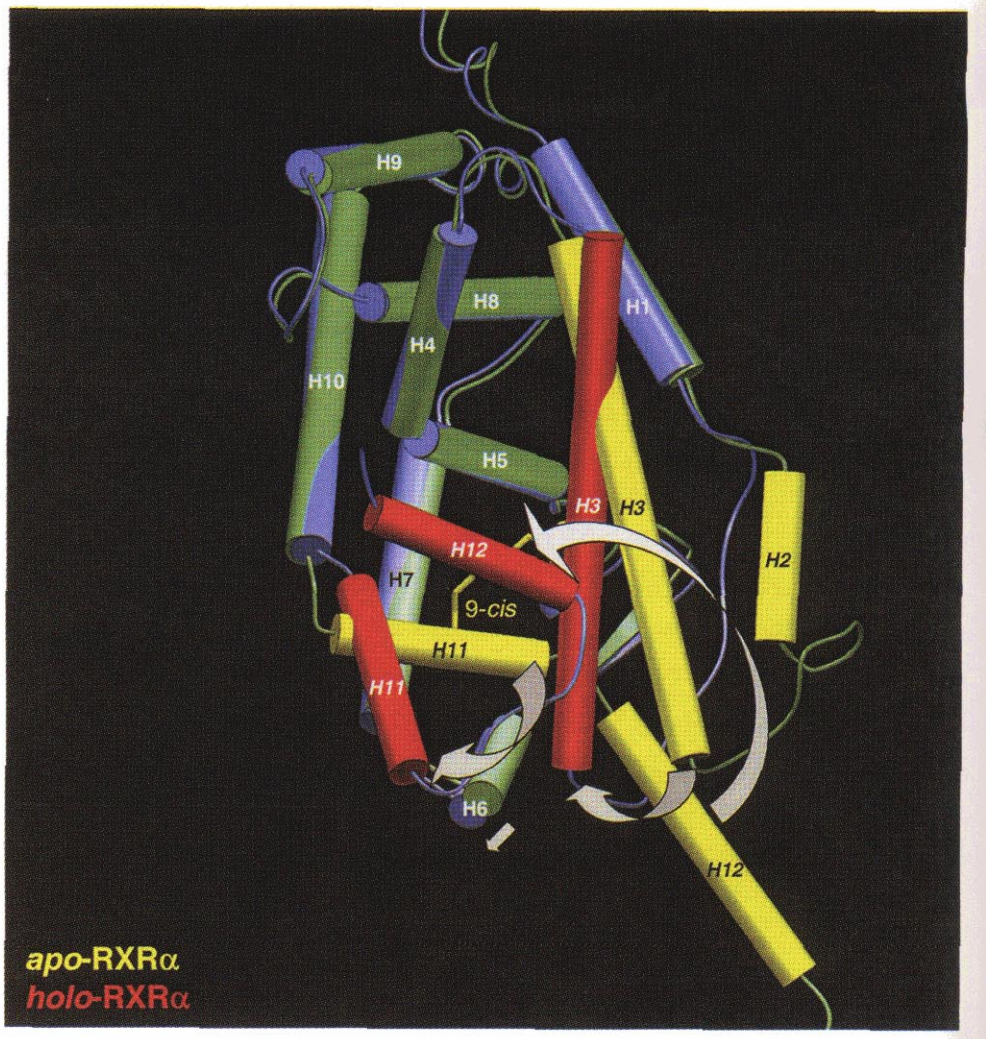


Figure 7.22. Schematic representation of apo- and holo-RXR ligand binding domain. Helices 1–12 (H1–H12) are indicated. Helices indicated in yellow and red represents the apo- and holo-forms of the receptor, respectively, whereas helices indicated in blue and green are positioned similarly in both forms of the receptor. The arrows represent movement of the helices to accommodate 9-cis-RA in the binding pocket (indicated). This figure was kindly supplied by Dr. Pascal Egea.

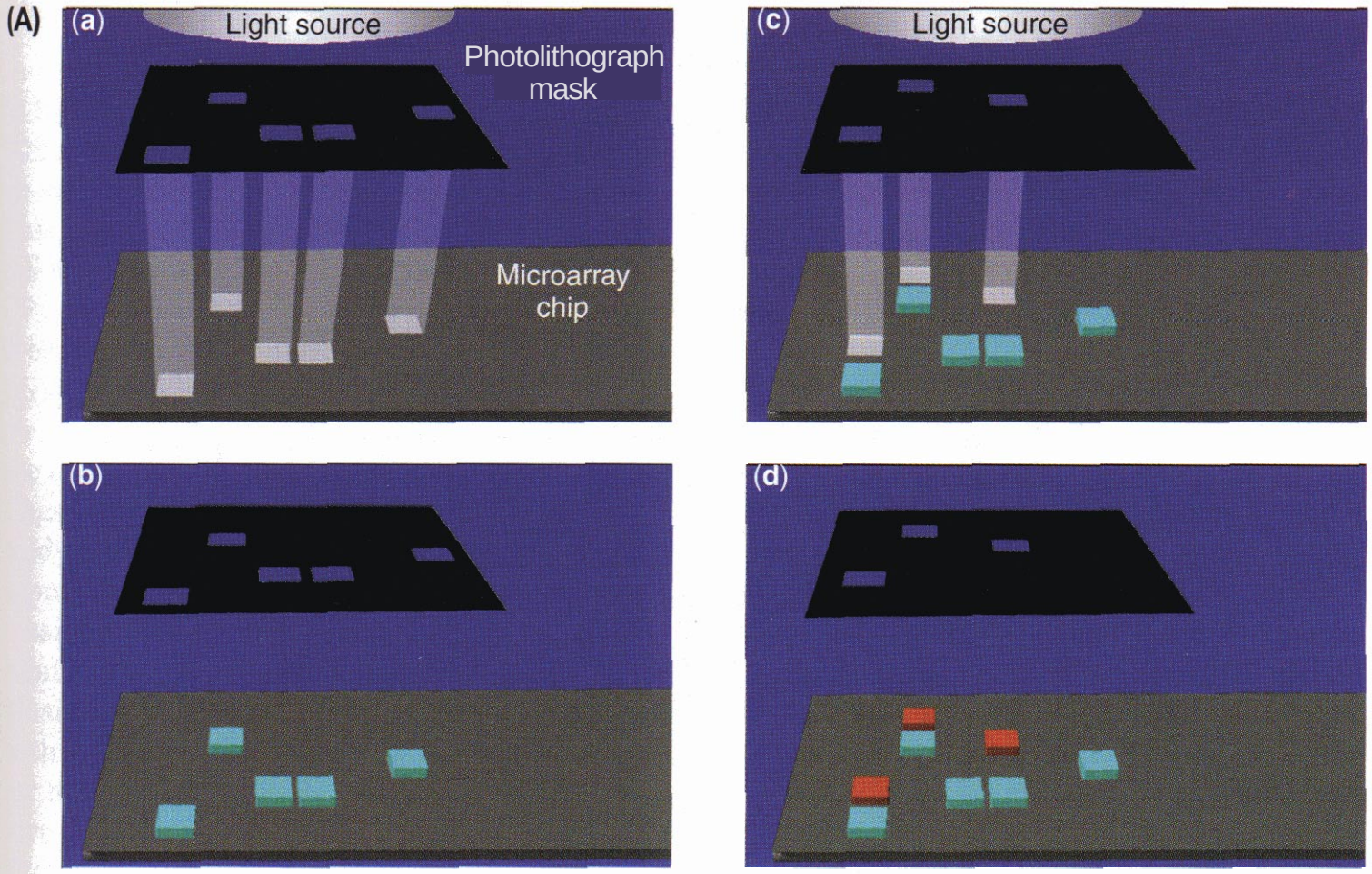


Figure 11.2. Photolithographic process for on-chip synthesis of oligonucleotides. A: the steps in this process in two cycles of nucleotide addition. **A** lithographic mask and light source are shown in panel a that results in exposure of specific spots on the microarray chip. The light activates reactive groups on the chip such that nucleotide coupling can occur only on the specific spots as shown in panel b. The nucleotide added is shown by green blocks and is thymidine for this example. Panel c shows a new mask allowing exposure of new spots to light in a second round of light activation of reactive groups. Panel d shows the next round of nucleotide addition in which the red blocks represent the next nucleotide, for example adenosine. This process is repeated until all spots have the desired DNA sequence.

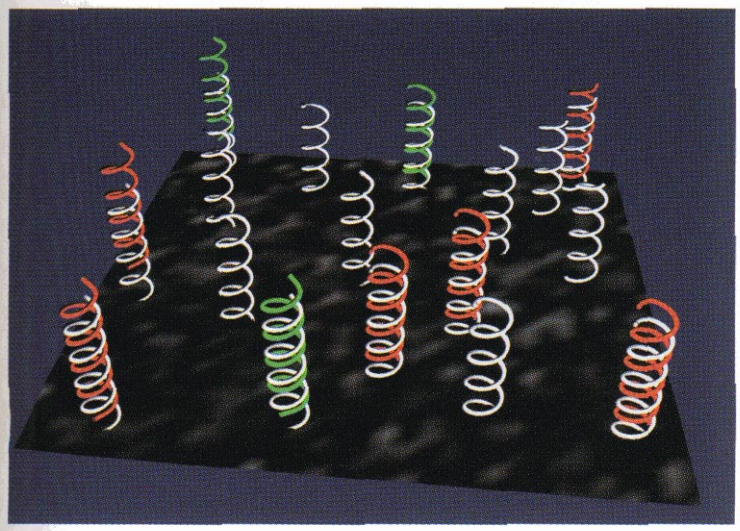


Figure 11.3. Hybridization of two differentially labeled cDNA sequences to arrayed DNA. This diagram shows a single array spot with probe DNA strands (in gray) bound to the chip. cDNA sequences from two different cell samples, one labeled with Cy3 (green) and one labeled with Cy5 (red), and hybridized to the spotted DNA.

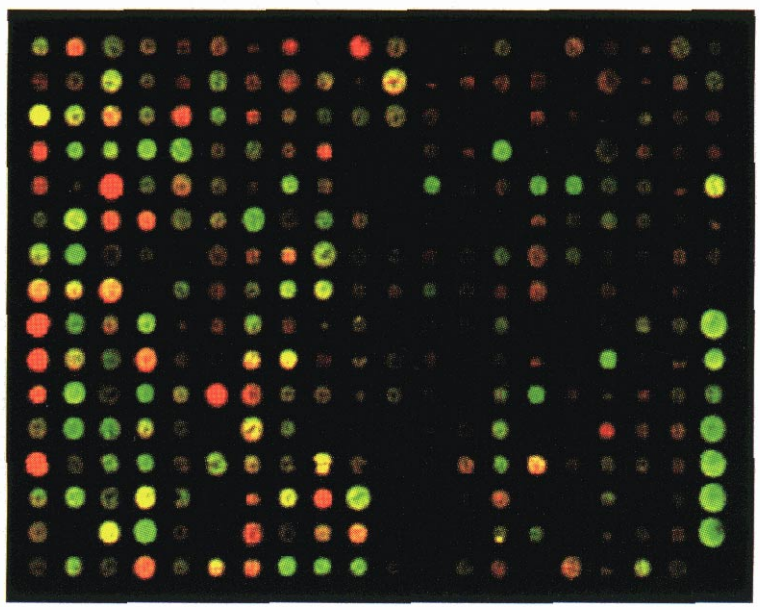


Figure 11.4. A spotted DNA array with two-color detection of hybridization. An example of a spotted DNA array (16 × 20 elements) is shown after hybridizaton with two differentially labeled cDNA preparations, Cy3 (pseudo-colored green) and Cy5 (pseudo-colored red). The overlaying of the green and red images produces the image shown. The hue of each spot, ranging from green to red, indicates the relative expression level for the gene specific for each spot (image courtesy Packard Biochip Technologies).

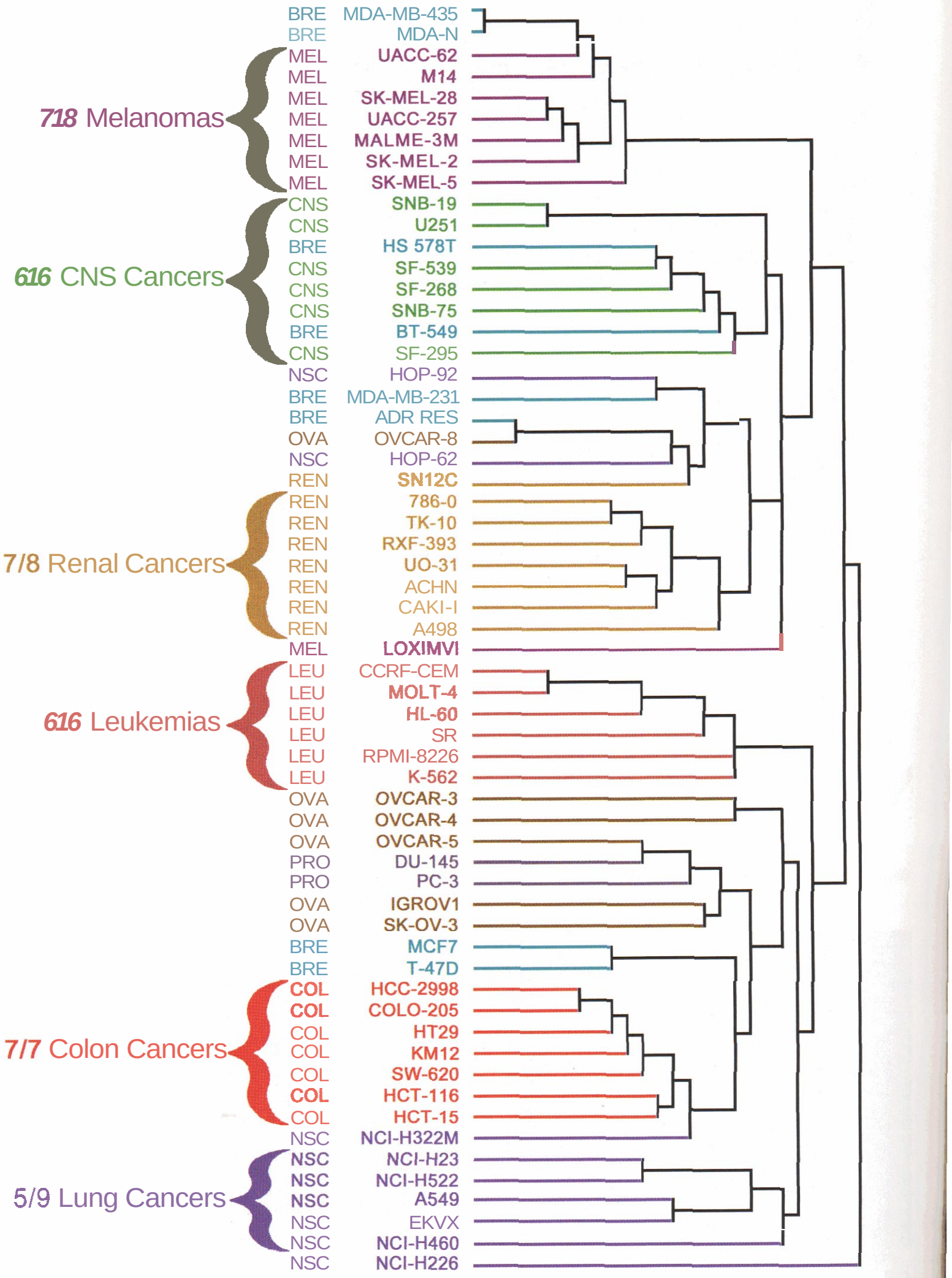


Figure 11.5. Cluster analysis of the NCI **60** cell lines. Gene expression data from the Ross et al. (12) study was filtered to contain only genes with complete data for all cell lines and genes with the highest variance across cell lines. Only 548 genes were selected for this analysis based on genes with variance of greater than twice the variance for the entire dataset. The cluster distance or similarity calculation was based on the Pearson correlation coefficient, and clusters were linked by average linkage using the software SPSS. Most cell lines can be seen to cluster based on tissue of origin, shown by the labels on the left and the fraction of the total number of cell lines for each tissue. The abbreviations for tissue types are as follows: BRE, breast cancer; CNS, central nervous system cancers; COL, colon cancers; LEU, leukemias; MEL, melanomas; NSC, non-small cell lung cancers; OVA, ovarian cancers; PRO, prostate cancers; REN, renal cancers.

to mouse glomeruli of the kidney (65). Furthermore, it seems that this receptor may be upregulated in response to cell stimulation. This has been demonstrated in a macrophage cell line in response to lipopolysaccharide (69). EP, receptor knockout mice have also been used to further highlight the functions of PGE_2 in the cardiovascular system. For example, Kennedy and colleagues (70) examined the effects of PGE_2 and receptor selective analogs in both wild-type and $EP_2^{-/-}$ mice. In wild-type animals, the EP_2 receptor selective agonist, butaprost, evoked a transient hypotension, which was not observed in mice deficient in the EP, receptor. However, the hypertensive effects of sulprostone were similar in both groups of animals. Hence, the EP_2 receptor may be important, in the mouse vasculature at least, in mediating some of the vasodilatatory responses to PGE_2.

EP_3 receptors are probably the best studied of all the EP receptors. They are present in gastrointestinal, uterine, and vascular smooth muscle, where they cause smooth muscle contraction. In autonomic nerves they cause inhibition of neurotransmitter release, and in adipocytes, they inhibit lipolysis (71). In gastric mucosal cells, they inhibit gastric acid secretion (72), and E series prostaglandins are well-characterized stimulators of mucus and bicarbonate secretion. Several PGE analogs have been developed to exploit this role as it was thought that EP_3 receptor agonists might be useful in the treatment of gastric ulceration and also prophylaxis of the side effects associated with long-term use of aspirin and other NSAIDs (see Section 4.2.4). In renal medulla they regulate the inhibition of water reabsorption (73). Distribution studies using Northern blotting and in *situ* hybridization reveal mRNA expression in the kidney and uterus, as well as the stomach, spleen, brain, and lung (74). Furthermore, this receptor subtype has been localized to the tubules in the medulla and also the macula densa and distal tubules within the kidney itself (75). The receptor distribution has also been examined in neuronal tissue, and has been reported in both dorsal root ganglion and trigeminal ganglion tissue and also within the brain in areas including the hypothalamus, hippocampus, locus coeruleus, and raphe nuclei (75, 76). Within the hypothalamus, EP_3 receptor transcripts have been detected in cells surrounding the organum vasculosa lamina terminalis (OVLT), and EP_3 receptor activation here has been associated with fever generation. E series prostaglandins are potent fever inducers when injected into the brain, and their involvement in the central mediation of fever was proposed as early as 1970 (77). At the spinal cord level, it has been suggested that PGE, may induce thermal hyperalgesia through EP, and EP_3 receptors (78). Furthermore, it has been proposed that brain-derived PGE_2, at lower doses, produces hyperalgesia through its actions on EP_3 receptors, whereas at higher doses, hypoalgesia is produced through actions on EP, receptors (79–81). Further evidence for involvement of EP_3 in mediating the hyperalgesic effects of PGE_2 comes from Xin and colleagues (82). They report that the intracerebroventricular injection of GR63799X (an EP_3 receptor agonist) caused fever and hyperalgesia in rats, which could be blocked by an anti-sense, but not a mis-sense oligonucleotide directed against the EP_3 receptor. In addition, the use of EP knockout mice has suggested that only mice lacking the EP_3 receptor failed to exhibit febrile responses to PGE_2, interleukin-1β, and lipopolysaccharide injections, thus providing further evidence for EP_3 receptor involvement in mediating the febrile response (83).

The most recently characterized EP receptor, the EP, receptor, also shows a widespread distribution and is found in most tissues examined. It has been shown to exist in piglet saphenous vein (29) and also in the jugular vein of the rabbit, hamster uterus, and rat trachea (84–86). In the kidney, it is expressed in the glomerulus, mediating the effects of PGE_2 on glomerular filtration. Within the brain, EP_4 receptor mRNA has been found in the hypothalamus and lower brain stem. It has also been suggested that the EP_4 receptor may be involved in bone remodeling and the induction of osteoclasts that are involved in bone resorption. For example, Sakuma and colleagues (87) found that osteoclast formation was stimulated most potently by PGE, analogs that display agonist activity at EP_4 receptors. Although it has been suggested that EP_4 receptors are important in maintaining a

patent ductus arteriosus (88), $EP_4^{-/-}$ mice died from heart failure within 3 days of birth of pulmonary congestion and a fully patent ductus (89). This result remains to be explained, given the previously demonstrated dilatatory effects in the ductus of EP_4 receptor activation.

It is also interesting to note that, given the clinical use of exogenous PGE, (dinoprostone) in cervical ripening and labor induction (see Section 5), the expression of the different EP receptors has been shown to alter with time in mice undergoing pseudopregnancy (90).

A role for E (and I) series prostaglandins in inflammatory pain is well established, not least because of the anti-nociceptive effects of NSAIDS, but also because of observations that exogenous prostaglandins can induce allodynia and hyperalgesia (91). In the periphery, sensitizing effects of PGE, have been recorded on several aspects of sensory nerve function, including ion channels and neuropeptide release. The EP receptors involved in mediating these effects remain poorly characterized, but these responses have been correlated with increases in cyclic AMP, which would be suggestive of EP,, EP_4, or possibly, EP, receptor involvement (see Section 3.4). Further studies will hopefully address these issues and may aid in the design of future anti-nociceptive medicines.

2.3.3 Prostaglandin $F_{2\alpha}$. Although PGF,, can act through EP and TP receptors, it is particularly potent at FP receptors. FP receptors are mostly concentrated in corpora lutea, where, in domestic animals, they mediate luteolysis stimulated by PGF,,. It has also been shown that, like uterine EP receptors, FP receptor expression changes during the oestrus cycle, becoming most abundant just before the luteal cells undergo apoptosis (92). In addition, FP receptor–deficient mice do not undergo parturition even after the administration of oxytocin. Therefore, it seems that FP receptor activation is required for normal parturition and labor in mice (93). This function of PGF,, is used widely in veterinary practice, and the receptors also seem to be present in this capacity in humans. However, initial hopes for FP receptor–specific agonists being useful in controlling human fertility (94) were dashed, because, unlike in animals, prostaglandins are not central in the destruction of the corpus luteum. Hence, $PGF_{2\alpha}$ and its analogs have proved ineffective as human luteolytics. However these agents are used successfully in animal husbandry to help synchronize the oestrus cycles of farm animals.

Functional FP receptors have also been demonstrated in the myometrium of some rodents and also humans (95, 96). However, in dogs, FP receptor agonists are lethal—probably as a result of the presence of contractile FP receptors on airway smooth muscle (61). This has yet to be reported in any other species. FP receptors are also present in iris sphincter muscles, and the ocular actions mediated by these receptors have been exploited to lower intraocular pressure and in glaucoma treatment in humans.

Messenger RNA distribution studies have identified FP receptor transcripts in the uterus, further confirming the role of $PGF_{2\alpha}$ in this tissue. In *situ* hybridization has also revealed that expression in the ovary is confined to the large luteal cells of the corpus luteum (97). No labeling was observed in the ovarian follicle. Receptor mRNA was also found in the kidney, lung, heart, and stomach, and these studies are entirely consistent with the actions of $PGF_{2\alpha}$ in mediating luteolysis and mesangial cell contraction in the glomerulus of the kidney (98).

2.3.4 **Prostaglandin I,.** The primary role of prostacyclin is thought to be in the local control of vascular tone and also the inhibition of platelet aggregation (99). It is synthesized mostly by vascular endothelial cells. In agreement with this, IP receptors have been located in both platelets and vascular smooth muscle cells (100). IP receptor knockout mice, generated by homologous recombination, did not display a hypotensive response to the potent, selective IP receptor agonist, cicaprost (see Section 4), but their basal blood pressure was unaltered when compared with controls. The mice survived normally, but following endothelial damage, an increase in thrombosis was observed, lending weight to assertions that PGI_2 acts as an antithrombotic agent (67).

IP receptors have also been found on nervous tissue. PGI, is a potent hyperalgesic, act-

ing on peripheral sensory neurones and may be more potent than PGE_2 in this respect, at least in rodents (101). IP receptors have been localized to specific subsets of dorsal root ganglion (DRG) neurons (102). In *situ* hybridization signals were detected in 40% of DRG neurons (60% small; 15–25 μm) but not in glia. Seventy percent of IP-positive neurons also contained mRNA for preprotachykinin A (PPTA), a substance P precursor. In addition, around 25, 41, and 24% of IP-positive neurons co-expressed EP$_1$, EP$_3$, and EP$_4$ transcripts, respectively, suggesting a possible overlapping role of IP and EP receptors in mediating pain sensation.

Mice lacking IP receptors have also been used to suggest a role for PGI_2 in inflammation. For example, following carageenan administration, $IP^{-/-}$ mice exhibited paw swelling that was similar to that observed in wild-type mice treated with indomethacin (67). Similar studies have also further suggested a role for prostacyclin in mediating peripheral hyperalgesia (67).

2.3.5 Thromboxane A$_2$ As far as is known, TXA_2 acts exclusively through TP receptors. TP receptors exhibit a widespread distribution in platelets and vascular smooth muscle, where they mediate platelet aggregation and also smooth muscle contraction (103,104). TP receptors are also present in airway smooth muscle (105), where they mediate bronchoconstriction. Several studies have examined the distribution of TP receptor mRNA. In the mouse, TP receptor transcripts have been detected abundantly in the thymus, spleen, and lung (106). In human tissues, TP receptor transcripts were found in the placenta and the lung (107). Therefore, it seems that TP receptors are present in immune related organs (e.g., spleen and thymus) as well as those rich in smooth muscle, such as the lung. Ushikubi and colleagues (108) have further investigated the TP receptor population in the thymus. Using radioligand binding, they found TP receptor expression was greatest in immature CD4–8– and CD4+8+ cells, and that receptor expression decreased as cells matured. They also suggested the presence of functional TP receptors on immature thymocytes, as the addition of a TP receptor agonist induced apoptosis in this cell population that was sensitive to TP receptor antagonism. Hence, in addition to roles in the cardiovascular and respiratory systems, TP receptors may also act in thymocyte development.

Recently, mice deficient in TP receptors have been generated to further elucidate potential roles of thromboxane A$_2$. These $TP^{-/-}$ mice exhibited an increased tendency to bleed and were unresponsive to the intravenous injection of the TP receptor specific agonist, U46619 (109). The increase in bleeding tendency has also been reported in human patients with a point mutation (Arg-60 to Leu) in the first cytoplasmic loop of their TP receptor (110). These patients display a defective platelet aggregatory response to TXA_2, which further suggests a role for TP receptors in hemostasis.

3 PROSTANOID RECEPTOR CLASSIFICATION AND CHARACTERIZATION

The general scheme proposed by Kennedy and colleagues (see Section 1) is still in use today (1), and has been confirmed and expanded by the use of modern techniques in molecular biology, which have made possible the cloning and molecular characterization of all the known prostanoid receptors.

The current system terms prostanoid receptors P receptors, which is preceded by a letter to indicate the most potent natural prostanoid at that particular receptor (hence the receptor at which PGD$_2$ is the most potent natural agonist is called the DP receptor and so on). This system was based initially upon a rigorous quantitative comparison of the agonist potencies of each of the natural prostanoids in tissue preparations chosen to avoid the problems associated with multiple receptor types. In addition, being aware of the limitations of agonists in receptor classification (111), efforts were made to identify specific prostanoid-receptor antagonists. A summary of the current state of prostanoid receptor classification is shown in Table 6.2.

Table 6.2 Second Messenger Couplings, Amino Acid (AA) Numbers, and Chromosomal Locations of the Cloned Prostanoid Receptors

Receptor	Subtype	Isoforms	Receptor Coupling	Species	Number of AA[a]	Chromosomal Location	Refs.
DP	DP_1	1		Human	359	N.D.	49
			G_s, ↑ cAMP	Mouse	357	14	112
				Rat	357	N.D.	113
EP	EP_1	2		Human	402	19p3.1	114,115
			↑ $[Ca^{2+}]_i$ through unidentified G-protein	Mouse	405	8	64
				Rat	405	N.D.	116
	EP_2	1		Human	358	5p13.1	117
			G_s, ↑ cAMP	Mouse	362	N.D.	68
				Rat	357	N.D.	116
	EP_3	10		Human	365	1p31.2	118
			G_i, ↓ cAMP[b]	Mouse	365	3	119
				Rat	366	N.D.	116,120
	EP_4	1		Human	488	5p13.1	121
			G_s, ↑ cAMP	Mouse	513	15	122
				Rat		N.D.	116
FP		2		Human	358	1p31.1	123
			G_q ↑ $[Ca^{2+}]_i$ with ↑ PI turnover	Mouse	366	3	97
				Rat	366	N.D.	123
IP	IP_1	1		Human	386	19q13.3	124,125
			G_s/G_q and ↑ cAMP/↑ IP_3	Mouse	417	7	126
				Rat	416	N.D.	127
TP	TP_1	2		Human	343	19p13.3	107
			G_q, ↑ $[Ca^{2+}]_i$ with ↑ PI turnover	Mouse	341	10	106
				Rat	341	N.D.	128

[a]Owing to C-terminal splicing, amino acid numbers are variable for EP_3 receptors. The AA numbers shown are for the $hEP_{3\text{-}III}$ receptor, the $mEP_{3\alpha}$ receptor, and $rEP_{3\alpha}$ receptors.

[b]Multiple C-terminal splice variants of EP_3 receptors exist that couple to different G-proteins. For example, bovine EP_3 receptors can couple to G_i, but also to G_s and G_q.

PI, phosphatidylinositol; ↑, increase; ↓, decrease.

3.1 Prostanoid Receptor Subtypes and Isoforms

Although the subclassification of EP receptors is well accepted and verified by numerous studies, the evidence for subtypes of DP, IP, and TP receptors is less clear. There is some evidence for two subtypes of DP receptor (129, 130), although this has yet to be proven unequivocally. However, recently, it has been shown that PGD, may act through a novel, putative chemoattractant receptor, termed CRTH2, which is preferentially expressed in human T helper type 2 cells, eosinophils, and basophils (131). This receptor is particularly interesting in that it is not a member of the general prostanoid receptor family; rather, it

is related to other chemoattractant receptors, such as that for *N*-formylmethionyl-leucyl-phenylalanine (fMLP). Although PGD, clearly has affinity for this receptor, it is not clear whether it is the natural ligand, and so for the time being, its identification as a ***bone fide*** prostanoid receptor must remain tentative. There is also some functional evidence for a subdivision within IP receptors (19, 132). For example, Wise and colleagues (133) found the PGI, mimetic, BMY45778, to be a potent and full IP receptor agonist in human platelets and rat neutrophils. In contrast, this compound was a weak partial agonist in the rat colon, suggesting the possible existence of more than one subtype of IP receptor.

Although it is unclear whether there are several subtypes of TP receptors (134, 135), this receptor does seem to have at least two isoforms, generated by alternative splicing, both of which have been cloned in humans (136). These differ in the lengths of their C-terminal tails distal to Arg-328. Similar alternative splicing has been reported in both EP, (1), EP_3 (137), and FP receptors (138). This has been particularly well characterized in the case of the EP_3 receptor. The EP_3 receptor exists in several splice-variants, and it has been proposed that at least six splice-variants could exist in each species. These variants all occur at the same relative amino acid in the C-terminus of the EP, receptor, thus altering the length of the C-terminal tail. This has recently been shown to alter potential signal transduction pathways and localization of the EP_3 receptor (118, 139, 140). Despite these differences, the binding affinities for the natural prostanoids seem similar, and it is unclear at present whether other EP receptor selective ligands display differences in affinity and agonist potency between different EP_3 receptor splice-variants, because no detailed comparative studies have been carried out under similar experimental conditions.

3.2 Prostanoid Receptors as G-Protein–Coupled Receptors

The first prostanoid receptor to be cloned was the TXA_2 receptor (107), and since then, homology screening has been used to clone prostanoid receptors from several species, including mice, rats, humans, and cows (137). Hydrophobicity and homology analyses of these receptors have revealed them to have seven putative transmembrane domains, suggesting they are all G-protein–coupled, rhodopsin-like receptors (GPCRs) belonging to the main subclass (2.1) (141). Overall homology between the different receptor types is quite low (20-30%), whereas homology between species orthologues is much higher (70–90%) (137). A summary of the gene locations and amino acid lengths of the cloned prostanoid receptors is shown in Table 6.2. Recent reports have also suggested that functional prostanoid receptors are present at the nuclear envelope, as well as at the cell surface. For example, nuclear EP_3 receptors can induce increases in intranuclear Ca^{2+} and inducible nitric oxide synthase (iNOS) gene expression (142, 143).

Despite the structural differences in receptor subtypes, strong conservation exists in several areas. There are 28 residues conserved around the putative transmembrane domains, 8 of which are shared by other families of GPCRs, and these are believed to be involved in structure/function relationships of GPCRs in general. Thus, two cysteine residues in the first and second intracellular loops are conserved, and these may form a disulphide bridge necessary for the stabilization of receptor conformation to allow ligand binding (144). Also conserved is an aspartate, in the second transmembrane region, which has been shown in other receptors to be important in coupling ligand binding to G-protein activation (145). Concensus sequences for *N*-glycosylation are also present, for example, in the second extracellular loop of EP_3 and EP, receptors. In addition, serine and threonine residues, putative phosphorylation sites, are widely distributed in the cytoplasmic portions of the receptors and may play a role in receptor desensitization, as has been reported for human TP receptors (146,147).

Specific to the prostanoid receptors, the most conserved region is the seventh transmembrane domain, where the sequence L-X-A-X-R-X-A-SIT-X-N-Q-I-L-D-P-W-V-Y-I-L is shared. Two further sequences, G-R-Y-X-X-Q-X-P-G-T/S-W-C-F and M-X-F-F-G-L-X-X-L-L-X-X-X-A-M-A-X-E-R, are also present in the second extracellular loop and third transmem-

brane domain, respectively. These have been suggested to be involved in prostanoid binding, with particular attention being paid to the conserved Arg in the seventh transmembrane domain, which is located at the analogous position to the retinal binding site, Lys^{296}, of the rhodopsin receptor. It was suggested that this residue might serve as the binding site for the ω-carboxyl group of prostanoid molecules (148). Site-directed mutagenesis experiments have also been conducted to elucidate the binding sites for various prostaglandin receptor-selective ligands, and these aspects will be covered in more detail (Section 4).

Computer-assisted sequence analyses have been used to construct phylogenetic trees to infer the evolutionary pathways of the prostanoid receptors. For example, Toh and colleagues (149) examined the molecular evolution of the lipid mediator receptors. They reported prostanoid receptors forming a distinct cluster within the rhodopsin-like GPCRs. In contrast, the receptors for platelet activating factor and lipoxygenases were in another cluster with the receptors for tachykinins and bradykinin. Moreover, the prostanoid receptor cluster could be further subdivided (Fig. 6.3). Hence, the $DP/EP_2/EP_4$ and IP receptors, which predominantly exert their effects through increasing intracellular cAMP levels, seem to be more closely related to each other than to EP_1/FP and TP receptors, which predominantly act through increasing intracellular Ca^{2+}, or to EP_3 receptors, which, although often causing decreases in intracellular cAMP, have been reported to act through multiple signaling pathways (see Section 3.4.4). Other groups (117, 149) have also proposed similar evolutionary pathways. These studies are consistent with the proposal that the PGE system and receptor may have evolved before the individual EP receptor subtypes, which mediate specific signaling pathways. Additionally, the construction of phylogenetic trees suggests that the prostanoid receptors evolved separately from the receptors for other lipid-signaling molecules.

3.3 Radioligand Binding Studies

The proposed scheme of receptor classification has been further supported by the existence of high affinity binding sites for each natural prostanoid, both in native tissues and heterologous systems. These studies have also been furthered by the generation of radioligands from ligands that show selectivity for particular prostanoid receptors, such as the recent development of [^{3}H]-BWA868C to detect DP receptor-like binding sites (150).

3.3.1 PGD_2-Specific Binding Sites. [^{3}H]-PGD, binding sites have been identified in human platelets, and these sites are bound, with high affinity, by the DP receptor selective agonist, BW245C (see Section 12). In contrast, BW245C does not displace [^{3}H]-prostacyclin binding from human platelets, suggesting that the binding sites are different. Likewise, the other natural prostaglandins show lower affinities in displacing [^{3}H]-PGD_2 binding than PGD, or BW245C (151,152). Thus, these data are consistent with functional studies suggesting that platelets, in humans at least, contain DP receptors. Binding sites for [^{3}H]-PGD_2 have also been reported in synaptic membranes from the rat (153). Recently, [^{3}H]-BWA868C has been reported and characterized as a new radioligand to aid the identification of PGD, binding sites (150). This radioligand showed high affinity binding in human platelets that were displaced by other ligands known to act through the DP receptor. Under the same conditions, EP, FP, IP, and TP receptor selective ligands were poor competitors, with K_i values in the micromolar range. This could be an important advance, because the use of a specific DP receptor antagonist should allow the closer correlation of affinities and potencies from functional studies, without the added complications of differing agonist efficacies between preparations.

3.3.2 PGE_2-Specific Binding Sites. The use of [^{3}H]-PGE_2 as a radioligand has proved useful in the determination of PGE, binding sites that may or may not represent functional EP receptors. However, because of the non-selective nature of this ligand and also the lack of truly selective EP receptor agonists and antagonists, this approach to subclassifying EP receptor subtypes has been limited. Nevertheless, high affinity binding sites ($pK_d > 8$) have been reported in adipocytes, brain, dorsal root ganglia, kidney,

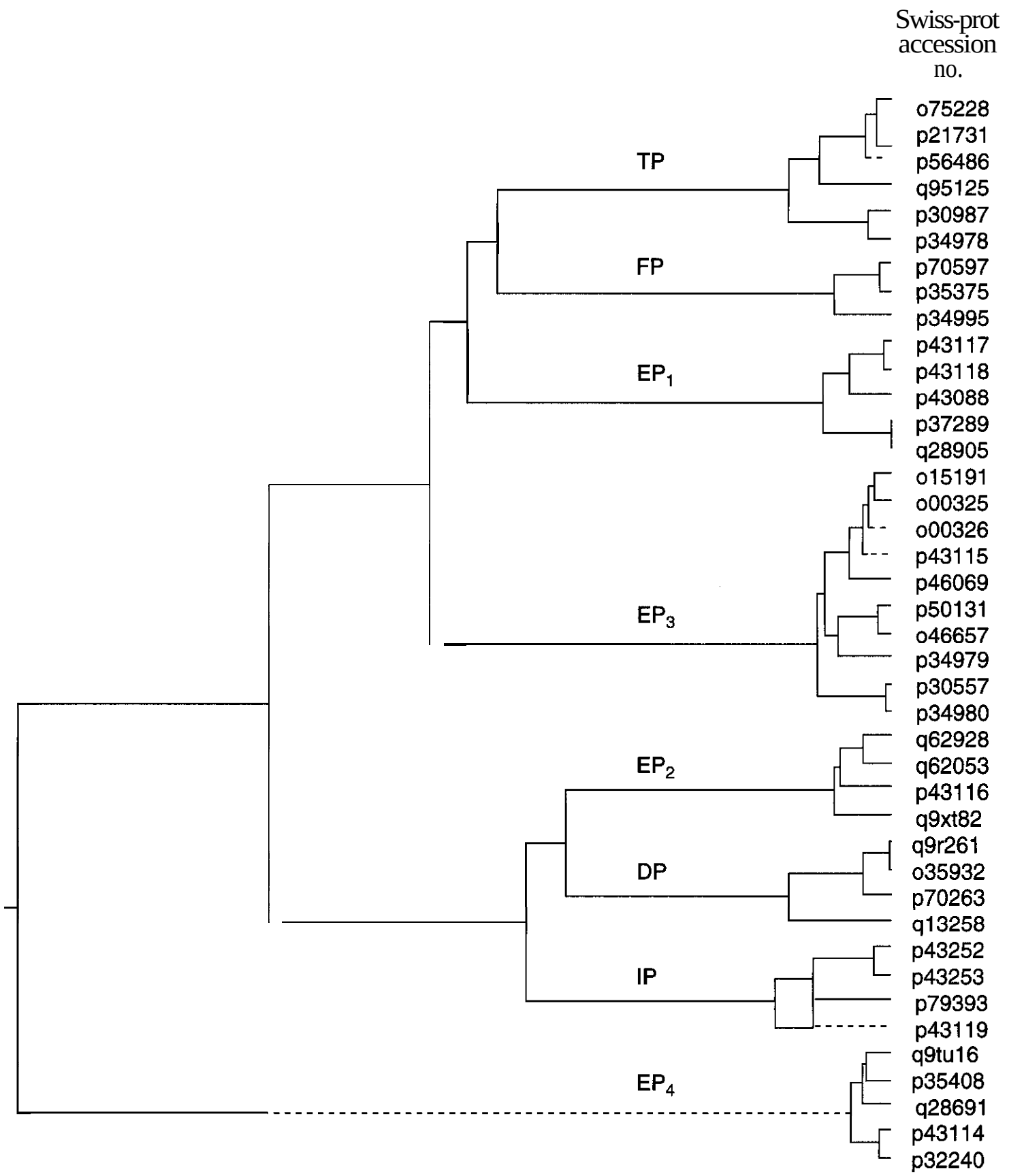


Figure 6.3. Molecular grouping of the prostanoid receptors. The dendogram was constructed from sequence comparison of the published sequences of the cloned prostanoid receptors from various species. The sequences used are identified by their accession numbers shown within the figure. The length of the solid lines represent evolutionary diversity.

corpus luteum, myometrium, gastrointestinal tract, liver, heart, and platelets **(32).** Furthermore, in some cases, PGE analogs have been used to attempt to identify the EP subtype involved. For example, competition studies using [^{3}H]-PGE_2 and sulprostone or AY23626 conducted in membranes prepared from rat adipocytes and renal collecting tubules have suggested the presence of EP, receptors. Encouragingly, the binding affinities obtained under these conditions can be correlated to the relative agonist potencies obtained for these compounds in other prototypical EP,, EP,, and EP, receptor–containing tissues.

3.3.3 $PGF_{2\alpha}$-Specific Binding Sites. The majority of studies using [^{3}H]-$PGF_{2\alpha}$ have been conduced on the corpora lutea of several species, including the rat (154), rabbit (155), and sheep (156). The detection of high affinity binding sites in these areas is entirely consistent with the effects of PGF$_{2\alpha}$ as a luteolytic agent in several species of animal and suggests the presence of functional FP receptors in mediating prostaglandin-induced luteolysis. In addition, this binding is displaced specifically by unlabeled $PGF_{2\alpha}$, and studies using fluprostenol and cloprostenol, both highly selective and potent PGF analogs (see Section 4.3), reveal a good correlation between binding affinity and agonist potency at FP receptors. In addition, when expressed in recombinant systems, there seems to be a good correlation between rank orders of affinity between recombinant FP receptors and those believed to be present in corpora lutea (157). [^{3}H]-17-phenyl $PGF_{2\alpha}$ has also been reported as a radioligand useful in the determination of FP receptor binding sites (158).

3.3.4 PGI_2-Specific Binding Sites. IP receptors have been identified in a wide variety of tissues, and PGI binding sites reflect this. Hence, binding studies have been performed on platelets, vascular smooth muscle, and nerve cells, as well as the guinea pig lung, generally using [^{3}H]-prostacyclin, [^{3}H]-iloprost, or [^{3}H]-PGE_1 as the radioligand (159, 160).

In all the preparations studied, a similar order of agonist potency was obtained in competition studies. Hence, iloprost $\cong$ PGI_2 > PGE_1 (10-fold weaker) > PGD_2 = PGE_2 = $PGF_{2\alpha}$ (32). Furthermore, if PGD_2, for which there are known binding sites on human blood platelets (161), is omitted, there is a clear association between the rank order of competitive potency and the relative potencies of these agonists to inhibit platelet aggregation. These data thus suggest that the [^{3}H]-PGI_2 binding sites identified in the above tissues are likely to be IP receptors of the same type.

In recombinant systems, the rank orders of competitive potency for a wide range of prostaglandin receptor ligands are similar to those obtained in platelets and other tissues, thus providing further evidence for the existence of IP receptors in these tissues (126, 162–164).

3.3.5 TXA_2-Specific Binding Sites. Radioligand binding studies have been performed using both TP receptor selective agonists and antagonists. Agonists used include [^{3}H]-U44069 (165), [^{3}H]-U46619 (166), and [^{125}I]-I-BOP (167), and antagonists used include [^{3}H]-13-APA, [^{125}I]-PTA-OH, and more recently, [^{3}H]-SQ29548 (168), [^{3}H]-GR32191B (169), and [^{125}I]-S-145 (170), all of which have pK_d values of >8. The latter compounds are particularly potent TP receptor blockers and are thus ideally suited for use in radioligand binding studies. Several studies have been performed on platelets, and in general, a good correlation in ligand potencies has been observed between functional and binding studies. For example, in human platelets, [^{3}H]-U44069 binding was displaced by U46619 and also TXA_2 (171). Similarly, identical rank orders have been obtained for the TP receptor antagonists, SQ29548, ONO3708, BM13177, and 13-APA in human platelets using both functional and ligand binding techniques (166). Interestingly, the binding properties of [^{3}H]-SQ29548 and [^{3}H]-GR32191B (vapiprost) seem to reflect some of their pharmacological properties in man. Thus, [^{3}H]-SQ29548 shows rapid association and dissociation kinetics (168), whereas [^{3}H]-GR32191B exhibits slow dissociation kinetics (169), which correlates with this compound's pharmacological profile in platelets where it seems to act as a partially insurmountable receptor blocker (172). The binding properties of recombinant TP receptors have also been extensively examined and the rank orders of affinity obtained are in good agreement with those obtained in membranes derived from endogenous TP receptor–containing tissues (157,164).

3.3.6 Summary. Thus, it can be seen that specific binding sites for all of the main natural prostaglandins have been identified in both membranes prepared from tissues and also in recombinant systems. These studies, showing binding sites at which each of the natural prostanoids displays the highest binding affinity, are consistent with the proposed system of receptor classification. Likewise, the good correlation between the orders of affinity in competition studies and agonist potency from functional studies suggest that binding sites

identified are functional receptors and that radioligand binding is a useful tool to aid in receptor identification and classification.

3.4 Signal Transduction

The coupling of prostanoid receptors to intracellular signalling pathways has been examined by several methods using both recombinant systems and in *vitro* preparations to determine the Gproteins and second messengers involved.

3.4.1 DP Receptors. DP receptors couple through G_s to increases in intracellular cyclic AMP. Indirect evidence for this was obtained by Simon and colleagues (173), who showed that D, E, and I series prostaglandins all stimulated adenylate cyclase in human colonic mucosa. These early observations have been confirmed by studies using recombinant DP receptors, where it has been demonstrated that both PGD, and the DP receptor selective agonist, BW245C, concentration dependently increase cyclic AMP levels in Chinese hamster ovary (CHO) cells expressing recombinant DP receptors (49, 112).

3.4.2 EP, Receptors. The G-protein involved in EP_1 receptor signaling has yet to be identified (1). Studies in recombinant cells suggest the involvement of Ca^{2+} mobilization. Watabe and colleagues (64) showed PGE, caused an increase in intracellular [Ca^{2+}] in CHO cells expressing this receptor, an event that was dependent on extracellular Ca^{2+} and accompanied by a very weak inositol trisphosphate (IP_3) response. This agrees with Creese and Denborough (174) who showed a similar extracellular Ca^{2+} effect in guinea pig trachea, a preparation known to contract through EP_1 receptors (175).

3.4.3 EP, Receptors. EP_2 receptors are coupled to G_s and mediate increases in intracellular cAMP concentrations. Evidence for this was first obtained by Hardcastle and coworkers, who reported a positive association between EP, and cAMP generation in enterocytes (176) and more recently from recombinant systems.

3.4.4 EP, Receptors. EP_3 receptors have been shown to couple to several different signal transduction systems. The major coupling is to G_i to reduce adenylate cyclase activity, but there are several reports of differential coupling of splice variants, including coupling to increases in intracellular calcium (139). This is consistent with responses observed in tissues, where EP_3 receptor activation can inhibit gastric acid secretion (see below) and lipolysis, two actions classically mediated by decreases in cellular cAMP. In addition, it also seems that EP_3 receptors can mediate smooth muscle contraction, consistent with increases in intracellular Ca^{2+} (27). In addition, the EP_3 receptor seems to be involved in the inhibition of arginine vasopressin water reabsorption through a pertussis toxin-sensitive pathway, suggesting the involvement of G_i (177, 178). Likewise, the EP_3-mediated inhibition of acid secretion in the stomach is also pertussis-sensitive (72).

Four EP_3 receptor isoforms have been cloned from cDNA generated from a bovine adrenal gland, and these display coupling to multiple second messenger pathways. Thus, the bovine EP_{3A} isoform couples to G_i, the EP_{3B} and EP_{3C} isoforms to G_s and the EP_{3D} isoform to G_s, G_i, and G_q (179). Isoforms of the mouse EP_3 receptor, $EP_{3\alpha}$ and $EP_{3\beta}$, differ in their responses to GTPγS and also in potency at inhibiting forskolin-induced cAMP accumulation, with EP3γ requiring threefold lower concentrations of agonist than $EP_{3\beta}$ to evoke a 50% inhibition (119).

3.4.5 EP, Receptors. This receptor subtype, like the EP, receptor, is known to couple to G_s and mediate increases in intracellular cAMP levels (116). There is some confusion in the early literature regarding the identity of the "true" EP_4 receptor. The "EP_2" subtype, originally cloned by Honda and colleagues (122) and Bastien and co-workers (121) has since been identified as the EP_4 receptor, on the basis of its insensitivity to the EP_2 receptor–selective agonist, butaprost (see Section 4), and sensitivity to the EP_4 receptor antagonist, AH23848B. The EP_2 receptor cloned by Regan and colleagues (117) is regarded as the "true" EP, receptor subtype.

3.4.6 FP Receptors. This receptor type mediates increases in inositol triphosphate for-

mation through G_q and phospholipase C activation. It has been known for some time that stimulation with $PGF_{2\alpha}$ is coupled to increases in phosphoinositide turnover and the elevation of intracellular calcium (180, 181). In mouse fibroblasts, the effect of $PGF_{2\alpha}$ to elevate intracellular calcium is pertussis toxin–insensitive, suggesting a lack of involvement of G_i, and correlates with IP_3 formation (182). Similar results have been obtained using recombinant FP receptors, where FP receptor activation resulted in a concentration-dependent increase in IP_3 formation (97).

3.4.7 IP Receptors. IP receptors mediate an increase in cAMP levels through G_s (163), but recombinant studies in CHO cells have also revealed a phosphatidyl inositide response that is cholera- and pertussis toxin–insensitive, suggesting the involvement of G_q (126).

3.4.8 TP Receptors. TP receptors are generally regarded as signaling through G_q to cause an increase in the concentration of intracellular calcium (183). However, there are reports that TP receptors may also couple to G_{11}, G_{12}, and G_{13} (184–186). Furthermore, there have also been reports that TP receptor splice variants, while both coupling to G_q, may interact differently with adenylate cyclase. Thus, the $TP\alpha$ receptor isoform induces increases in intracellular cAMP, whereas the $TP\beta$ isoform induces decreases in intracellular cAMP levels (187).

4 PROSTANOID RECEPTOR-SELECTIVE LIGANDS AND STRUCTURE-ACTIVITY RELATIONSHIPS

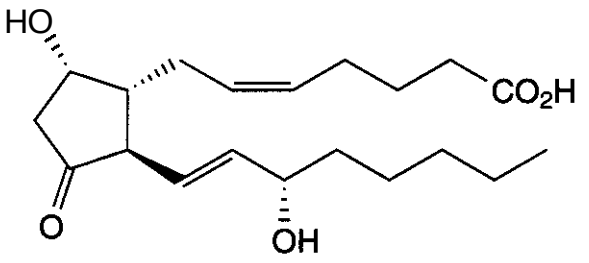
From the desire to produce novel therapeutics, several ligands that are selective for subtypes of prostaglandin receptors have been developed, and these are detailed below.

4.1 DP Receptors and Selective Ligands

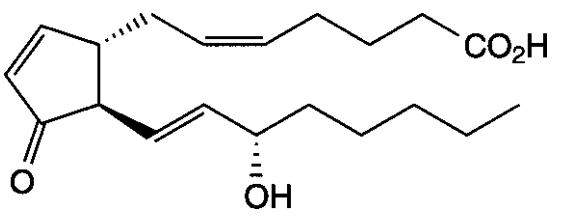
The structures of some DP receptor ligands are shown in Fig. 6.4. PGD, is not an inherently selective agonist; it also has FP and TP receptor activity (188). The dehydration product of PGD_2, PGJ_2 (9-deoxy-Δ^9-PGD_2), (189) is equipotent to the natural agonist in producing vasodilation and inhibiting of platelet aggregation and more selective with respect to EP and FP receptor activity. PGJ_2 has also been shown to be of similar potency to PGD_2 in heterologous systems (190). Furthermore, PGJ_2 and its derivative, 15-deoxy,12,14-PGJ_2, are also agonists at the peroxisome proliferator-activated receptor-gamma ($PPAR\gamma$) (191). The most widely described DP receptor agonist is the synthetic prostanoid, BW245C (192). This is a hydantoin prostanoid analog that is at least one order of magnitude more potent than PGD, as a DP receptor agonist, but is considerably less active at TP and FP receptors. It displaces [^{3}H]-PGD_2, but not [^{3}H]-prostacyclin binding form in bovine platelets (152), and its inhibitory action on human platelets can be blocked by the DP receptor antagonist, AH6809. Furthermore, BW245C has been shown to potently inhibit the aggregation of human platelets and relax the rabbit transverse stomach strip (193). Another DP receptor selective agonist is the 9-chloro analog of PGE_2, ZK110841 (194). SQ27986 has also been described as a potent and highly selective DP receptor agonist (195). Interestingly, introducing a 15-cyclohexyl group into the a-side chain of PGD_2 and other prostaglandins seems to enhance DP receptor-like selectivity. Finally, and most surprisingly, is RS93520. This is an analog of PGI,, but is far more potent as a DP receptor agonist, being only a modest IP receptor agonist on the human platelet (196). Recently, L-644698 has been described as a novel DP receptor selective agonist (190), and it has been reported to be at least 300-fold more selective for human DP receptors over human EP_2 receptors and more than 4000-fold more selective over the other human recombinant prostanoid receptors. It also exhibits similar efficacy to PGD, and BW245C, as evaluated by the accumulation of cAMP in recombinant cells, suggesting this may prove a useful tool in DP receptor characterization in the future. The agonist and antagonist potencies of some ligands active at the DP receptor are shown in Table 6.3 and affinity constants at both mouse and human recombinant receptors in Table 6.4.

The first compounds with recognized DP receptor blocking activity were the phloretin

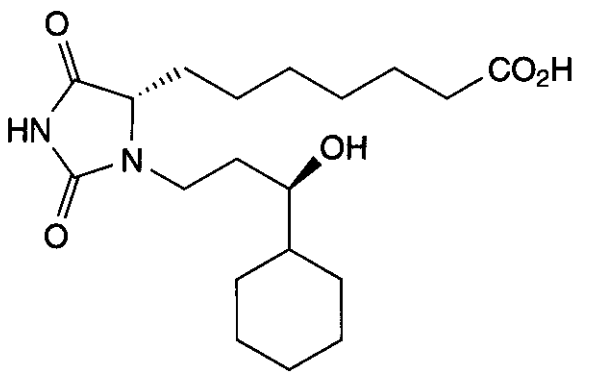
Agonists



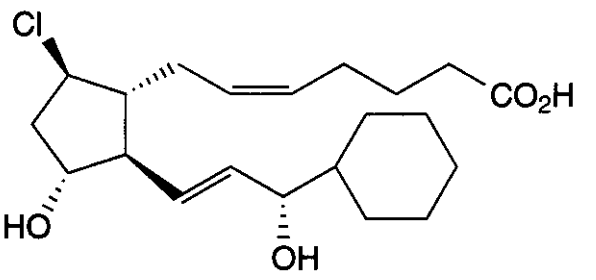
PGD_2



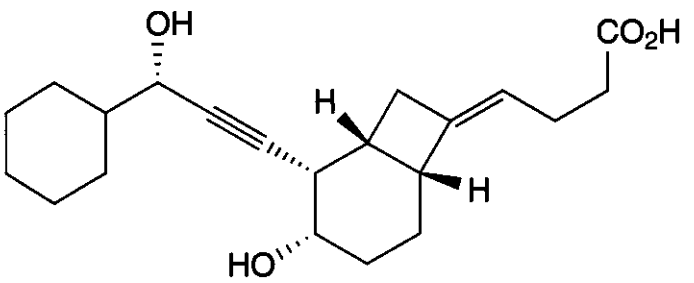
PGJ_2



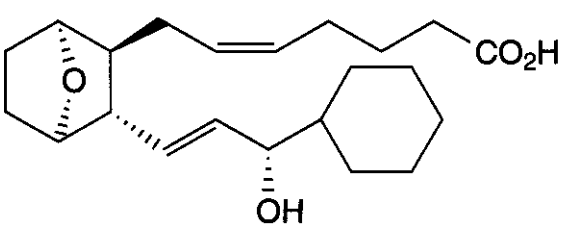
BW245C



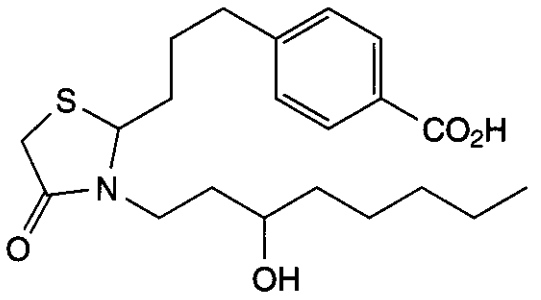
ZK110841



RS93520

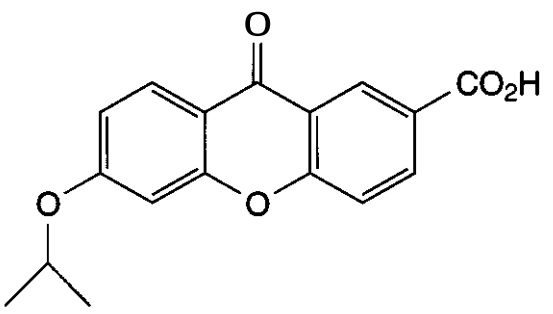


SQ27986

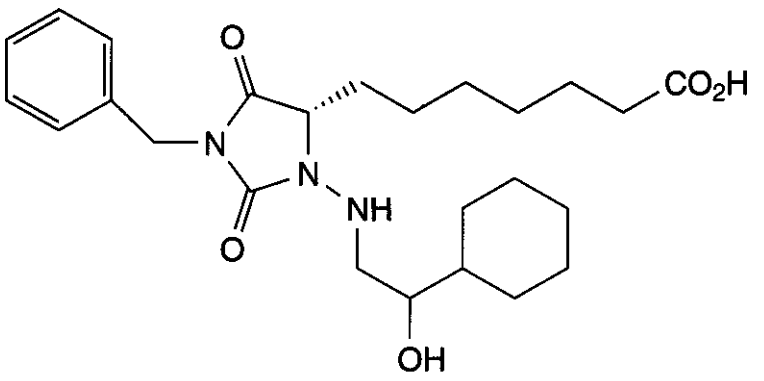


L644698

Antagonists



AH6809



BWA868C

Figure 6.4. The structures of some DP receptor-selective ligands.

Table 6.3 The Potencies of Some DP Receptor Agonists and Antagonists

Agonists	Equieffedive Concentration Relative to PGD_2 (=1.0)	Refs.
BW245C	0.03–0.7	49, 112, 152, 193
ZK110841	0.2–1.0	190, 194
RS93520	1.0	196
SQ27986	0.3	195, 197
L-644698	1–6	56, 173
Antagonists	**pA_2**	**Refs.**
AH6809	6.0–6.6	198, 199
BWA868C	9.3	200

derivatives, N-0164, N-0057, and N-0161. N-0164 antagonized the anti-aggregatory actions of PGD, and BW245C on human platelets and also inhibited the concomitant PGD_2-induced cAMP elevation (201). However, N-0164 also possesses activity in other systems. For example, it antagonizes PGE_2- and $PGF_{2\alpha}$-induced gastrointestinal smooth muscle contraction (202) and has also been shown to have inhibitory effects on thromboxane synthase (203) and cAMP phosphodiesterases (204), in addition to blocking TP receptors

Table 6.4 Affinities of Some Prostanoid Receptor Agonists and Antagonists (*) at Recombinant Human and Mouse DP, FP, IP, and TP Receptors

	Human [K_i (nM)]				Mouse [K_i (nM)]			
Ligand	DP	FP	IP	TP	DP	FP	IP	TP
PGE_2	307	119	**	>10000	***	***	***	***
SC19220*	N.D.	N.D.	N.D.	N.D.	***	***	***	***
Butaprost FA	>10000	**	>10000	>10000	***	N.D.	N.D.	N.D.
Butaprost ME	>10000	>10000	>10000	>10000	N.D.	***	***	***
Sulprostone	**	198	**	**	***	580	***	1300
M&B28767	\g10000	510	\g10000	343	***	124	***	***
GR63799	>10000	1241	>10000	>10000	***	***	***	***
Enprostil	>10000	88	>10000	>10000	***	***	***	***
Misoprostol FA	>10000	>10000	**	**	***	N.D.	N.D.	N.D.
Misoprostol ME	**	**	**	**	N.D.	***	1000	***
11-deoxy-PGE_1	N.D.	N.D.	N.D.	N.D.	***	N.D.	N.D.	N.D.
AH23848B*	1380	>10000	>10000	592	N.D.	***	***	***
PGD_2	1.7	6.7	**	6602	21	47	***	***
BW245C	0.4	>10000	>10000	>10000	250	1700	***	***
ZK110841	0.3	1670	2138	1121	***	***	***	***
BWA868C*	N.D.	N.D.	N.D.	N.D.	220	***	***	***
$PGF_{2\alpha}$	861	3.2	>10000	8700	***	3.4	***	***
Fluprostenol	**	2.3	**	>10000	***	3.8	***	***
Cloprostenol	>10000	0.47	**	6123	N.D.	N.D.	N.D.	N.D.
Latanoprost	555	**	**	>10000	N.D.	***	11	***
Iloprost	1035	619	11	6487	***	***	10	***
Cicaprost	>1340	>1340	17	>1340	***	***	110	***
Carbaprostacyclin	132	360	282	>10000	***	***	***	67
U46619	3970	241	>10000	35	1000	***	***	13
SQ29548*	**	**	**	4.1	***			

Human data taken from Abramovitz et al. (157). Mouse data from Kiryama et al. (164).
**K_i estimate > 100,000 nM.
***Unable to displace 50% of their radioligand at 10 μM.

(205). Indeed, N-0164 was first identified as an EP receptor blocker, where it antagonizes PGE, and $PGF_{2\alpha}$-induced smooth muscle contractions (200). AH6809 is also a DP receptor blocker, but this compound also exhibits antagonist activity at TP, EP_1 (and human EP_2) receptors (164, 206), having an estimated pA_2 value of 6.5 in both cases. A more potent and selective DP receptor antagonist was described by Giles and colleagues (200): BWA868C is a derivative of the DP receptor agonist, BW245C and is the most useful DP receptor antagonist currently available. It has also been shown to possess activity *in vivo* (207). This compound displays potent antagonist activity against the functional effects of PGD_2 in human platelets (208), rabbit jugular and saphenous vein (200, 209), and human myometrium (210). Recently, S-5751 has also been described as a DP receptor selective antagonist (211).

[^{3}H]-BWA868C has also recently been described (195). This radioligand displayed high affinity binding in human platelets and the DP receptor selective ligands described above all exhibited high affinities in competition experiments in the same tissue. Ligands active at the other prostanoid receptors were, in contrast, poor competitors. Thus, it seems that this antagonist may prove useful for future DP receptor characterization and autoradiographic studies.

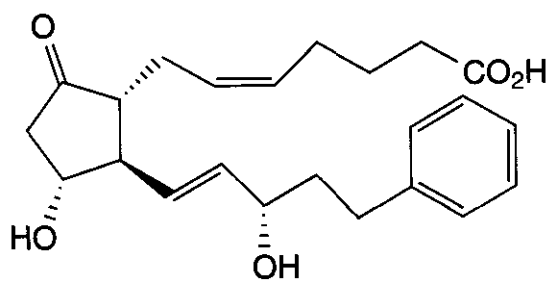
4.2 EP Receptors and Selective Ligands

4.2.1 General Considerations. The identification of PGE analogs with selective functional profiles illustrates the progress that has been made into determining structure-activity relationships for EP receptor selectivity, and it seems that further structural features confer EP receptor subtype selectivity. For EP agonist activity, the length of the a-chain is crucial, with the alteration of this by a single carbon atom conferring loss of EP receptor selectivity (212). Substitutions at C-1 seem to reduce agonist potency at EP_2 receptors. Hence replacing the carboxylic acid group with for example, esters, methanesulfonamido, and imino groups, seems to maintain agonist potency at EP_1 and EP, receptors only (213, 214). In contrast, substitutions at C-2 to C-6 considerably reduce agonist potency at EP receptors. Furthermore, an alkynic link between C4 and C5 reduces EP_3 agonist potency, whereas an interphenylene at C-3–C-7 increases selectivity for the EP, receptor (215, 216). The cyclopentyl ring also seems critical for EP receptor selectivity, because substitution with a cyclohexyl group produces a significant loss of activity at EP receptors in general (212). Likewise, a carbonyl group at C-9 is preferred for EP receptor agonist activity, although a methylene group allows EP_1 and EP_3 receptor activity (217–219). The hydroxy group at C-11 seems critical for EP_1 receptor activity, but not at other EP receptors. However, removal of this group seems to decrease agonist potency at all EP receptor subtypes and may also result in a relative increase in TP receptor agonist activity.

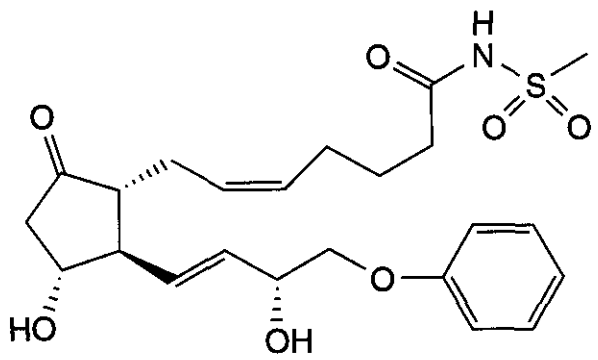
The length of the ω-chain does not seem crucial in conferring EP receptor agonist activity, and there are several cases in which the length of this chain has been altered without inducing differences in agonist activity (32). Likewise, the C-13–C-14 double bond is not critical, as long as it is present in the *trans* conformation. In contrast, a hydroxy group at C-15 is necessary for EP receptor agonist activity, but the presence of this group at both C-15 and C-16 can reduce EP, receptor potency. When a methyl group is introduced at C-15 or C-16, TP receptor activity seems to increase (220). The substitution of the final two to four carbon atoms of the methyl chain with a cyclic structure also seems to confer some EP receptor selectivity, with 17-phenyl-trinor-PGE, probably one of the best-documented examples. This agonist has some EP_1 receptor selectivity (32).

Recently, Ungrin and colleagues (221) performed a study to determine the structure activity relationships of 55 prostanoid-like compounds at the recombinant human EP_1 receptor. They examined the effects of these analogs on both receptor binding and activation. On the basis of this, they suggested that the C-11 and C-15 hydroxy group are essential for agonist activity, along with the presence of a carbonyl group at C-9, rather than an ester moiety. Furthermore, they found that the alteration of the ω-tail could enhance the potency of otherwise weak compounds. In sum-

Agonists

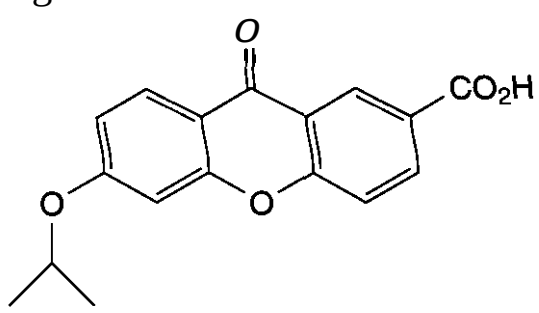


17-phenyl-trinor-PGE_2

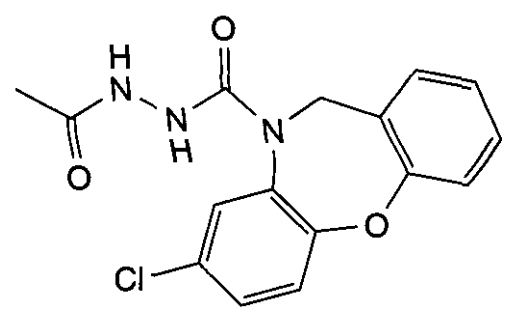


sulprostone

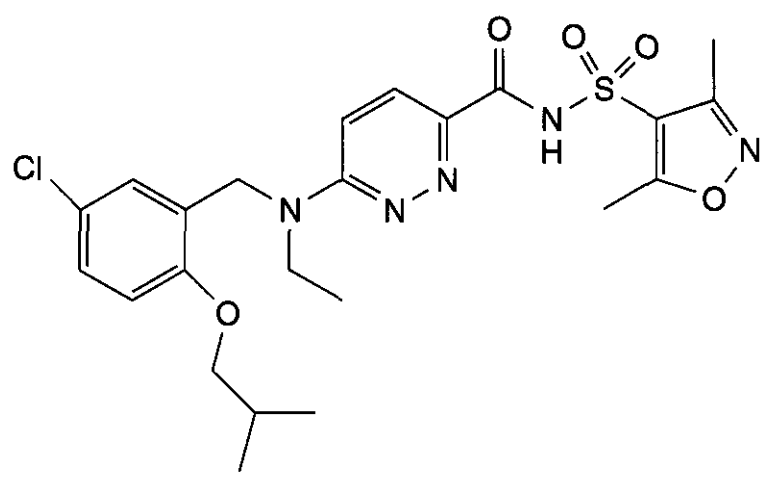
Antagonists



AH6809



SC-19220



ZM325802

Figure 6.5. The structures of some EP, receptor-selective ligands.

mary, in conferring EP receptor selectivity, the following elements are important: an α-chain of seven carbon atoms, a cyclopentyl ring substituted at C-9, preferably with a carbonyl group, and a hydroxy group at C-15 or C-16.

4.2.2 EP, Receptor-Selective Ligands. The structures of some EP, receptor selective ligands are depicted in Fig. 6.5. To date, no highly selective, potent EP_1 receptor agonists have been described, and it is only recently that potent EP_1 receptor antagonists have been reported. 17-phenyltrinor-PGE_2 (see above), 16,16-dimethyl PGE_2, ICI 80205, and 9-methylene PGE_2 all show moderate EP_1 receptor selectivity but are also active at other EP receptors (222). ICI 80205 also has TP receptor activity. Sulprostone was also identified as an EP_1 receptor selective agonist, but it is actually more potent as an EP_3 receptor selective ligand (157). Iloprost is a stable analog of PGI_2, but this behaves as a potent partial agonist at EP, receptors. Thus, compounds do not have to be PGE derivatives to display EP_1 agonist activity. Iloprost is also relatively selective for EP_1 receptors over the other EP receptor subtypes, displaying very little functional activity in EP_2 and EP_3 containing preparations.

Several compounds have been synthesized that demonstrate EP_1 receptor blocking activ-

ity in functional studies. The first of these compounds to be described is the weak antagonist SC-19220 (223), with pA_2 values of 5.2-5.6 in EP, receptor–containing preparations. This compound and its derivatives have been tested for EP_1 antagonist activity in both the guinea pig ileum and the rat fundus. It was found that increasing the length of the acetyl chain increases potency but reduces selectivity. SC-19220 has also been found to be active in *vivo*, showing antinociceptive effects in a variety of animal pain tests (224). However, SC-19220 is highly insoluble and has also been shown to exhibit some local anesthetic activity (225). Although SC-19220 is regarded as a weak competitive EP_1 antagonist on the basis of functional studies, it has negligible binding affinity for the recombinant mouse EP_1 receptor (164). AH6809 also shows weak EP, blocking activity, although it is more potent than SC-19220. However, this compound is also a blocker of the human EP_2 receptor (206) and DP receptors. No binding of this compound to the recombinant mouse EP, receptor was detected, and at the human receptor, AH6809, displayed similar weak affinities for EP_1, EP_{3}, EP_4 and DP receptors (157,164). In addition, the usefulness of AH6809, especially in vivo, is compromised by its avid binding to albumin; it is approximately 97% protein bound in the presence of 4% bovine serum albumin (226). Recently, Shaw and colleagues have reported a new EP_1 antagonist, ZM325802, which they have shown to have a K_i of 0.25 nM at human recombinant EP, receptors and to be 8400-fold more selective over EP, and EP, receptors. Binding at EP_2 receptors was not determined, but this compound failed to antagonize the relaxant effect of PGE_2 in the guinea pig isolated-trachea preparation, a model of EP_2 receptor activity (63). They also found that this compound displayed anti-nociceptive properties in in vivo tests in the rat, further suggesting a possible role for EP, receptors in prostaglandin-induced hyperalgesia. A summary of the agonist and antagonist potencies, as well as the inhibition constants of ligands that have EP, receptor activity, are shown in Tables 6.5 and 6.6, respectively.

It should be noted that, whereas most of the agonists described at EP, receptors are obvious derivatives of PGE_2 (with the exception of iloprost), none of the antagonists thus far described bear any clear chemical resemblance to E series prostaglandins.

4.2.3 EP, Receptor-Selective Ligands. The structures of some compounds that are active at EP_2 receptors are shown in Fig. 6.6. Several compounds have been described that seem to be specific EP_2 receptor agonists. The first compound identified, but later found to possess some EP_3 receptor activity as well, was the PGE, analog, AY23626 (228). Misoprostol and 19(R)-OH PGE, (229) are also EP_2 receptor agonists, but the former is more potent at EP_3 receptors (222) and the latter at EP, receptors (116). Despite a lack of absolute selectivity, both these agents have been useful tools in preparations lacking EP_3 receptors. More specific for the EP, receptor are AH13205 (230) and butaprost (TR4979) (231). Butaprost, although not possessing great potency, is highly selective for the EP_2 receptor subtype

Table 6.5 Potencies of Some EP, Receptor Agonists and Antagonists

Agonists	Equieffective Concentration Relative to PGE, (=1.0)	Refs.
17-phenyl-trinor PGE,	1	227
Sulprostone	4–6	228
Iloprost	1[a]	60,116
Antagonists	**pA_2**	**Refs.**
SC-19220	5.2–5.6	226,228
AH6809	6.4–7.0	226,228
ZM325802	9.6[b]	63

[a]Partial agonist.
[b]pK_i value.

Table 6.6 Affinities of Some Prostanoid Receptor Agonists and Antagonists (*) at Recombinant Human and Mouse EP_1, EP_2, EP_3, and EP_4 Receptors

	Human [K_i (nM)]				Mouse [K_i (nM)]			
Ligand	EP_1	EP_2	EP_3	EP_4	EP_1	EP_2	EP_3	EP_4
PGE,	9.1	4.9	0.33	0.79	20	12	0.85	1.9
SC19220*	N.D.	N.D.	N.D.	N.D.	***	***	***	***
Butaprost FA	>10000	91	1643	>10000	***	110	***	***
Butaprost ME	>10000	3513	>10000	>10000	N.D.	N.D.	N.D.	N.D.
Sulprostone	107	**	0.35	7740	21	***	0.6	***
M&B28767	419	988	0.14	10	120	***	0.68	500
GR63799	329	>10000	4.77	149	***	***	1.9	480
Enprostil	82	>10000	12	>10000	***	***	***	***
Misoprostol FA	>10000	34	7.9	23	120	250	67	67
Misoprostol ME	>10000	10249	319	5499	N.D.	N.D.	N.D.	N.D.
11-deoxy PGE_1	N.D.	N.D.	N.D.	N.D.	600	45	1.5	23
AH23848B*	>10000	>10000	4419	13727	***	***	***	***
PGD,	5280	2973	421	1483	***	***	280	***
BW245C	>10000	219	>10000	132	***	***	***	***
ZK110841	1.8	6.0	−503	41	***	***	***	***
BWA868C*	N.D.	N.D.	N.D.	N.D.	***	***	***	***
$PGF_{2\alpha}$	547	964	38	288	1300	***	75	***
Fluprostenol	>10000	**	708	>10000	***	***	***	***
Cloprostenol	815	**	4.4	9137	N.D.	N.D.	N.D.	N.D.
Latanoprost	1750	>10000	6503	**	N.D.	N.D.	N.D.	N.D.
Iloprost	11	1870	56	284	21	1600	22	2300
Cicaprost	>1340	>1340	255	44	***	1300	170	***
Carbaprostacyclin	23	942	14	352	***	1600	31	2300
U46619	>10000	>10000	>10000	2013	***	***	***	***
SQ29548*	**	**	**	**	***	***	***	***

Human data from Abramovitz et al. (157). Mouse data from Kiryama et al. (164).
**K_i estimate > 100,000 nM.
***Unable to displace 50% of radioligand at 10 μM.

over other EP receptors, making it a useful experimental tool. Like misoprostol it displays much higher affinity for recombinant EP, receptors when in its free acid form (116, 157).

To date, there are no reports of compounds that act as specific EP_2 receptor blockers, although AH6809 is an antagonist at human EP, receptors. A summary of the affinities and potencies of EP, receptor ligands obtained in both functional and binding studies are shown in Tables 6.6 and 6.7, respectively.

4.2.4 EP_3 Receptor-Selective Ligands. The structures of some ligands active at the EP_3 receptor are illustrated in Fig. 6.7. EP_3 receptors are believed to be important in mediating the beneficial effects of prostaglandins in the stomach, where they seem to inhibit gastric acid secretion. Several EP_3 receptor agonists have been developed to protect against the gastroduodenal damage and increased risk of ulceration associated with NSAID use (233). Examples include misoprostol, rioprostil, M&B28767, sulprostone, and nocloprost (84, 234, 235). However, these compounds also seem to be active at other EP receptors. Misoprostol and rioprostil both have well-documented agonist activity at the EP_2 receptors, although not at EP, receptors, whereas sulprostone, which has been widely used in EP_3 receptor characterization studies, is also active at EP, receptors (228). In contrast, enprostil and GR63799X are more selective for the EP, receptor (72, 236). In addition, both compounds are highly potent in functional assays. These functional assays have also been backed up by the results of studies, both functional and radioligand binding in recombinant receptors (116, 157, 164). Probably the most selective agonist at the EP_3 receptor is SC46275

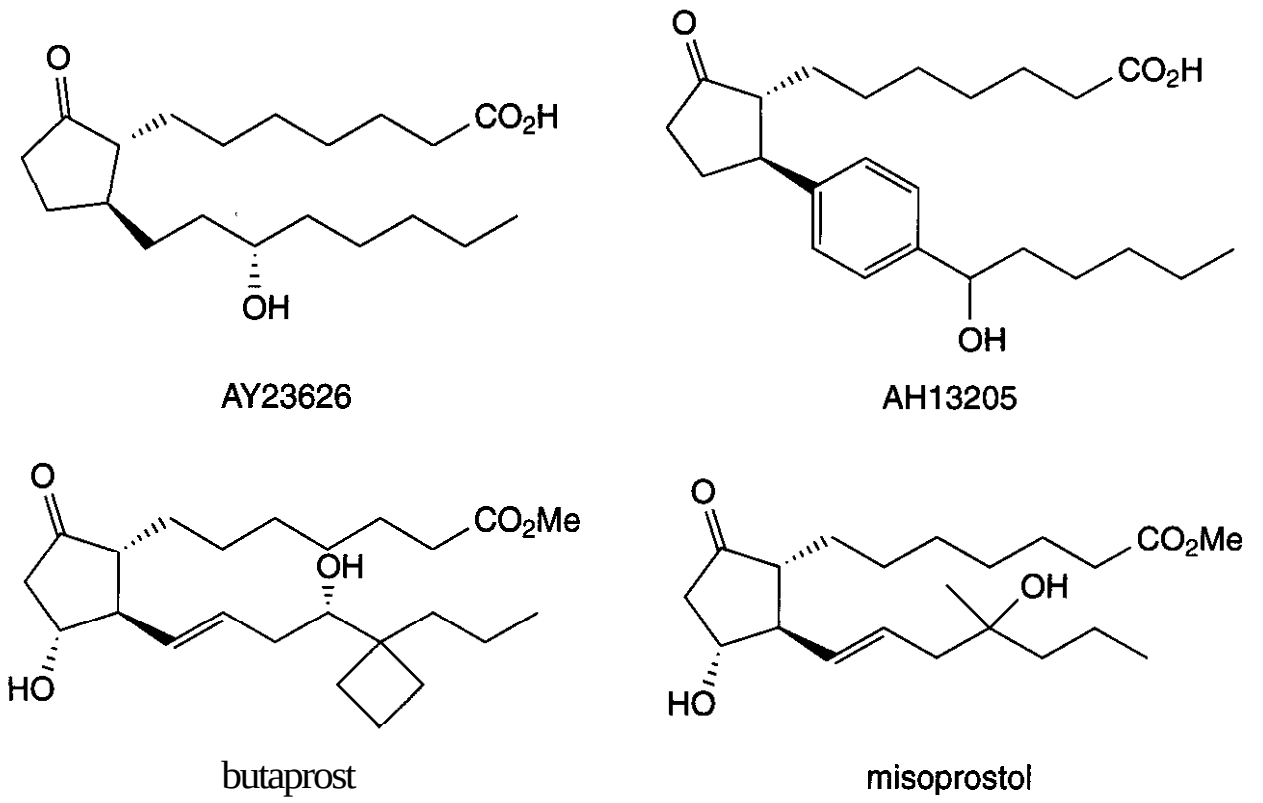


Figure 6.6. The structures of some EP, receptor-selective ligands.

(237), which displays nanomolar potency in smooth muscle preparations containing EP, receptors, such as the guinea pig vas deferens, but not those containing EP, or EP_2 receptors (237).To date, there are no well-documented examples of potent, selective antagonists at the EP_3 receptor, although a series of compounds that seem to be EP_3 receptor antagonists have recently been reported (238). A summary of the affinities obtained in recombinant binding studies and functional potencies for EP, receptor active compounds is shown in Tables 6.6 and 6.8, respectively.

4.2.5 EP, Receptor-Selective Ligands. The EP_4 receptor is the most recently identified subtype (232). To date, there are no well-documented examples of potent, selective EP_4 receptor agonists, but 11-deoxy-PGE_1 and 19R(OH)-PGE_2 have been identified as agonists that display around 30-fold selectivity for EP_4 receptors over the EP_2 receptor subtype (116). However, recently, series of compounds that show apparent EP_4 receptor selectivity have been described (241, 242) The TP receptor blocking drugs, AH23848B and AH 22921, are both weak EP_4 receptor antagonists, with pA_2 values of around 5.4. Despite its low potency, AH23848B is considered a relatively specific antagonist (232). The structures of some EP_4 receptor selective compounds, along with their binding affinities and functional potencies are shown in Fig. 6.8 and Tables 6.6 and 6.9, respectively.

4.3 FP Receptors and Selective Ligands

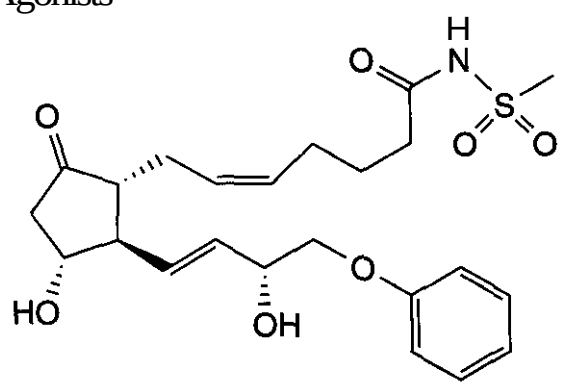
$PGF_{2\alpha}$ is a potent FP receptor agonist, but it also displays some activity at EP and TP re-

Table 6.7 Potencies of Some EP_2 Receptor Agonists

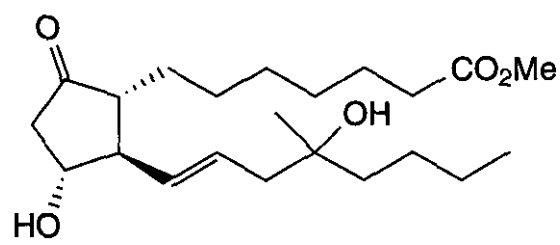
Agonists	Equieffective Concentration Relative to PGE, (=1)	Refs.
AH13205	30–100	230
Misoprostol[a]	14	232
19(R)-OH PGE_2[b]	40	229
AY23626	2–14	228
Butaprost	6–30	116,231

Note. there are no selective EP, receptor antagonists (1).
[a]Also has activity at EP, receptors.
[b]Also has activity at EP, receptors.

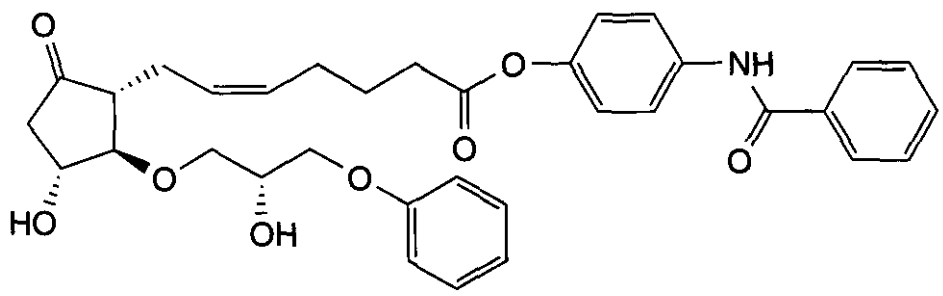
Agonists



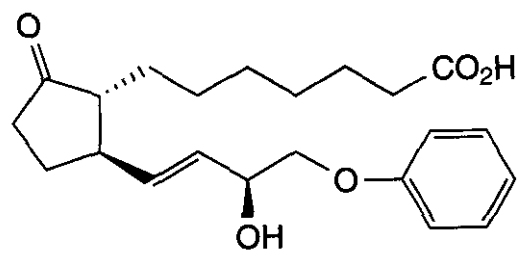
Sulprostone



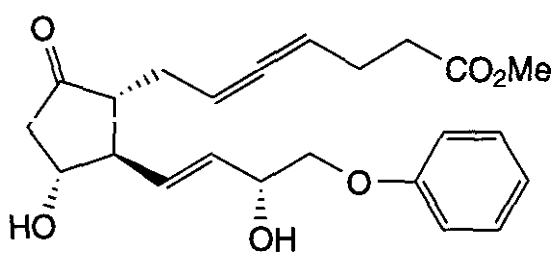
Misoprostol



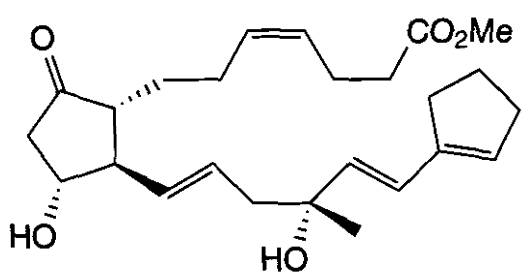
GR63799X



M&B28767



Enprostil



SC46275

Figure 6.7. The structures of some EP, receptor-selective ligands.

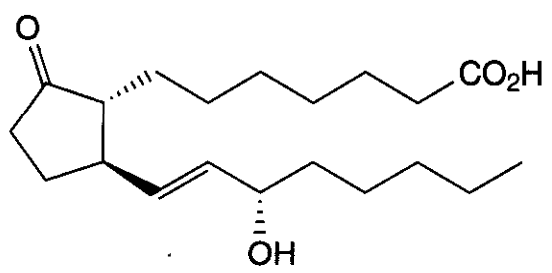
ceptors. $PGF_{2\alpha}$ is a potent luteolytic agent and is used in veterinary practice for this purpose. Also potent luteolytics in several animal species are two 16-phenoxy analogs of $PGF_{2\alpha}$, known as fluprostenol and cloprostenol (244). Their contractile actions on FP receptor preparations, the iris muscle of the dog and the cat, confirmed their identity as potent FP receptor agonists. Of the two compounds, fluprostenol is the most FP receptor selective, being almost inactive at all the other prostanoid receptors, whereas cloprostenol displays some weak activity in EP, receptor-containing preparations (232). Several other agents, all analogs of $PGF_{2\alpha}$, have also been synthesized as FP receptor selective ligands. These include pros-

Table 6.8 Potencies of Some EP, Receptor Agonists

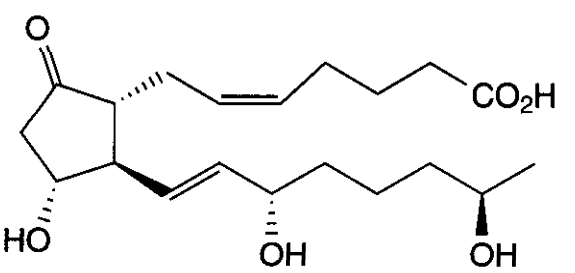
Agonists	Equieffective Concentration Relative to PGE_2 (=1)	Refs.
Misoprostol[a]	0.2–1.0	72,227,236
Rioprostol	0.9–1.1	72
M&B28767	0.1–0.6	74,227
Nocloprost	1	239
Enprostil	0.02–0.1	71, 72,236
Sulprostone[b]	0.1–1.0	1,116,240
GR63799X	0.1	236

[a] Also has activity at EP, receptors.
[b] Also has activity at EP, receptors.

Agonists

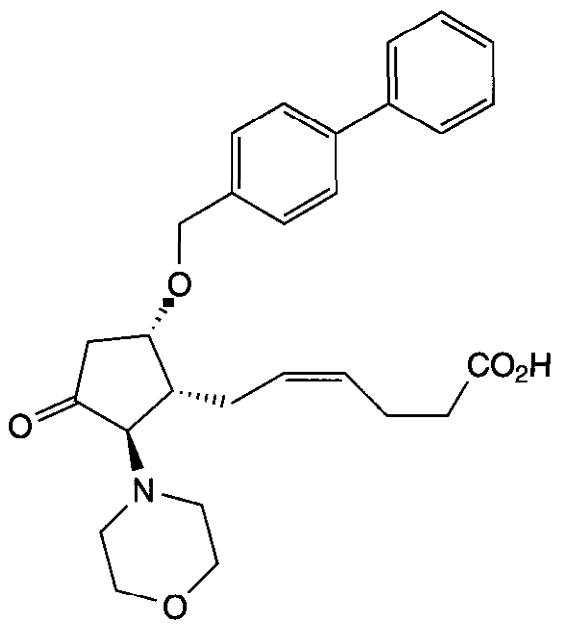


11-deoxy-PGE_1

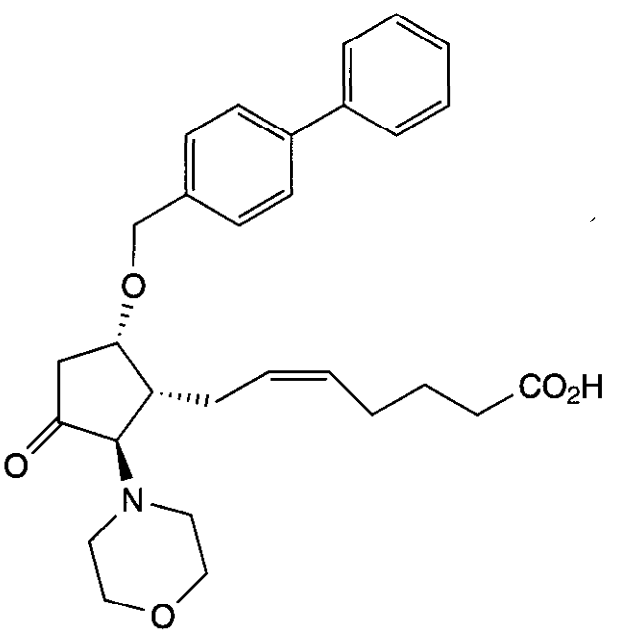


19R(OH)-PGE_2

Antagonists



AH23848B



AH22921

Figure 6.8. The structures of some EP_4 receptor-selective ligands.

talene, fenprostalene, and tiaprost (245). In addition, no specific FP receptor antagonists have been reported. Several agents, 14-dide-hydro-20 methyl $PGF_{2\alpha}$, *N,N*-dimethylamino-$PGF_{2\alpha}$, and *N,N*-dimethylamido-$PGF_{2\alpha}$, have exhibited antagonistic activity in preparations thought to contain FP receptors, but it is unclear whether these effects are mediated by FP receptor blockade (232). The structures of some FP receptor ligands are shown in Fig. 6.9 and the binding affinities and functional potencies are shown in Tables 6.4 and 6.10, respectively.

Crossley (250) has investigated the structure activity requirements for FP receptor activation. Alteration of the length of the β-chain, which can be altered from 8-11 carbon atoms, has little effect on activity. In addition, replacement of the carbon atoms in positions 17-21 in the p-chain with oxygen alters

Table 6.9 The Potencies of Some EP_4 Receptor Agonists and Antagonists

Agonists	Equieffective Concentration Relative to PGE, (=1)	Refs.
11-deoxy-PGE_1	1	116
19R(OH)-PGE_2[a]	30	116
Antagonists	**pA_2**	**Refs.**
AH23848B	4.9–5.4	232, 243
AH22921	5.3	232, 243

[a] Also has activity at EP_1 receptors.

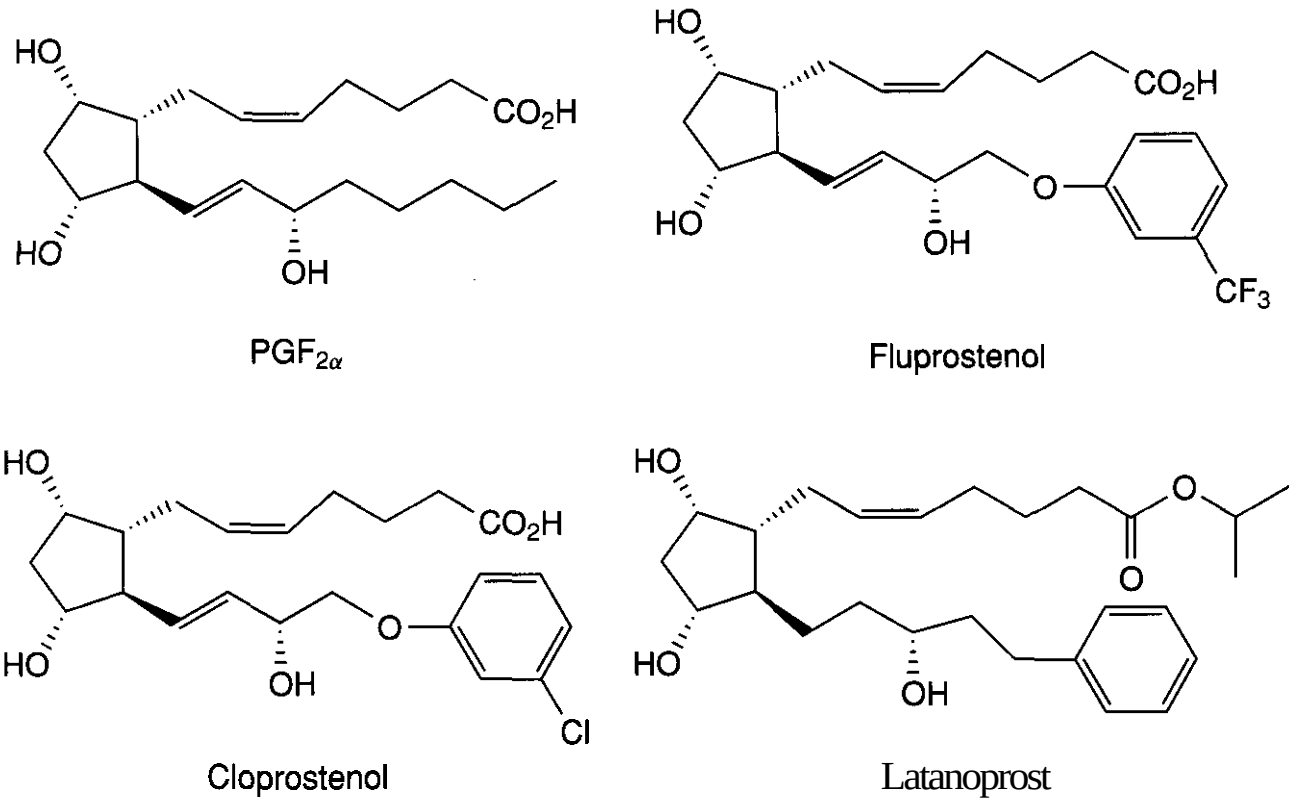


Figure 6.9. The structures of some FP receptor-selective ligands.

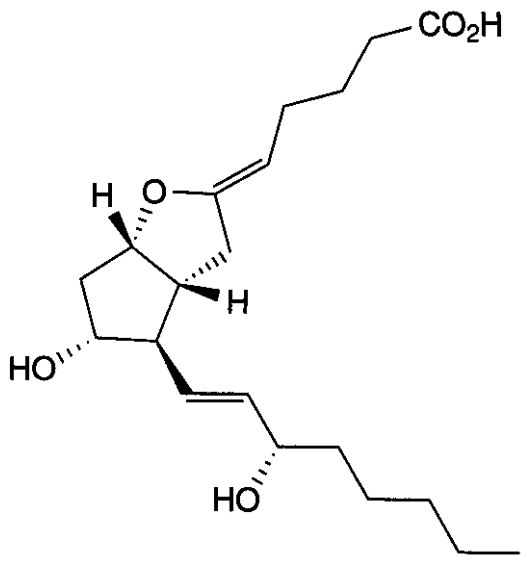
potency. The 17-keto derivative is the most potent FP ligand, also displaying the greatest selectivity over EP receptors. Furthermore, replacement of the β-chain C-17–C-20 atoms with phenoxy groups carrying further *meta* and para substitutions also affects FP receptor interactions. The most potent FP receptor agonist produced using this procedure is the p-fluorophenoxy analog, but it also displayed significant activity at TP receptors. However, the rn-trifuoromethylphenoxy (fluprostenol) and *m*-chlorophenoxy (cloprostenol) substituted analogs are both highly potent and selective FP receptor agonists (see above).

4.4 IP Receptors and Selective Ligands

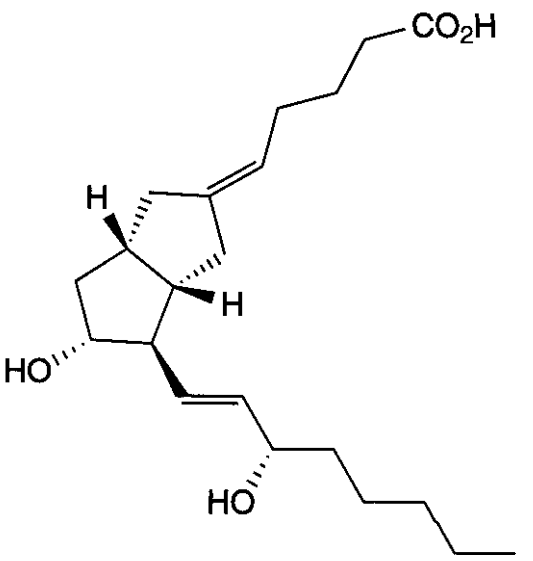
The structures of some compounds with activity at IP receptors are shown in Fig. 6.10. PGI_2 itself is chemically very unstable and is only moderately selective, possessing agonist activity at EP, and TP receptors, as well as at IP receptors (60). Several IP receptor ligands have been synthesized, with the aim of increasing its stability and maintaining the potentially beneficial platelet anti-aggregatory actions of prostacyclin, but not its hypotensive properties. The chemical instability of prostacyclin stems from the close proximity of the strained enol structure and carboxylic group. Hence, efforts to produce stable analogs have focused on the substitution of the enol ether structure and also altering the length of the a-side chain. Substitution of the oxygen in the enol group with less reactive sulphur, nitrogen, and carbon atoms has improved stability. An example is carbaprostacyclin [(5E)-6a-carba-PGI_2] (251). To retain functional activity, it seems that the 1-carboxy and 11- and 15-hydroxy groups and unsaturation of C-5 are critical. Functional activity is enhanced by the introduction of a methyl group at C-16 or C-17. In addition, the presence of an alkyl group at C-18 confers increased stability. This feature is present in the first stable prostacy-

Table 6.10 Potencies of Some FP Receptor Agonists

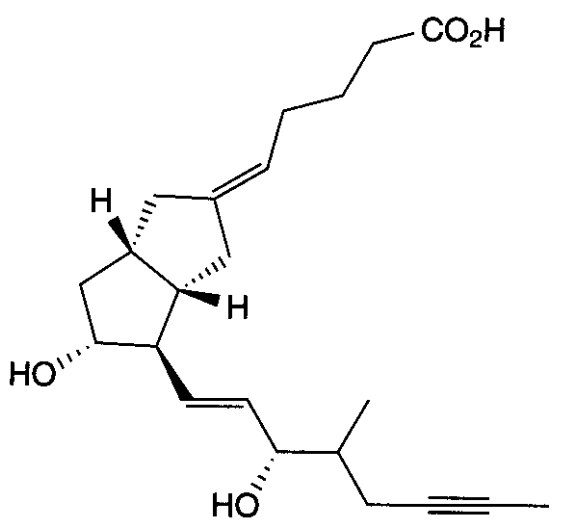
Agonists	Equieffective Concentration Relative to $PGF_{2\alpha}$ (=1)	Refs.
Fluprostenol	0.2-1.0	246, 247
Cloprostenol	0.3-0.5	248
Prostalene	0.7-2.7	248
Fenprostalene	1	249



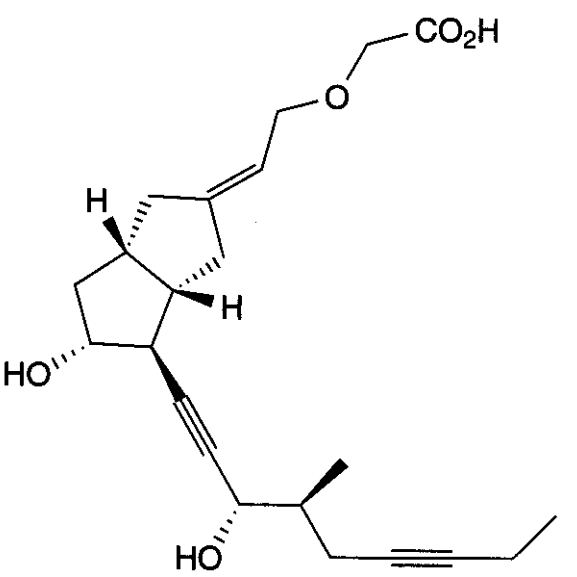
PGI_2



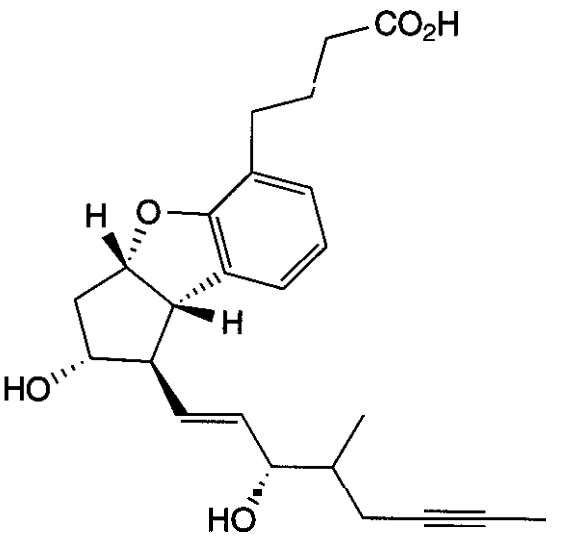
Carbaprostacyclin



Iloprost



Cicaprost



Beraprost

Figure 6.10. **The structures of some IP receptor-selective** ligands.

clin analog described, **iloprost,** a molecule in which the **enol** ether oxygen present in PGI, has been substituted with a methyl group and the lower side-chain altered, including the introduction of a triple bond at **C-18,** to improve stability (252). **A** summary of the alterations made to the structure of PGI, that increase stability and potency at IP receptors is shown in Fig. 6.11. **Iloprost** is as potent as **prostacyclin** in mediating the inhibition of platelet aggregation and the relaxation of vascular smooth muscle (252), but it is not purely IP

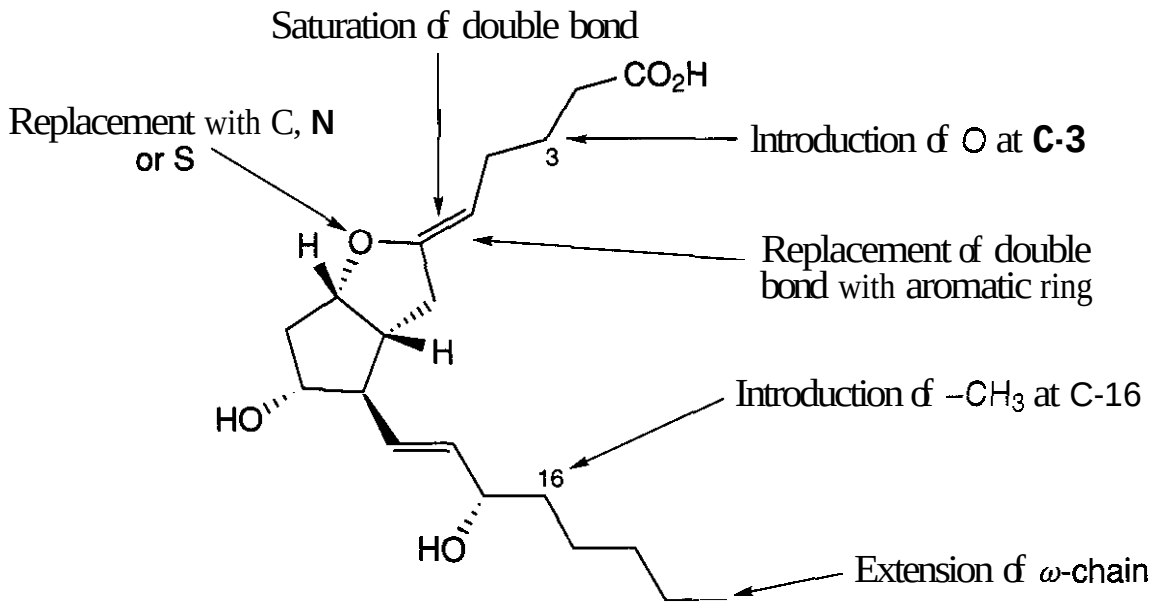


Figure 6.11. Alterations that have been made to increase the stability of prostacyclin.

receptor-selective, having partial agonist activity at EP_1 receptors (60). Therefore, caution must be used when interpreting functional data from this compound in systems known or suspected to contain EP_1 receptors.

However, the upper side-chain of iloprost is susceptible to β-oxidation, resulting in a bioavailabiltiy of only around 20% after oral dosing (253). With this is mind, the same group from Schering also produced cicaprost, which is probably the most selective IP receptor agonist currently available (254). In this compound, they replaced the β-methylene group at C-3 in the upper side-chain with an oxygen atom to block the β-oxidation pathway, and further altered the lower side-chain to produce this highly potent, stable compound. These changes included the introduction of a second triple bond at C-13 and an additional methyl group at C-20. It exhibits little functional activity in EP receptor–containing preparations (227) and is more potent than both parent compounds at inhibiting platelet aggregation and as a smooth muscle relaxant. Cicaprost has also been suggested to have anti-metastatic effects on tumors (253). Other analogs have also been described, including beraprost, in which the enol ether portion of the PGI_2 molecule is incorporated into an aromatic ring, and the lower chain is similar in structure to that of iloprost. The affinities and potencies of some IP receptor agonists are shown in Tables 6.4 and 6.11, respectively.

In addition, several "non-prostanoid PGI, mimetics" have been described as IP receptor agonists. From the use of these agents, including octimibate, EP157, and BMY45778, which all lack a typical prostanoid ring, it has been suggested that subtypes of IP receptor may exist (19). To date, despite the array of selective IP receptor agonists, no specific antagonists have been described.

4.5 TP Receptors and Selective Ligands

The structures of some compounds that are active at TP receptors are shown in Fig. 6.12. TXA_2 itself is inherently unstable, having a half-life in solution at pH 7.4 and 37°C of around 30 s, making it unsuitable for rigorous pharmacological studies. However, there are several examples of stable TP receptor agonists, the most useful of which are analogs of

Table 6.11 Potencies of Some IP Receptor Agonists

Agonists	Equieffective Concentration Relative to PGI_2 (=1)	Refs.
Carbaprostacyclin	3.5–30	159, 251
Iloprost	0.4–3.5	159, 256, 257
Cicaprost	0.5–1.2	159, 254
Octimibate	29[a]	258

[a]Primate IP, receptors only.

PGH_2. U44069 and U46619 (259) are 9,11-epoxymethano and 9,11-methanoepoxy derivatives of PGH_2, respectively. These compounds are the most widely characterized TP receptor agonists in use today. U46619 is the best characterized of the two ligands, and it displays similar properties to TXA_2 in several preparations (104). In addition, it also causes platelet aggregation and smooth muscle contraction with similar potency to TXA_2 in vitro (260). Other TP receptor agonists that have been synthesized include EP171, SQ26655, I-BOP, and STA_2 (32).

Agonists at TP receptors can be broadly divided into two groups: those that are analogs of TXA_2, such as STA_2, and those that are analogs of PGH_2, such as U46619. Replacing the acetal moiety of TXA, not only stabilizes the compound, but also may produce ligands that display either agonist or antagonist properties. Hence, STA_2, the thia analog of TXA_2, is a full agonist at TP receptors (261), whereas the 9(11),11a-dicarba derivative, CTA_2, displays partial agonist properties on human platelets (262).Similarly, replacing the dioxygen bridge in PGH_2 with isosteric moieties produces compounds with TP receptor agonist activity. For example, replacing either oxygen with a methylene group produces U44069 and U46619 (259).Furthermore, it seems that the incorporation of a p-fluorophenoxy moiety into the terminus of the ω-chain, as in EP171, increases agonist potency.

Compared with the other prostanoid receptors, TP receptors are unusual in that numerous antagonists have been identified, several of which are structurally unrelated to TXA_2 itself. Some of the first identified drugs with apparent TP receptor blocking activity in blood platelets were the TXA_2 analogs, 9,11-azoprosta-5,13-dienoic acid (263), 9,11-epoxyimino-5,13-dienoic acid (264), and pinane TXA_2 (265). However, these compounds all act as partial agonists in smooth muscle preparations. Further work produced compounds more distinct in structure from TXA_2, such as the 7-oxabicyclo[2.2.1]heptane analog, SQ29548 (266), AH19437, AH23848B, and GR32191B (vapiprost) (104, 172,267). EP045 and EP092 (263, 268) were generated as PGH_2 receptor analogs. Compounds structurally unrelated to prostanoids, such as BM 13505, which is a benzylsulfonamido analog (269), and the indole-2-propanoic acid derivative, L-655240 (270), have also been described as potent TP receptor antagonists. Another potent, non-prostanoid TP receptor antagonist is BAYu3405 (271). The affinities and potencies of some TP receptor agonists and antagonists are shown in Table 6.4 and Table 6.12, respectively.

4.6 Prostanoid Receptors: Location of Ligand Binding Sites

Recent advances in molecular biology have meant that the critical regions of amino acids to confer high affinity ligand binding are now beginning to be determined. For example, the importance of arginine at position 329 in the seventh transmembrane domain of all EP receptors has been examined by point mutations in the rabbit EP_3 receptor. Thus, mutation of this amino acid to alanine or glutamate abolished the binding of [^{3}H]-PGE_2, whereas the mutation of aspartate 388 was without effect on ligand binding, but altered signal transduction, such that the EP_3 receptor agonist, sulprostone, was without effect even at high concentrations (279). Similar results have been obtained using mouse receptors: mutation of the equivalent arginine 309 to glutamate/valine produced a loss in ligand binding, whereas switching it to lysine produced higher affinity binding (280). These data suggest that the seventh transmembrane domain of prostaglandin receptors is involved in both ligand binding and signal transduction.

Taken together with evidence that suggests that modification of the carboxyl group on the a-chain of prostaglandins tend to reduce agonist potency (219), the above findings suggest that this conserved arginine could be involved in interacting with the a-chain of prostaglandin ligands. Indeed, it has been shown that ligands containing methyl esters at C-1 tend to display lower binding affinities than those containing negatively charged hydroxy groups. An example is the comparison of ligand binding of misoprostol methyl ester and free acid to the EP_3 receptor (157). Furthermore, the rabbit EP_3 receptor displays a 370-fold decrease in affinity for PGE, methyl ester over PGE_2 (279). On the other hand, the same authors demonstrated that sulprostone, which contains a sulfonamide at C-1 that is bulkier

Agonists

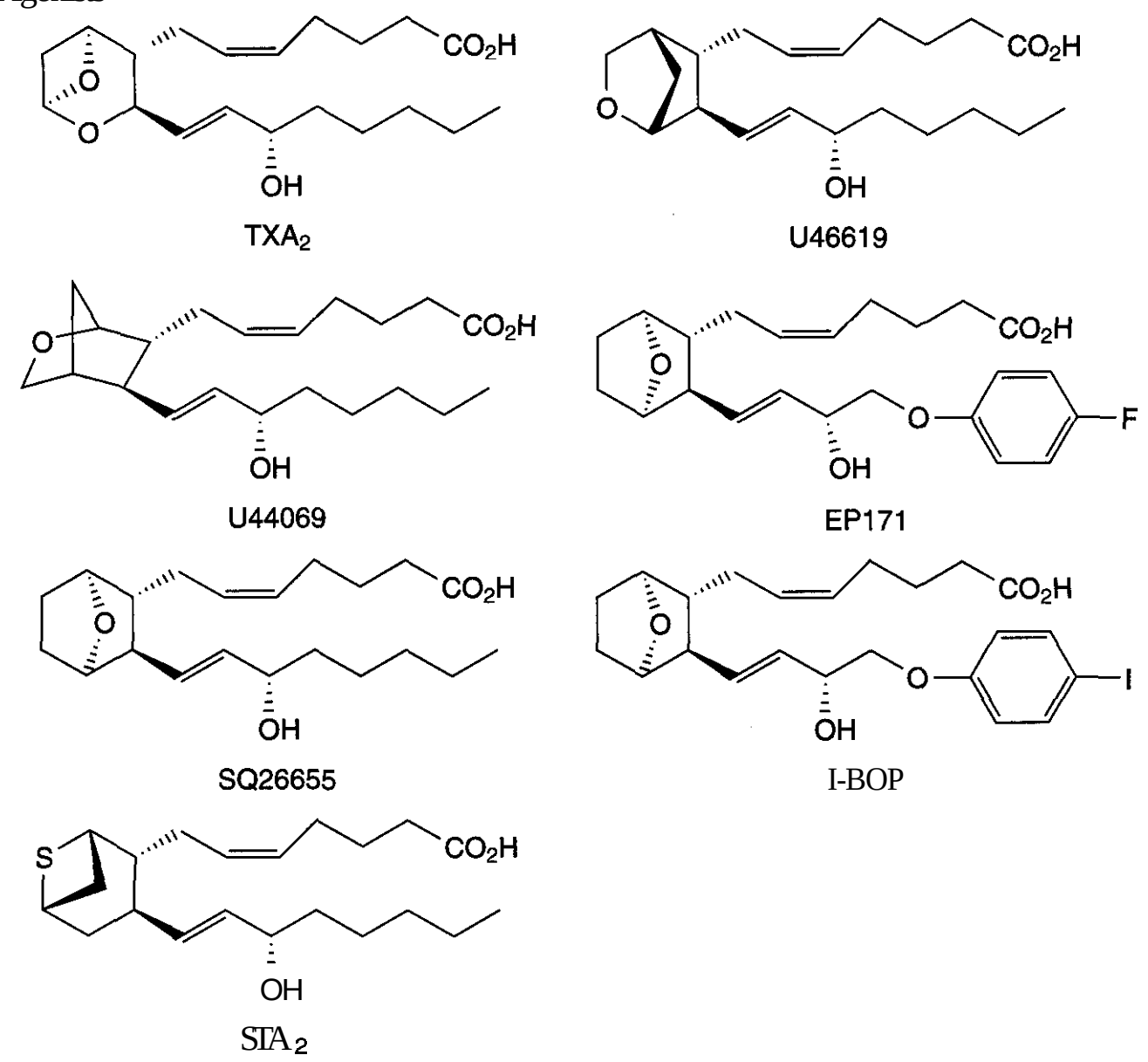


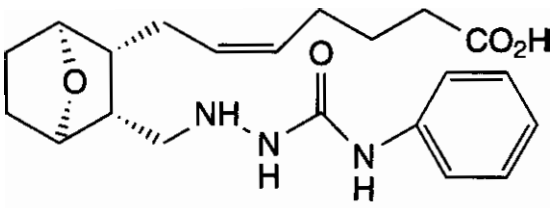
Figure 6.12. The structures of some TP receptor-selective agonists.

than a carboxyl group but still carries a negative charge ($pK_a = 5.25$ cf 5.19 for the carboxyl group of PGE_2), exhibited a threefold higher affinity than PGE_2. These data suggest that a negative charge on C-1 is important for high affinity ligand binding to EP receptors. Chang and colleagues (281) have investigated the relative contribution of the conserved arginine with respect to hydrogen bonding and ionic interactions that may be involved in ligand binding. They mutated the arginine to non-charged but polar glutamine or asparagines, or the non-polar leucine, to see how these changes affected the binding of PGE_2. The mutation to leucine decreased binding affinity by around 40-fold, whereas binding was almost unaffected by the other two mutations. On the basis of this, they have suggested that hydrogen bonding may be enough for high affinity ligand binding.

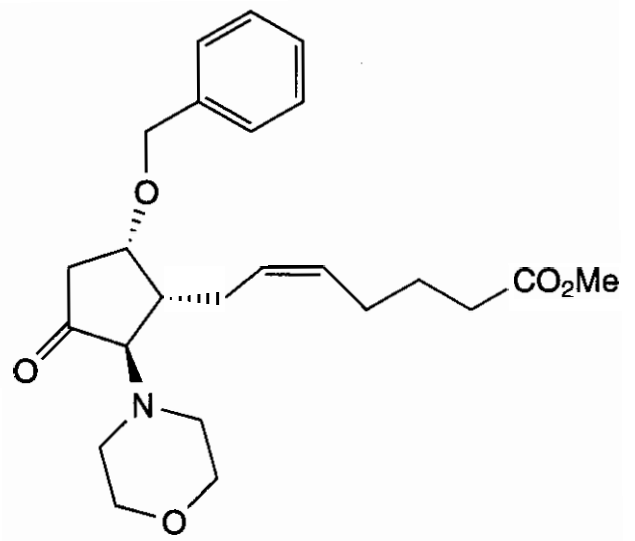
It is also interesting to speculate whether the cysteine residue in the second extracellular loop forms a disulphide bridge that is important for receptor conformation. In the rabbit, mutation of this cysteine 204 to the uncharged alanine did not affect PGE_2 binding (279). This is in contrast to results reported in human TP receptors, where mutation of the equivalent cysteine and another in the first extracellular loop abolished ligand binding (282, 283). Also, as the TP receptor fails to display ligand binding after reduction with dithiothreitol (284), and in other rhodopsin-like receptors equivalent cysteine residues form a disulphide bridge, it has been suggested that cysteine 105 and cysteine 183 may form a disulphide bond essential to ligand binding.

Studies with human TP receptors have also highlighted several regions that are thought

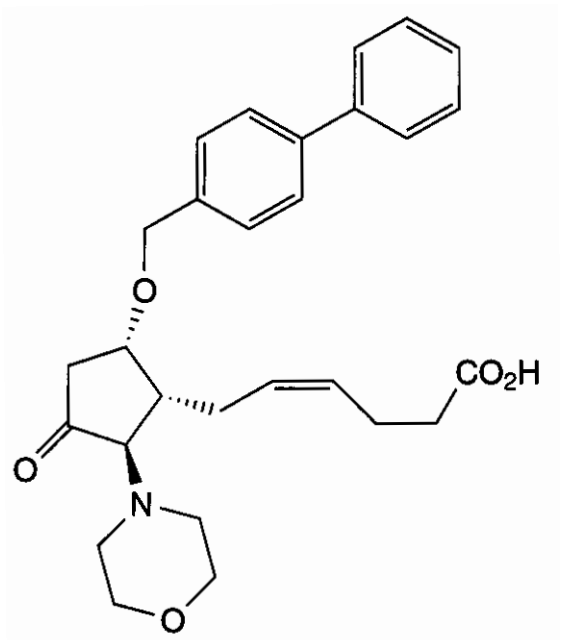
Antagonists



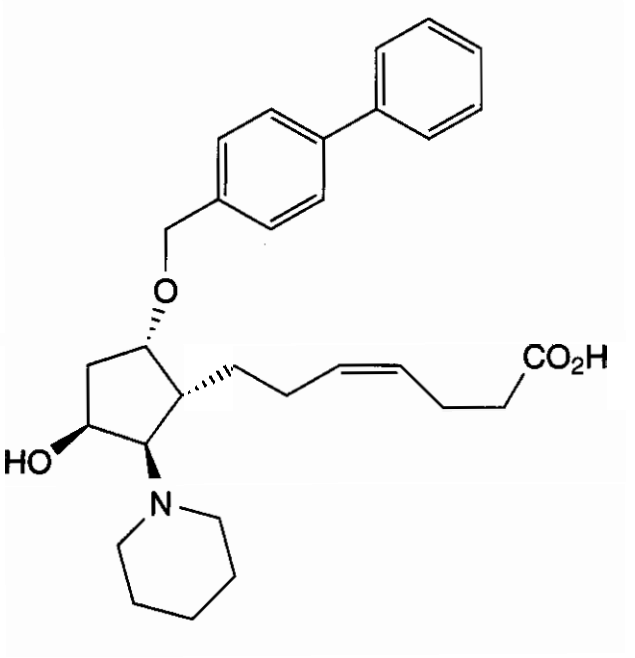
SQ29548



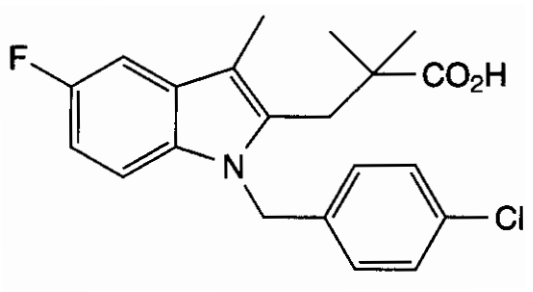
AH19437



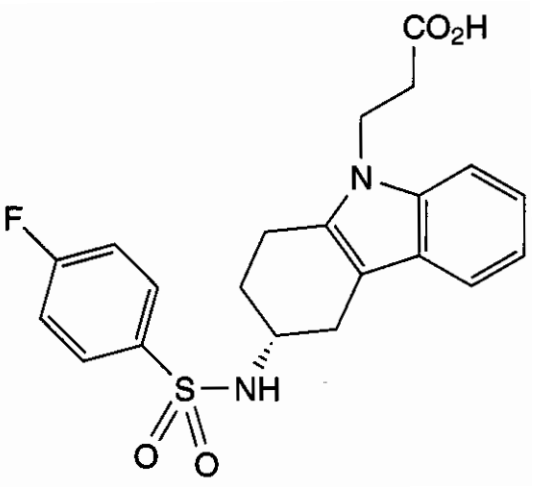
AH23848B



GR32191 (vapiprost)



L655240



BAYu3405

Figure 6.12. (Continued.)

to be important in ligand binding. For example, the mutation of tryptophan 299, in the seventh transmembrane domain, to leucine produces a receptor that can bind the TP receptor agonist, U46619, but not the antagonist, SQ29548 (285). Furthermore, mutation of the conserved arginine 295, also in the seventh transmembrane domain of the human TP receptor, causes a reduction in ligand binding (285). Likewise, the importance of cysteine residues in the human TP receptor has also been investigated by mutational studies,

Table 6.12 Potencies of Some TP Receptor Agonists and Antagonists

Agonists	Equieffective Concentration Relative to U46619 (=1)	Refs.
EP171	0.008-0.03	172,273
SQ26655	0.14-0.47	172
I-BOP	0.12–0.25	273,274
STA_2	36	275
Antagonists	**pA_2**	**Refs.**
SQ29548	7.8–9.5	172, 276, 277
AH19437	5.9–6.6	104, 172
AH23848B	7.8–8.6	172, 273
GR32191	7.9–8.8	172, 277
L-655240	8.0–8.6	269, 277
BAYu3405	8.1–8.9	278

which have stressed the importance of those residues in the first and second extracellular loops (283). It also seems that glycosylation of the TP receptor may be important for ligand binding: when two extracellular glycosylation sites are mutated, the resulting receptor displays a loss of ligand binding (286).

The use of chimeric receptors has also been employed to attempt to dissect out regions of receptors important in ligand binding. For example, Kobayashi and colleagues (287) used chimeric DP and IP receptors to study possible domains involved in DP and IP receptor ligand binding. In substituting different regions of the mouse IP receptor with equivalent regions from the mouse DP receptor, they suggested that the sixth and seventh transmembrane domains of the IP receptor confer specificity to IP receptor ligands and that the third transmembrane domain of the DP receptor confers the specific binding of PGD,. They suggested that the IP receptor recognizes both the side-chains and cyclopentane rings of the ligands it binds. PGI, has a unique a-chain structure, caused by the presence of an additional ring attached to the cyclopentyl ring present in all prostanoids. It has been suggested that the IP receptor can recognize PGE,, but not PGE_2, because of the lack of the C-5–C-6 double bond, which allows it to mimic the configuration of the side-chain in I series prostaglandins. Likewise, the binding of I- and E-series, but not D- and F-series prostaglandins, was explained by the lack of cyclopentane ring recognition for the latter two prostaglandin series. Kedzie and co-workers (288) employed a similar approach, mutating EP, receptor residues common to EP_2 and EP, receptors, but not IP receptors to those present in IP receptors. They showed that mutating the human EP_2 receptor leucine 304 in the seventh transmembrane domain to tyrosine resulted in an increase in potency of iloprost of around 100 times in a cAMP-dependent reporter gene assay. Likewise, mutation of the critical arginine 302 resulted in a loss of potency for all prostanoids tested. This may be consistent with the above assertion that amino acids in the sixth and seventh transmembrane domains are relevant in the binding of the a-chain of IP ligands. Hence it can be seen that some progress is being made into the regions of prostanoid receptors that confer high affinity ligand binding.

5 CLINICAL USE OF AGENTS

The current drugs available in the clinic are mostly natural prostaglandins themselves and a few closely related analogs, and it is only very recently that efforts have again increased to find highly selective synthetic prostaglandin receptor ligands, which might be useful medicines. One barrier to this effort, at least with respect to pain and inflammation, is that aspirin and the competitive COX inhibitors, which inhibit prostaglandin synthesis *per* se, are already available and have proved to be remarkably efficacious. However, they do cause unwanted gastrointestinal side effects, especially the non-selective COX inhibitors. It

remains to be seen whether selective prostanoid receptor antagonists might provide better medicines for the treatment of pain and other therapeutic indications. A summary of the available agents and their indications is presented in Table 6.13. The therapeutic areas in which these agents are used are diverse and are a consequence of the widespread physiological actions of the natural prostaglandins. Prostaglandins used in the termination of pregnancy, cervical ripening, and labor induction include dinoprost ($PGF_{2\alpha}$) and carbaprost (15-methyl-PGF$_{2\alpha}$), dinoprostone (PGE_2), sulprostone, gemeprost, and misoprostol. Alprostadil (PGE_1) is used to treat erectile dysfunction. Prostaglandins used in cardiovascular conditions include alprostadil and the PGE, analog, limaprost, as well as epoprostenol (prostacyclin) and its analog, iloprost. Misoprostol is used clinically, with some success, as an inhibitor of gastric acid secretion. Prostaglandins, particularly of the F series, are widely used as luteolytics in veterinary medicine, but a discussion of this is beyond the scope of this chapter. However, the FP receptor agonist, latanoprost, has found use in the treatment of glaucoma (289, 290). D-series prostaglandins are not used in the clinic at present. Intravenous administration of PGD_2 in humans causes facial flushing and intense nasal congestion. No effects were seen with respect to ADP-induced platelet aggregation, suggesting PGD, is unlikely to be a useful anti-thrombotic in man (291). Each clinically available prostanoid is described below, beginning with the natural prostaglandins and then the selective ligands that have been developed with the aim of producing more stable compounds, with a longer duration of action and a more specific effect. At present, the clinically available prostanoids are all receptor agonists. The structures of some of the clinically available prostanoids not covered in Section 4 are shown in Fig. 6.13.

5.1 Natural Prostaglandins

5.1.1 Alprostadil (Prostaglandin E$_1$). Alprostadil is the name given to an injectable formulation of PGE_1, which when administered exogenously causes vasodilatation and the inhibition of platelet aggregation. It exerts its actions primarily through EP and IP prostanoid receptors. The main clinical usesare in the treatment of male erectile dysfunction and congenital heart disease. Alprostadil is largely inactivated by the lungs on its first passage through the pulmonary circulation. Its metabolites are excreted in the urine within approximately 24 h.

Alprostadil is available in several formulations for the diagnosis and treatment of impotence caused by erectile dysfunction. The most common formulation is for intracavernosal injection (5–20 μg). For intracavernosal injections, it is recommended that the dose given does not exceed 60 pg, although doses of up to 100 pg have been reported. Dose-related responses have been reported (292). Following intracavernosal alprostadil injection, the incidence of pain at the injection site has been reported to be dose-dependent (293). In addition, penile fibrosis and priapism has also been observed when prostaglandins are used for this purpose. In cases of priapism, corrective therapy should be administered rapidly. In comparative studies, intracavernosal alprostadil was as effective at producing erections as papaverine alone or in combination with the non-selective α-adrenoreceptor antagonist, phentolamine (294, 295). It has also been administered in combination with these two agents. Other administration routes include urethral suppositories, but the dose required is higher (125–1000 pg rather than the usual dosing range of between 5 and 20 μg) (296). In a recent trial, intraurethral alprostadil was reported to have an overall success rate of around 50% (297).

Alprostadil is also used to treat several aspects of cardiovascular disease. For example, it is administered intravenously to neonates with congenital heart disease to maintain the patency of the ductus arteriosus before surgery can take place. In neonates treated with alprostadil for congenital heart defects, several side effects were observed in a 3-year study examining almost 500 children. These included some cardiovascular events, and in some individuals, respiratory depression (298). Alprostadil has also been used in the treatment of peripheral vascular disease, especially Raynaud's syndrome, which is characterized by digital ischemia induced by cold and

emotion (299). In addition, several studies have shown it to be superior to placebo in peripheral arterial occlusive disease (PAOD) (300). However, such uses do not constitute mainstream therapies. Alprostadil is one of several drugs that have been used to combat circulatory disturbances in ergotamine poisoning (301) and has also proved effective in

Table 6.13 Agents in Clinical Use That Are Active at Prostanoid Receptors

Generic Name	Trade Name(s)	Originator	Synonyms	Dose
Alprostadil	Caverject	Pharmacia Upjohn	PGE,	Ductus arteriosus: 50–100 ng kg^{-1} min^{-1} Erectile dysfunction: 5–20 μg intracavernosally
	Muse	AstraZeneca		Erectile dysfunction: 125–1000 μg intraurethrally
Dinoprostone	Prepidil	Pharmacia Upjohn	PGE_2	Cervical ripening: 500 μg in 2.5 ml (gel), Labour induction: 1–2 mg per 2.5 ml (gel), 0.5 mg h^{-1} orally
	Prostin E_2	Pharmacia Upjohn		Termination of pregnancy: 100 μg intra-amniotically, 5 μg ml^{-1} IV
Limaprost	Opalmon	Ono	ONO-1206	Anti-thrombotic
Gemeprost	Cervagem	Rhone-Poulene	16,16 dimethyl PGE_1	Cervical ripening: 1 mg pessary vaginally
Misoprostol	Arthotec	Searle	SC-29333	Anti-ulceration: 800 μg orally
	Cytotec	Searle		Labour induction: 25–50 μg vaginally
Sulprostone	Nalador	Schering AG	CP-34089; SHB 286; ZK-57671	Cervical dilation: 500 μg per 3–6 h IV Termination of pregnancy: 100–500 μg h^{-1} IV
Latanoprost	Xalatan	Pharmacia Upjohn	PhXA-41; XA-41	Glaucoma: 0.005% solution topically
Epoprostenol	Flolan	GlaxoSmithKline	Prostacyclin; PGI_2	Dialysis: 5 ng kg^{-1} min^{-1} IV Pulmonary hypertension: continuous infusion after dose-ranging
Iloprost	Ilomedin	Schering AG	ZK-36374	Peripheral vascular disease: 0.5–2 ng kg^{-1} IV as trometamol salt

Table 6.13 (Continued)

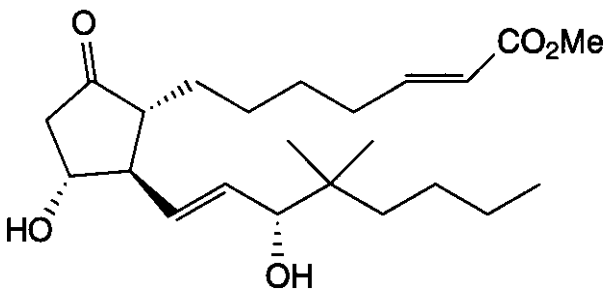
Generic Name	Trade Name(s)	Originator	Synonyms	Dose
Carbaprost	Hemabate	Pharmacia Upjohn	15-methyl $PGF_{2\alpha}$; U-32921	Termination of pregnancy: 250–500 μg IM
	Prostinfenem	Pharmacia Upjohn		Postpartum haemorrhage: 250 μg IM
Dinoprost	Prostin $F_{2\alpha}$	Pharmacia Upjohn	$PGF_{2\alpha}$	Termination of Pregnancy: 40 mg intra-amniotically, 25–50 $\mu g\ min^{-1}$ IV Labour induction: 2.5–20 $\mu g\ min^{-1}$ IV
	Prostarmon F	Ono		Ileus: 0.3–0.5 $\mu g\ kg^{-1}\ min^{-1}$ IV

the management of hemorrhagic cystitis in children following bone marrow transplantation (302).

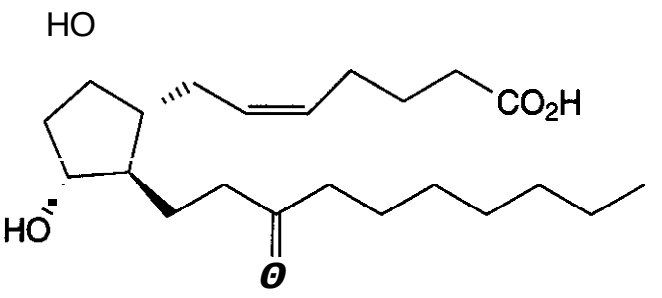
Alprostadil also seems to be beneficial in the treatment of some hepatic disorders, with some promise being shown in patients with fulminant or subfulrninant viral hepatitis (303). In these cases it has also been administered with dinoprostone and the EP_3 and EP_4 receptor agonist, misoprostol.

In addition to the side effects outlined above, administration of exogenous PGE_2 may produce some irritation at the infusion site, headache, and vasodilation. The vasodilatory effects also occur with prostacyclin analogs and are often more pronounced in these cases. The rare occurrence of gastric mucosal hyperplasia has also been reported in children receiving long-term treatment with prostaglandins (304).

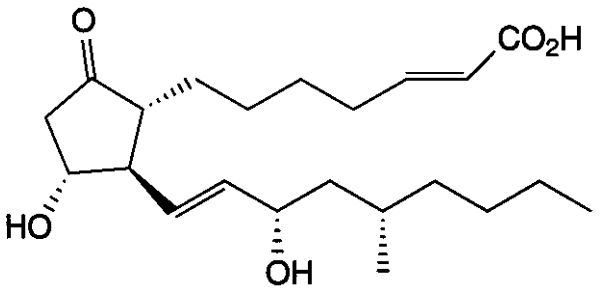
5.1.2 Dinoprostone (Prostaglandin E_2). Dinoprostone is the name given to pharmaceutical formulations of PGE_2, and the action most ex-



Gemeprost



Unoprostone



Limaprost

Figure 6.13. The structures of some compounds in clinical use that are active at prostanoid receptors.

ploited in the clinic is its ability to contract smooth muscle. It exerts its actions predominantly through interactions with EP receptors. It is widely used in the induction of labor but also for the termination of pregnancy, hydatiform mole, and missed abortion. The main route of administration is vaginal, but this drug can also be dosed intravenously, orally or extra-amniotically. Intravenous infusion is associated with a large incidence of adverse effects (305).

In labor induction, dinoprostone is used to soften and dilate the cervix before the membranes are breached and labor is induced. It may also be administered either vaginally or orally to induce labor. However, the oral route is not common, because it is associated with a greater incidence of gastrointestinal adverse effects. Likewise, intravenous dosing has also been employed, but this is no longer generally recommended.

Dinoprostone is also used in the termination of pregnancy in the second trimester. For this purpose, it is usually dosed extra-amniotically through a suitable catheter, with the dose given being dependent on patient response. Intravenous infusions have also been used for this purpose, as well as in cases of hydatiform mole and missed abortion. In the United States, the preferred route of administration for this purpose is through vaginal pessary. Although they are effective alone, because of the incidence of adverse effects, prostaglandins are normally used at low doses in combination with mifepristone (306).

Along with alprostadil (see above), dinoprostone is also used in the short term to maintain ductus arteriosus patency before surgery. Oral dinoprostone, when used longer, may be beneficial in allowing later surgery by facilitating infant growth. This has been reported by both Silove and colleagues (307) and Thanopoulus and co-workers (308) in 60 and 22 infants, respectively. Initial treatment regimens typically continue for 4 weeks, and dosing is typically oral, but can be intravenous if gastrointestinal absorption is poor.

Like alprostadil and other prostaglandins, dinoprostone has also been used in the management of hemorrhagic cystitis, especially that caused by cyclophosphamide (309), and in cardiovascular disease, including Raynaud's syndrome. However, the latter therapy is not widely used. Furthermore, dinoprostone has proved useful in treating the oral lesions in patients with pemphigus vulgaris.

Side effects occurring following dinoprostone seem to be related to the dosing system used, and the intravenous route is associated with more adverse reactions. Common problems include nausea, diarrhea, and abdominal pain, along with short-term cardiovascular effects such as headache, hypotension, and facial flushing, as seen with alprostadil. However, a report cataloguing 3313 pregnancies in which dinoprostone was used in cervical ripening or labor induction reported that side effects were rare and were generally limited to diarrhea, vomiting, and fever (310). The most common adverse effect seen with the use of prostaglandins combined with mifepristone is excessive vaginal bleeding.

5.1.3 Dinoprost (Prostaglandin $F_{2\alpha}$). This is the name given to pharmaceutical formulations of $PGF_{2\alpha}$ and is widely used in the termination of pregnancy, because it exerts a contractile effect on uterine smooth muscle at any stage of the gestation period. However, it is also used, like dinoprostone, for missed abortion, hydatiform mole, and fetal death in *utero*. It is no longer generally recommended for routine use in the induction of labor because of the higher incidence of adverse effects encountered compared with dinoprostone.

For termination of pregnancy, the intra-amniotic route is preferred, because this results in fewer side effects than intravenous dosing. Dinoprost has also been used successfully to treat the ileus that results from vinca alkaloid administration (311).

Dinoprost produces similar adverse effects to dinoprostone, and epoprostenol and iloprost have also both been reported to produce similar cardiovascular effects (312).

5.1.4 Epoprostenol (Prostaglandin I_2; Prostacyclin). Epoprostenol is the name used to describe a prostacyclin formulation for exogenous administration. This drug has a very short half-life in the body and is rapidly broken down to 6-keto-$PGF_{1\alpha}$. Prostacyclin is a vasodilator and potent inhibitor of platelet aggregation and is used to treat pulmonary hy-

pertension and to prevent platelet aggregation in, for example, blood from patients undergoing kidney dialysis. Epoprostenol has also been evaluated for the treatment of heart failure. However, long-term use resulted in an increase in patient mortality, so development for this purpose has now been discontinued (313). Like alprostadil and dinoprostone, epoprostenol has also been used in the treatment of peripheral vascular disease.

Owing to its instability, epoprostenol is supplied as the sodium salt and must be administered by continuous infusion. As a consequence, a dose-ranging study must first be performed to evaluate the maximum tolerable infusion rate. Epoprostenol has also been used with some success in patients with acute respiratory distress syndrome (314, 315).

In pulmonary hypertension, epoprostenol was first introduced as a short-term treatment enabling patients to undergo heart-lung transplants. However, in some patients treated long term with the drug, an encouraging clinical improvement has been reported (316–318). The intravenous route of administration is difficult to manage because it requires continuous infusion, but as an alternative, inhalation of epoprostenol may provide a therapy with fewer adverse effects, and this regimen has been used successfully in adults with both primary and secondary pulmonary hypertension (319, 320). Epoprostenol has also been used in organ transplantation, finding use in both organ preservation and also in the treatment of some of the post-transplant complications that may occur.

Epoprostenol has also been used in patients with ischemic stroke. However, the pathophysiological involvement of endogenous prostaglandins in ischemic brain damage is not clear, and no studies showing significant, sustained therapeutic benefits with epoprostenol have been reported (321,322).

5.2 Synthetic Prostaglandins

5.2.1 Gemeprost. Gemeprost is a synthetic analog of PGE,. It is usually given vaginally as pessaries and is used therapeutically to soften and dilate the cervix and to stimulate uterine smooth muscle contraction in the termination of pregnancy. For the latter indication, gemeprost may be given intravaginally in conjuction with oral mifeprostone (306). Mifeprostone is a progesterone antagonist and serves to sensitize the uterus to prostaglandins. This facilitates the use of lower doses of prostaglandins in pregnancy termination, thus reducing the severity and incidence of unwanted adverse effects. However, gemeprost is expensive and is thermolabile, requiring refrigerated storage. It is not approved for use in the United States (306).

Side effects following intravaginally administered gemeprost are generally relatively mild, with systemic effects such as nausea and diarrhea reported. The effects of this drug on the fetus are not known, but once a prostaglandin has been used to initiate termination of pregnancy, if it is found to be unsuccessful, termination must be completed by another means.

5.2.2 Misoprostol. Misoprostol is a synthetic analog of PGE, and is regarded as a selective EP, and EP_3 receptor agonist. It has a higher affinity at the EP_3 receptor, and this has been demonstrated in recombinant systems using the cloned receptors of several species (157, 164). It is a mixture of four stereoisomers, with the 11R,16S isomer accounting for most of its activity, and it will be interesting to see if stereochemically pure formulations show greater therapeutic efficacy. Once administered, it is rapidly metabolized to its active form, misoprostol-free acid. It is further metabolized by oxidation in several organs and is excreted mainly in the urine. It has a plasma elimination half-life of around 20–40 min (323) and is about 85% serum albumin bound (324).

Misoprostol has several therapeutic uses, including the treatment of gastroduodenal ulceration and labor induction and has the advantage of being relatively inexpensive and stable enough to be stored at room temperature. Prostaglandin therapy for the induction of labor is a well-established practice, and misoprostol has been compared with dinoprostone and also oxytocin. It was found to be equieffective with oxytocin (325) and more effective than dinoprostone (326). It was also shown to be effective in 92% of women in combination with tamoxifen (327). However, mi-

soprostol is often not effective in the termination of pregnancy when used alone. Its misuse has led to reports of associated congenital abnormalities in neonates, including scalp, skull, and limb defects. It is possible that these teratogenic effects may be caused by an increase in uterine pressure caused by vascular spasm or uterine contractions **(328, 329).** However, no such events have been reported in animal studies **(330).** Misoprostol has also been shown to be effective in treating both gastric and duodenal ulcers but is no more efficacious than other established therapies, such as histamine H_2-receptor antagonism, and has more unwanted side effects **(331, 332).** One condition where it was thought it might offer superiority over standard antacid therapy was in ulcer prevention in patients on long-term NSAID treatment **(333).** Nevertheless, proton pump inhibitors, such as omeprazole, seem to be as effective and have fewer side effects.

Misoprostol may also prove useful in the treatment of postpartum hemorrhage and has also been used in organ transplantation. For example, it has been reported to improve kidney function in cyclosporin-treated patients who have received renal transplants **(334).**

The most common adverse effect seen with the use of misoprostol is diarrhea, but abdominal pain has also been reported, limiting the use of this and other similar compounds in the management of gastroduodenal ulceration. Effects on uterine contraction have also been reported, suggesting that misoprostol should not be given to pregnant women (335).

5.2.3 Sulprostone. Sulprostone is a synthetic derivative of PGE_2 and is primarily recognized as an EP, receptor agonist, although it also has activity at EP, receptors **(116).** It was developed as an abortifacient, and like other PGE analogs, it acts as a uterine stimulant. It has thus been used to stimulate cervical dilation before pregnancy termination during the first 3 months of gestation. Dosing is generally intravenous, although several other routes have been used, including intramuscular, although this is no longer recommended. It has also been used in the treatment of postpartum hemorrhage. Sulprostone's use as an abortifacient has been discontinued, because of a link with increased cardiovascular complications, such as hypotension and acute myocardial infarction **(336).** Sulprostone has a similar side effect profile to dinoprostone.

5.2.4 Latanoprost. Latanoprost is a derivative of $PGF_{2\alpha}$ that is used in the treatment of glaucoma and ocular hypertension, and increases the outflow of the aqueous humor, bypassing the obstructed site of normal drainage by opening an alternative drainage pathway through uveal and scleral tissues. It is used topically as eye drops, and as an adverse effect, it may induce an increase in brown pigmentation of the iris, corneal deposits, and, rarely, an increase in eyelash growth. This effect is caused by increased melanin formation in melanocytes. In addition, latanoprost also produces increased reductions in intraocular pressure when used with other anti-glaucoma agents, such as the muscarinic agonist, pilocarpine, and the carbonic anyhydrase inhibitor, acetazolamide **(337).** It has the advantage that it only requires once daily administration, thus improvingpatient compliance **(338).** Unoprostone is another $PGF_{2\alpha}$ analog that has also been used in the treatment of glaucoma, although mainly in the Japanese market (339).

5.2.5 Iloprost. Iloprost is a stable analog of prostacyclin, and like PGI_2, causes vasodilation and the inhibition of platelet aggregation. In addition to acting at IP receptors, it displays potent partial agonist activity at EP, receptors **(60).** It has found clinical use, along with other prostaglandins, in the treatment of peripheral vascular disease and several administration routes have been investigated for its use in the management of pulmonary hypertension, a disease where epoprostenol is an established therapeutic **(318). A** recent, uncontrolled trial has suggested that inhaled iloprost may provide a novel treatment option in patients with life-threatening pulmonary hypertension that is unresponsive to current therapies **(340).** In particular, it has been used to combat pulmonary arterial obstructive disorder, which tends to occur following artery narrowing and occlusion influenced by atherosclerosis. This therapy is usually well toler-

ated, with headache and facial flushing being the most common adverse effects (341).

5.2.6 Carboprost. Carbaprost is a 15-methyl analog of dinoprost (prostaglandin $F_{2\alpha}$) and is also known as methyldinoprost. Like dinoprost, it is a uterine stimulant, but because of the presence of the 15-methyl group, it has a more prolonged course of action, because this delays enzymatic dehydrogenation associated with inactivation.

Clinical use is generally for the termination of pregnancy, but it is also used in postpartum hemorrhage. Following administration to the bladder, it has also proved useful in cyclophosphamide-induced hemorrhagic cystitis in bone marrow patients (342).

5.2.7 Limaprost. Limaprost (ONO-1206) is a synthetic prostaglandin E_1 analog that has been used in the treatment of peripheral vascular disease. It is usually administered orally. Compared with PGE_2, limaprost has two additional methyl groups at C-17 and C-20 and also an extra double bond at C-2–C-3. It has also been shown to have some efficacy in the treatment of Raynaud's syndrome (343).

6 NEW DEVELOPMENTS

All of the clinically useful prostaglandins at present exert their actions as agonists at prostanoid receptors, and are in general, synthetically produced natural prostaglandins or closely related analogs. Hence, although there has been a trend towards the exploitation of prostanoid receptor subtype specific ligands as potentially useful medicines, the compounds that have been used to date have failed to fulfil their therapeutic promise. For example, it was hoped that EP_3 receptor agonists, such as GR63799X and misoprostol, might prove beneficial as cytoprotective agents, but they proved to be less effective, in this respect, than histamine H_2 receptor antagonists, such as ranitidine.

However, the recent success of both latanoprost and unoprostone in the treatment of glaucoma (338, 339) suggests that FP receptor agonists at least may have an important future role in the clinic. Recent evidence from knock-out mouse studies has further suggested the potential involvement of EP_2, EP_3, and IP receptors in mediating painful responses (67, 344). Coupled with this, the use of EP_1 receptor antagonists for the treatment of inflammatory pain has recently been suggested (63). In light of the possibility that DP and EP_1 receptor antagonists might prove useful in the treatment of allergic inflammation and migraine, respectively (211, 345), there is evidence to suggest that, in the future, potent specific prostanoid receptor antagonists may make effective medicines.

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CHAPTER SEVEN

Retinoids

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Contents

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1 INTRODUCTION

Retinoids are derivatives of vitamin A that have both diverse and essential actions in developmental and cellular differentiation processes, vision, and reproduction (1). The biological activity of retinoids has been appreciated since the 1920s **(2, 3),** and the huge strides made by synthetic chemists, and molecular, developmental, and structural biologists since the late 1980s have contributed dramatically to our understanding of the molecular processes that underlie the biological activities of retinoid compounds. Retinoids are now well established as valuable therapeutic agents in the treatment of a variety of skin and proliferative disorders. Indeed, the number of retinoid compounds approved by the Food and Drug Administration (FDA) for clinical use, seven, has more than tripled since the last edition of this text, and there are no indications that the wave of retinoid drug development has reached a crest.

Naturally occurring retinoids are characterized by three distinct structural elements (Fig. 7.1): (1) a lipophilic terminus composed of a β-ionone ring, (2) an isoprene side chain that is subject to both enzymatic and non-enzymatic isomerization (Fig. 7.2), and **(3)** a polar terminus that is the subject of oxidative modifications (Figs. 7.1 and 7.2). All of these processes occur in *vivo* with the superimposed complexity of a multitude of binding and transport proteins and receptors possessing differential affinities for these naturally occurring retinoids.

The objectives of this chapter are to describe (1) retinoids that are currently in clinical use, including the adverse effects associated with the therapeutic use of these compounds; (2) retinoid biosynthesis and metabolism; **(3)** the basis of action of retinoids in molecular, cellular, and organismal contexts including vision; and (4) new agents on the horizon of retinoid biology.

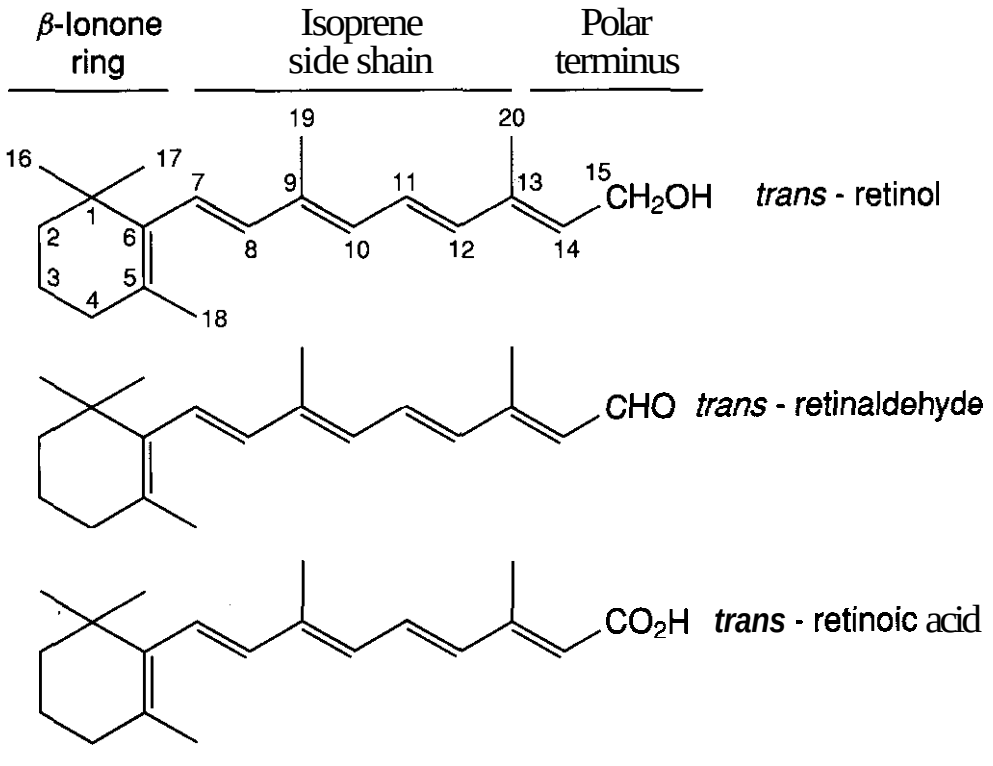
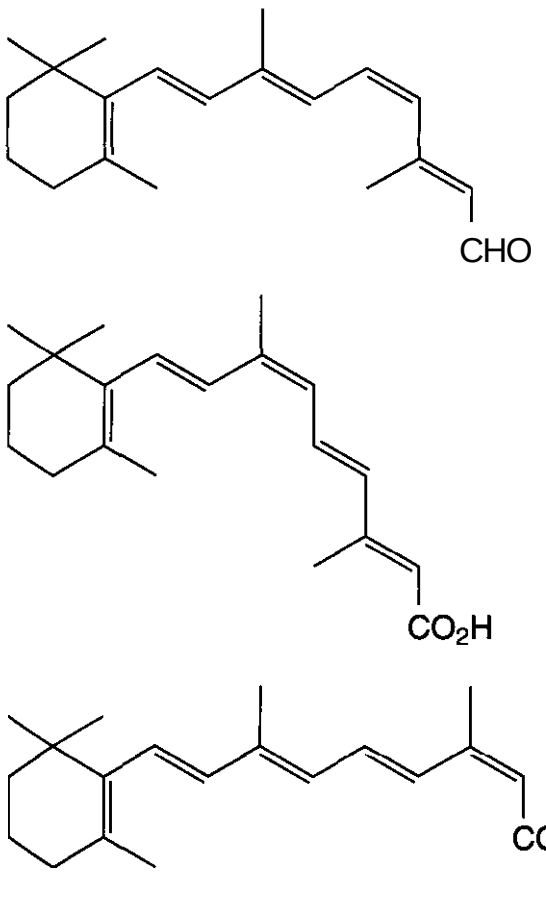


Figure 7.1. Naturally occurring trans-retinoids.



11-*cis*-retinal

9-cis-retinoic acid

13-cis-retinoic acid

Figure 7.2. Naturally occurring *cis*-reti-

2 CLINICAL APPLICATIONS

Early studies conducted by Wolbach and Howe suggesting that vitamin A plays a key role in limiting cellular proliferation and in the maintenance of the terminally differentiated phenotype of a number of cell types (2, 3) set the stage for the present use of naturally occurring retinoids and synthetic derivatives in the treatment of diseases of the skin (psoriasis, severe acne, and photodamage) and proliferative disorders (acute promyelocytic leukemia, cutaneous T cell lymphoma, and Kaposi's sarcoma). Retinoid compounds presently possessing FDA approval for clinical use are listed in Table 7.1 and structures are shown in Fig. 7.3. In addition, the reader is advised to consult governmental (http:// clinicaltrials.gov/) and Pharmaceutical Research and Manufacturers of America (http:ll www.phrma.org/searchcures/newmeds/webdb/ drugs.phtml) web pages that indicate all ongoing clinical trials that may ultimately lead to the use of retinoids to treat a wider spectrum of diseases including cancers of the skin, breast, kidney, bladder, prostate, and head-and-neck, neuroblastomas, acute myeloid leukemia, Hodgkin's lymphoma, and emphysema.

Adverse effects of retinoids in current use are generally severe and limit the clinical usefulness of these compounds. Systemic retinoid compounds are considered to be extreme teratogens, inducing severe malformations of the central nervous system, skull, external ear, eye, cardiovascular system, thymus, and parathyroid gland. (Table 7.2 describes teratogenicity associated with systemic isotretinoin use.)

Owing to the extreme teratogenicity of systemic retinoid compounds, the FDA and Roche Pharmaceuticals have taken stringent measures to prevent pregnant women from being administered systemic isotretinoin and to prevent women taking isotretinoin from becoming pregnant. All other systemic retinoid compounds bear similar warnings. Well-controlled studies on the potential human teratogenicity of topically applied retinoids have not been conducted; however, common sense dictates that topical use of retinoids during pregnancy should be avoided.

Non-teratogenic, adverse effects of retinoids are also severe and are dependent on route of administration and the nature of the disease being treated. Untoward effects of retinoid compounds administered by mouth are similar to those of hypervitaminosis A and include cheilitis and pronounced lipid disturbances such as hypertriglyceridemia, hypercholesterolemia, and reduction in high density lipoprotein levels, pancreatitis, vasculitis, con-

Table 7.1 Retinoids Currently Approved by FDA for Clinical Use

Generic Name	Trade Name	Route of Administration	Therapeutic Use
Adapalene	Differin	Topical	Acne vulgaris
Acitretin	Soriatane	Topical	Psoriasis
Alitretinoin (9-cis-retinoicacid)	Panretin	Topical	Kaposi's sarcoma
Bexarotene	Targretin	Topical	Cutaneous T-cell lymphoma
Isotretinoin (13-cis-retinoic acid)	Accutane	Topical Oral	Severe acne Severeacne
Tazarotene	Tazorac	Topical	Psoriasis
Tretinoin (*trans*-retinoic acid)	Renova	Topical	Skin photodamage including wrinkles, sun spots, and roughness
	Retin-A	Topical	Severe acne
	Vesanoid	Oral	Acute promyelocytic leukemia
	Solange (a combination of tretinoin and mequinol, a depigmenting agent)	Topical	Solar (actinic) lentigines

junctivitis, edema, lymphadenopathy, weight loss, GI disturbances including inflammatory bowel disease, dysregulation of blood glucose levels, benign intracranial hypertension, hearing impairment, fatigue, depression, decalcification of bone, calcification of cartilage and tendons, hair loss, and skin scaling.

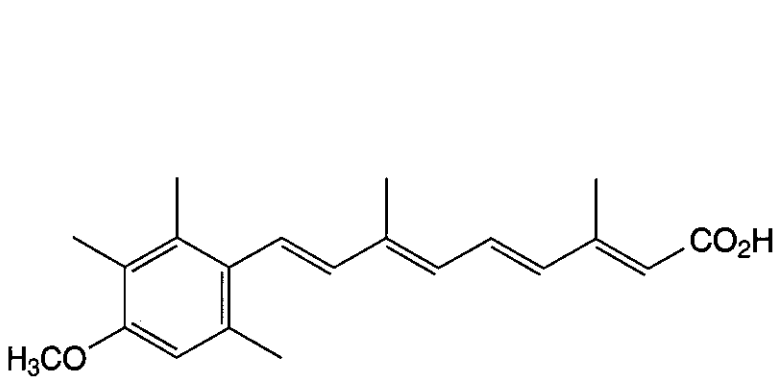
Adverse reactions associated with use of the systemic administration of tretinoin (Vesanoid) in the treatment of acute promyelocytic leukemia (APL) are particularly severe, resulting in what has come to be known as "Retinoic Acid-APL Syndrome." Approximately 25% of APL patients treated with tretinoin develop this syndrome, which is characterized by fever, dyspnea, weight gain, pulmonary infiltrates, and pleural or pericardial effusions resulting in compromised myocardial function and episodic hypotension. In addition, approximately 40% of patients undergoing oral tretinoin therapy for APL develop a rapidly evolving leukocytosis that can be associated life-threatening complications.

Topically administered retinoids, although generally free of systemic toxicity, cause moderate-to-severe adverse reactions at the site of administration, including irritation as indicated by development of a rash (erythema, scaling, irritation, redness, dermatitis), dryness, burning, pruritis, peeling, blistering, and edema. In many cases, initiation of topical retinoid therapy results in an exacerbation of the disease, such as acne, for which the retinoid is being used. This effect is generally temporary and subsides with continued therapy, but in severe cases, requires discontinuation of the retinoid agent.

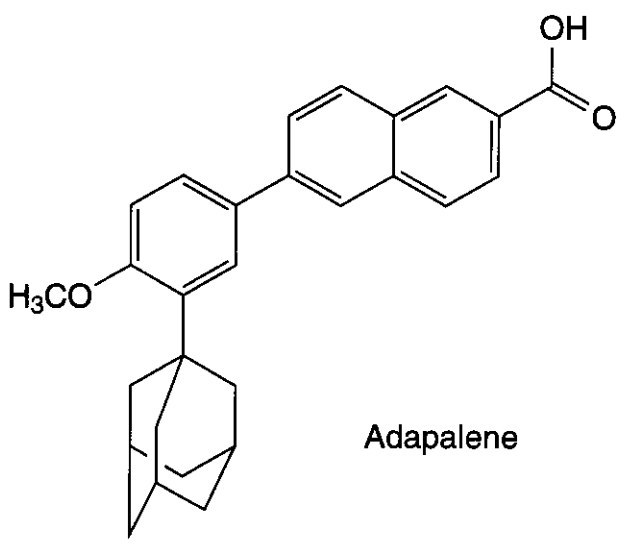
3 RETINOID BIOSYNTHESIS

3.1 Fate of Dietary Sources of Retinoids

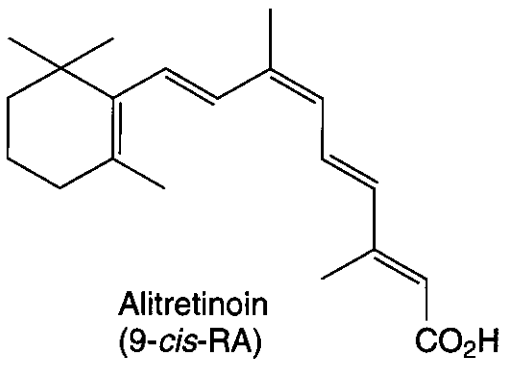
Vitamin A is essential for life, and although humans and other animals are capable of modifying retinoid compounds, these species are incapable of the de *novo* synthesis of retinoids. Thus, dietary intake of the retinoids is crucial for the maintenance of health and prevention of vitamin A deficiency. Dietary retinoids consist of two general types: (*1*) carotenoids, including principally β-carotene as well as potentially hundreds of other related compounds



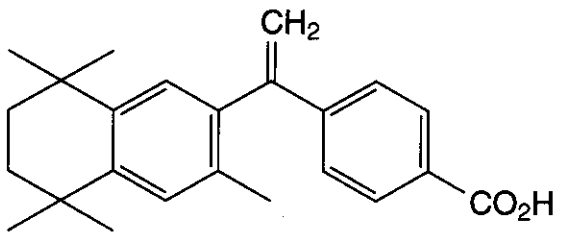
Acetretin



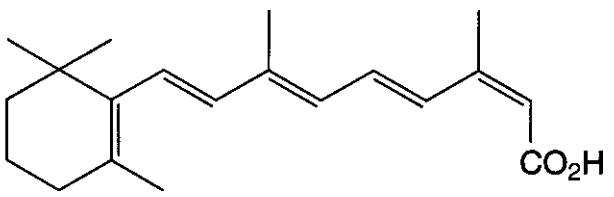
Adapalene



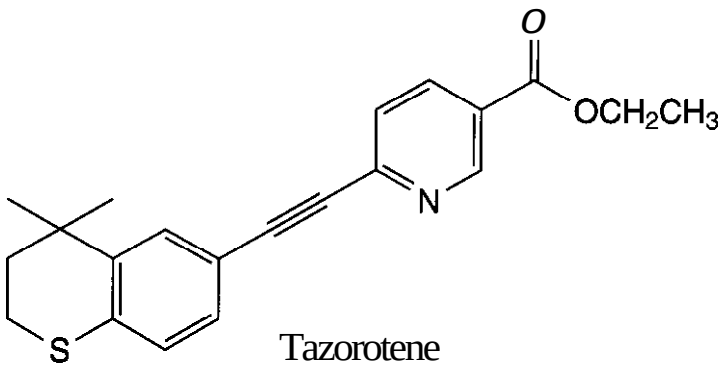
Alitretinoin
(9-*cis*-RA)



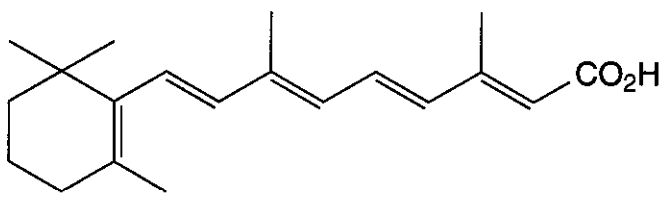
Bexarotene



Isotretinoin
(13-*cis*-RA)



Tazorotene



Tretinoin
(*trans*-RA)

Figure 7.3. Chemical structures of FDA-approved retinoids.

that are derived primarily from plant sources, and (2)several forms of **retinyl** esters that are derived from the consumption of other animals. Both of these precursors give rise to **retinol** that is the most abundant **retinoid** in blood, whereas **retinyl** esters in liver and other tissues represent the major storage form of retinoids in the body.

3.2 Symmetrical Cleavage of β-Carotene

The small intestine is a major site of **retinoid** metabolism, beginning **with** the oxidative cleavage of **β-carotene** into two molecules of retinaldehyde (Fig. 7.4). The subject of **β-carotene** cleavage has been quite controversial until recently because both symmetric and asymmetric cleavage of **β-carotene** has been reported in crude extracts, and at least in some systems, direct formation of **retinoic** acid from **β-carotene** has been observed (4). However, recent breakthroughs in this field have substantially clarified this area. These breakthroughs can be traced to the first identification of a maize **cDNA** encoding 9-cis epoxy-

Table 7.2 Human Teratogenicity Associated with Systemic Isotretinoin Therapy

Organ	Teratogenicity Observed
Central nervous system	Cerebral abnormalities Cerebellar malformation Hydrocephalus Microcephaly Cranial nerve deficits
Skull	Developmental abnormalities
External ear	Anotia Micropinna Small or absent external auditory canals
Eye	Microphthalmia
Heart and cardiovascular	Developmental abnormalities
Thymus	Developmental failure
Parathyroid gland	Functional deficits leading to parathyroid hormone deficiency

carotenoid dioxygenase, the enzyme responsible for the production of abscisic acid, a carotenoid cleavage product that functions as an important plant growth regulator (5, 6). Vogt and collaborators used cDNA encoding maize 9-cis epoxycarotenoid dioxygenase as a probe to isolate a metazoan (Drosophila) homolog of this enzyme and showed that the enzyme, β-carotene-15,15′-dioxygenase, symmetrically cleaved p-carotene into two molecules of retinaldehyde (7). Interestingly, mutations in the Drosophila gene, *ninaB*, encoding this enzyme, cause blindness, because this species (8), like humans, is dependent on retinoid chromophores for rhodopsin-based visual signal transduction (see below). The phenotype associated with this mutant can be rescued by dietary administration of retinal, the direct precursor of the Drosophila visual chromophore, 3-hydroxy-retinal (8), thus bypassing the need for dietary sources of carotinoids. Hunziker's group then isolated a cDNA encoding the chicken β-carotene-15,15′-dioxygenase (9), and the groups of Blaner and Cunningham isolated the mouse homolog (10, 11) and demonstrated that this enzyme catalyzed the symmetrical cleavage of p-carotene into two molecules of retinaldehyde.

3.3 Asymmetrical Cleavage of β-Carotene

Asymmetric cleavage p-carotene has been reported by several groups, and this controversy also seems to be solved as a result of the cloning of a cDNA encoding an enzyme that asym-

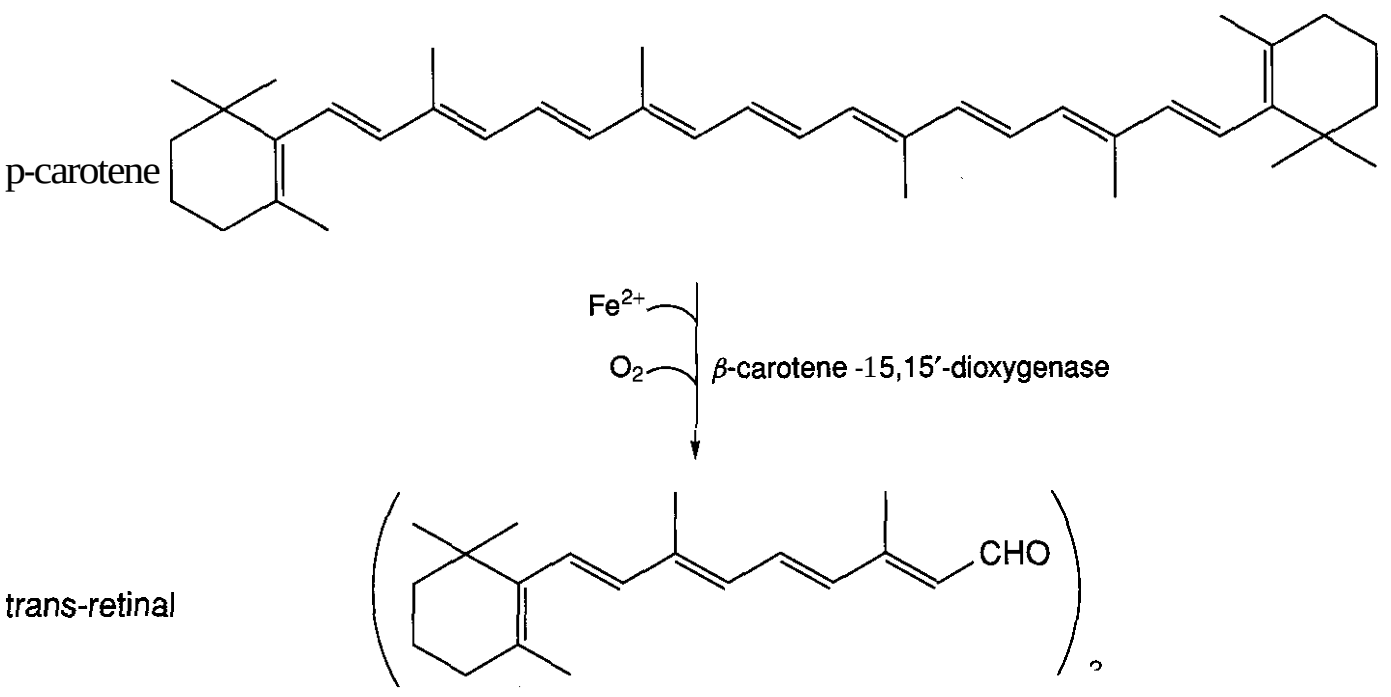


Figure 7.4. Symmetrical cleavage of p-carotene.

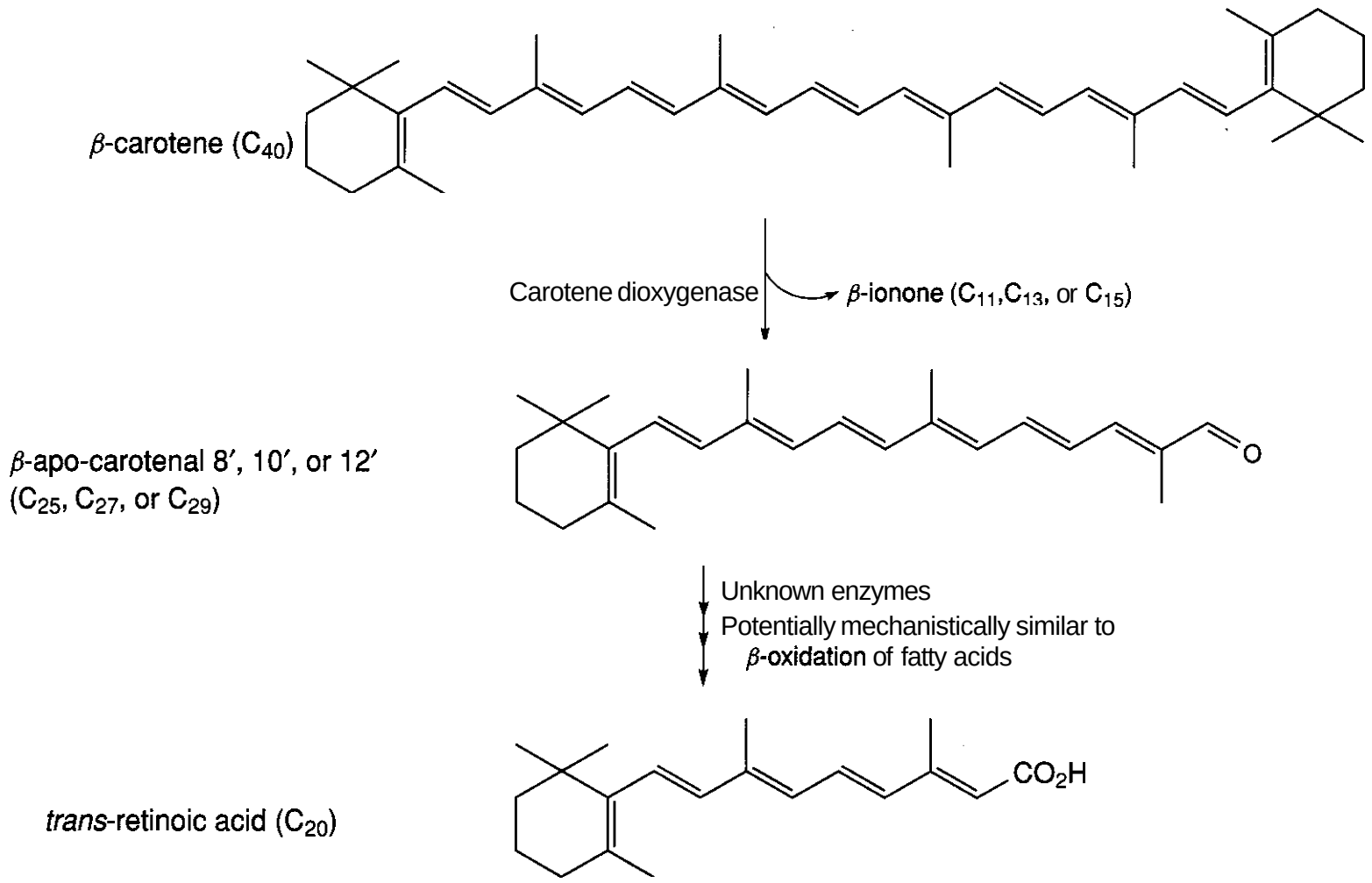


Figure 7.5. Asymmetical cleavage of β-carotene.

metrically cleaves p-carotene to yield two molecules of β-apocarotenal with different chain lengths (12) (Fig. 7.5). These β-apocarotenal cleavage products have been proposed to serve as precursors to retinoic acid in a retinal-independent pathway (13). In this proposed pathway, it has been suggested that the β-apocarotenal cleavage products are subjected to a series of chain-shortening reactions similar to β oxidation of fatty acids and then oxidatively metabolized to one molecule of retinoic acid (13); however, this has yet to be demonstrated directly. Nonetheless, it is now clear that at least two pathways of p-carotene metabolism are possible in humans and other mammals, whereas Drosophila use only the symmetrical cleavage pathway. The enzymes responsible for these cleavage reactions are present in intestinal mucosal cells that likely serves as an important site of p-carotene processing in humans. The enzymes are also present in several other tissues and seem to be expressed during the early stages of embryogenesis, suggesting that the localized production of retinoids during developmental processes is critical.

3.4 Formation of Retinyl Esters

Retinaldehyde, when bound to retinol binding protein II (CRBPII), serves as a substrate for retinal reductase resulting in the production of retinol (14), which then binds to cellular retinol binding protein (CRBP) forming holo-CRBP. Holo-CRBP seems to be the preferred substrate for an esterification reaction (Fig. 7.6) mediated by lecithin:retinol acyl transferase (LRAT), a microsomal enzyme that uses acyl groups donated from phosphatidylcholine (14). In cells not expressing CRBP, retinol esterification is carried out by a different enzyme, acyl CoA:retinol acyl transferase (ARAT).

Dietary retinyl esters are also processed in mucosal cells of the intestine through sequential de-esterification/re-esterification reactions (14). Retinyl esters are then incorporated into chylomicrons and pass from the intestine into the lymph fluid where lipolysis occurs, resulting in the formation of chylomicron remnants that continue to harbor retinyl esters. Chylomicron remnants containing retinyl esters en-

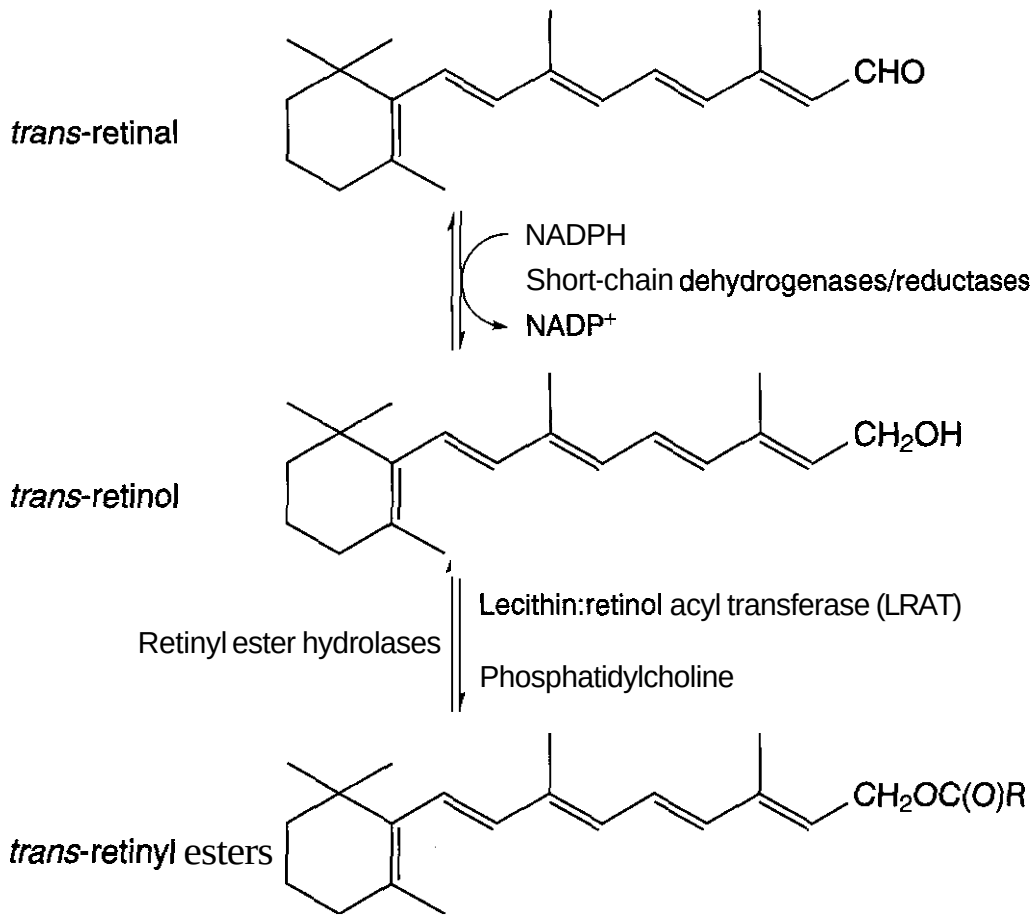


Figure 7.6. Reduction of retinal to retinol in gut and formation of retinyl esters.

ter the general circulation and are taken up primarily by the parenchymal cells of the liver and de-esterified to yield retinol. Cellular concentrations of free retinol in liver are quite low because most retinol binds immediately to CRBP or retinol binding protein (RBP), the latter of which is a serum transport protein belonging to a large family of fatty acid binding proteins. RBP-retinol is secreted by the parenchymal cells and taken up by the stellate cells of the liver or target tissues (15, 16). Within stellate cells, retinol is re-esterified and stored in lipid droplets in which *trans*-retinyl esters account for approximately 42% of total lipid present (15, 16). Palmitate retinyl esters comprise the majority retinyl esters present in lipid droplets with lower amounts of the corresponding stearate, oleate, and linoleate esters. Retinyl esters present in the liver stellate cells account for approximately 75% of total retinoid body stores and are extremely long-lived (17).

3.5 Mobilization of Body Vitamin A Stores

As extrahepatic demand dictates, retinyl esters stored in the stellate cells of the liver are mobilized (18). This process involves de-esterification by the action several retinyl ester hydrolases, rebinding to RBP, and transport to the target tissue(s). RBP seems to be critical for retinol transport to tissues, especially when the diet is deficient in vitamin A. Mice that are null for RBP expression exhibit reduced levels of serum retinol levels: however. these mice seem to function normally, at least with respect to vision, if retinol is supplemented in the diet (19). Retinol dissociates from RBP on reaching these extrahepatic tissues and likely enters the target cell by passive diffusion (18). Although a receptor-mediated form retinol uptake has been proposed (20, 21), it is unclear how this contributes to the overall cellular uptake of retinol which has been repeatedly shown to enter the cell rapidly by passive diffusion (18).

3.6 The Fate of Intracellular Retinol

The fate of intracellular retinol is dependent on cell type. In the eye, trans-retinol can be esterified by LRAT or isomerized to 11-*cis*-retinol by a cell-specific isomerase (22). 11-*cis*-retinol is then oxidized to 11-cis-retinal by 11-cis-retinol dehydrogenase (22). 11-cis-retinal binds to opsin by forming a Schiff base with an opsin lysine residue and the resulting complex is known as rhodopsin (22). Rhodopsin is a member of the seven transmembrane family of proteins that serves as receptor for photons

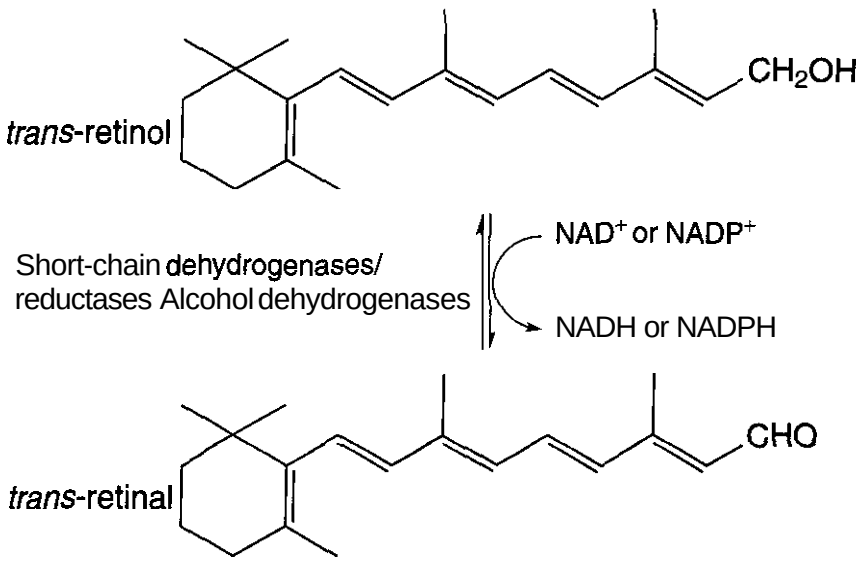


Figure 7.7. Oxidation of retinol to retinal in cells.

(23). Light-induced isomerization of 11-*cis*-retinal plays a central role in the conversion of visual signals, i.e., light, into nerve impulses known as the visual cascade (see below) (23).

3.7 Oxidation of Retinol

In many other cell types, trans-retinol binds to CRBP and this holo-CRBP serves as the substrate for an oxidative reaction in which the primary alcohol is converted into an aldehyde (Fig. 7.7), yielding retinaldehyde (trans-retinal) (18). CRBP plays an extremely important role both in protecting retinol from non-specific oxidative reactions and in the oxidative reaction to retinaldehyde (18). Indeed, CRBP physically interacts with the enzyme(s) responsible for this oxidation reaction, retinol dehydrogenase (RDH). Many enzymes, including class I, II, and IV cytosolic alcohol dehydrogenases, as well as microsomal RDHs, short-chain dehydrogenases, and cytochrome P450 isoforms, have been described that convert unbound retinol into retinal in a nicotinamide adenine dinucleotide phosphate ($NADP^+$)– or nicotinamide adenine diphosphate (NAD^+)–dependent manner (14, 24). With a bewildering number of enzymes capable of oxidizing retinol to retinal, this area remains a bit of a controversial topic in retinoid biology (14), but it is currently felt that the enzymes using holo-CRBP as a substrate (as opposed to free retinol) may be the most physiologically relevant of the large family of "retinol dehydrogenases." However, the crystal structure of CRBP bound by retinol has been solved at 2.1Å, revealing that the protein is composed of 10 anti-parallel β strands that fold into an orthogonal barrel (25). Within the barrel, trans-retinol was found to exist in a planar conformation with the free hydroxyl bonded to Gln^{108}. From these definitive, crystallographic studies, it is not clear how the hydroxyl group, which is sequestered deep within the protein, would be available for oxidation in a reaction catalyzed by a retinol dehydrogenase as proposed by Napoli (14). Clearly, the role of holo-CRBPI in the oxidation reaction needs to be clarified, and this will likely require co-crystallization of holo-CRBP and the retinol dehydrogenase. Thus, the complete picture is still emerging, and a potential role for the cytosolic alcohol dehydrogenases, which seem to use only free retinol as the substrate, in retinol oxidation cannot be ruled out at this time (24).

3.8 Formation of Retinoic Acid: A Major Signaling Retinoid

Oxidative conversion of retinal (RAL) to retinoic acid (RA; Fig. 7.8) is a much less contentious issue because two types of retinaldehyde dehydrogenases have been clearly delineated (14). Once formed, RAL binds to CRBP forming CRBP-retinal, which serves a substrate for both retinaldehyde dehydrogenase 1 and 2 (RALDH1 and RALDH2, respectively), that catalyzes the irreversible formation of RA (14). In addition, both enzymes also produce RA from free RAL, however, free RAL is essentially undetectable in all tissues excluding the retina (14). Both RALDH enzymes are expressed in multiple tissues and during embryogenesis; however, the expression pattern of RALDH2 seems to be much more restricted,

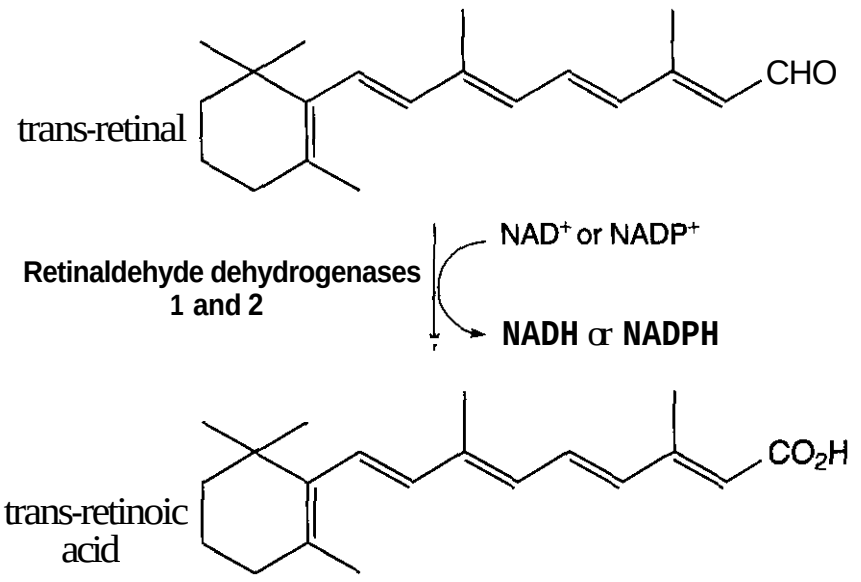


Figure 7.8. Oxidation of retinal to retinoic acid in cells.

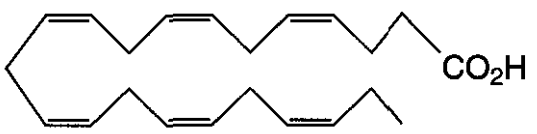
suggesting a role in the localized formation of RA during development and in the adult organism (26–28). Indeed, this hypothesis has been confirmed by genetic means that demonstrated the essential role of RALDH2 during embryogenesis (29). Although the two enzymes are highly related on the amino acid level and bind RAL with a reasonably similar affinity, two findings strongly suggest that RALDH1 and RALDH2 perform distinct functions in *vivo*. First, the expression of the two enzymes differs temporally during embryogenesis (26–28) and spatially during both embryogenesis and in adult animals (14). Second, the two enzymes are differentially regulated by apo-CRBP, which inhibits RALDHl (IC_{50} = 1.4 μM) but not RALDH2 (14). Third, RALDH1 and RALDH2 may be differentially regulated by RA, presumably acting through RARs and RXRs to regulate expression of the corresponding genes (see below) (14).

A third RALDH (RALDH3) has been identified in the developing chick retina (30, 31). This enzyme is capable of producing RA from RAL and appears to be the avian ortholog of human aldehyde dehydrogenase 6 (30, 31). In addition to the developing retina (which also expresses RALDH1), RALDH3 expression was also observed in the nasal region (30, 31), suggesting that this RALDH may be involved in both retinal and olfactory system development. The relative contributions of each RALDH enzyme to the overall production of RA likely depends on both cell type and developmental stage of the organism. Each of the RALDH enzymes display a high degree of inter-species conservation, suggesting a commonality of function and highlighting the important role of each. Furthermore, Napoli's group recently reported the isolation of another aldehyde dehydrogenase, ALDH12, capable of converting retinaldehydes into RA (see below).

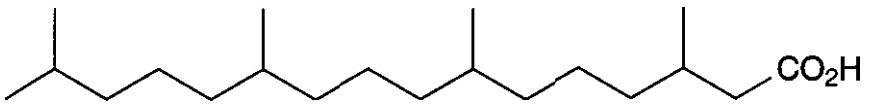
With the exception of the eye and possibly B and T lyrnphoctes (see below), trans-RA and its isomers are generally considered to be the most important retinoids with regard to cellular signaling. The retinoic acids bind directly to and activate the numerous members of the retinoic acid (RAR) and retinoid X (RXR) families of nuclear receptors, all of which function as ligand-dependent transcription factors. In turn, these receptors convey the retinoid signal by regulating the expression of literally hundreds of RA target genes encoding proteins that are involved in nearly all aspects of organismal function during development and in adult life. Indeed, all of the retinoid compounds approved by the FDA for clinical use activate one or both families of retinoid receptors. The RARs, RXRs, retinoid signaling mechanisms, and target genes are discussed in greater detail in following sections.

3.9 Active Isomers of RA

Some isomers of trans-RA have been reported to exert very potent biological effects. Most noteworthy of these is 9-cis-RA (see Fig. 7.2), which was originally demonstrated to be a retinoid that activates both RARs and RXRs, whereas trans-RA activates only RAR family members (32–34). Levin and colleagues originally isolated [^{3}H]9-*cis*-RA using RXRs to "trap" it in cells fed [^{3}H]*trans*-RA, implying



Docosahexaenoic acid



Phytanic Acid

Figure 7.9. Structures of alternative RXR agonists.

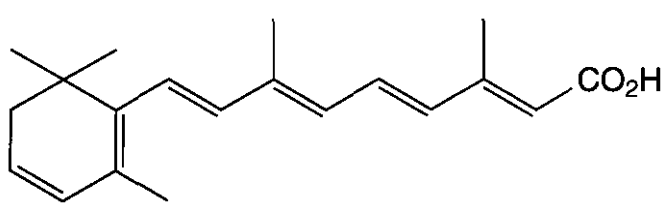
that the cells were capable of the isomerization step (32). Heyman and collaborators took a related approach to arrive at the same conclusion independently (35). However, the putative trans-RA isomerase has not been isolated and isomerization can occur non-enzymatically (36, 37). In addition, cisoid derivatives of β-carotene exist that may give rise to 9-*cis*-retinol, 9-*cis*-RAL, and ultimately, 9-cis-RA (38). Napoli and Lin recently isolated a novel retinal dehydrogenase activity corresponding to the previously known human aldehyde dehydrogenase, ALDH12 (39). ALDH12 was demonstrated to have a distinct preference for 9-*cis*-RAL relative to *trans*-RAL, suggesting that this enzyme may be important for the in vivo formation of 9-*cis*-RA (39). Moreover, ALDH12 expression was observed in fetal and adult tissues (liver and kidney) that contain relatively high levels of cis retinoid precursors such as 9-*cis*-retinol. Thus, the possible isomerization of trans-RA into the 9-cis-RA in vivo does not seem to be required for the generation of the latter compound.

Other groups have demonstrated that several chemical moieties may activate RXR family members leading investigators to speculate that 9-cis-RA may not be the only physiological ligand capable of activating RXRs in vivo (40, 41). Interestingly, neither of the other RXR-active compounds, phytanic acid and docosahexaenoic acid, is a retinoid, although both harbor structural motifs similar to naturally occurring retinoids (Fig. 7.9).

An unsaturated derivative of retinoic acid, 3,4-didehydroretinoic acid (Fig. 7.10), has been demonstrated by Eichele and Thaller to be an important signaling molecule in a model system of limb development, the chick limb bud assay (42). 3,4-Didehydroretinoic acid is derived from 3,4-didehydroretinol, which is also known as vitamin A2. However, with the exception of cultured human keratinocytes exposed to high concentrations of retinol (43), this form of retinoic acid has not been detected in mammalian tissues to any appreciable degree (14). Thus, the potential role of 3,4-didehydroretinoic acid in the vitamin A signaling pathways in mammals is not clear at the present time.

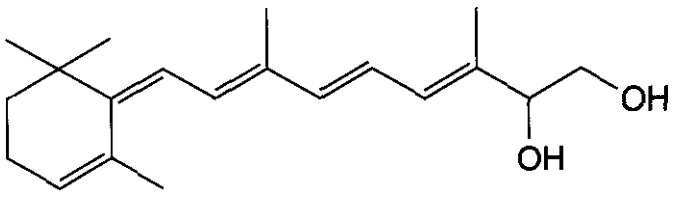
3.10 14-Hydroxy-4,14-retro-retinol

Hämmerling and his colleagues have identified a novel pathway of vitamin A action in lymphocytes that does not involve RA. These investigators originally discovered that retinol was required for B-cell proliferation (44) and T-cell activation (45) and that RAL, but not RA, would substitute for retinol in these actions. These investigators have subsequently discovered that 14-hydroxy-4,14-retro-retinol and 13,14-dihydroxyretinol (Fig. 7.11) bind to and augment the activation of both cRaf and protein kinase Cα by reactive oxygen (46, 47). cRaf and protein kinase Cα are serine-/threonine-kinases that play important roles in signal transduction cascades, such as that associated with activation of mitogen-activated protein (MAP) kinase. If these data are confirmed, both cRaf and protein kinase Cα can also be considered as receptors for retro-retinoids. Hammerling et al. has proposed that retro-retinoids may function to facilitate electron transfer allowing the efficient redox acti-

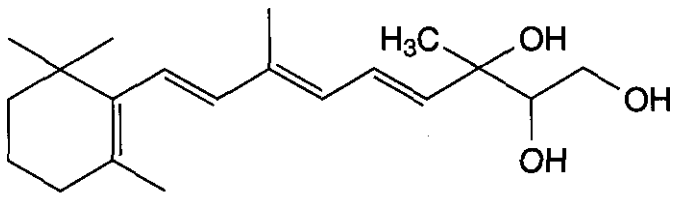


3,4-didehydro-*trans*-retinoic acid

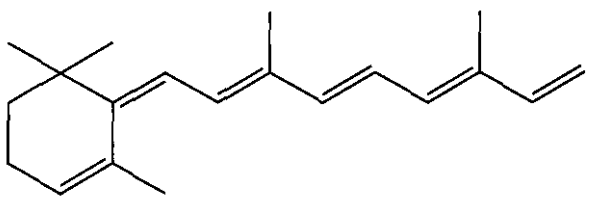
Figure 7.10. 3,4-didehydroretinoic acid.



14-hydroxy-4,14-*retro*-retinol



13,14-dihydroxy-retinol



Anhydroretinol

Figure 7.11. Retro-retinoids.

vation of both kinases (47). These investigators have also isolated another compound, anhydroretinol (Fig. 7.11), that functions as an antagonist of 14-hydroxy-4,14-retro-retinol action in the lymphocyte context (48). Anhydroretinol not only antagonizes the effects of 14-hydroxy-4,14-retro-retinol on B-cell proliferation and T-cell activation, but itself rapidly induces T cell death (48). Thus, it seems that 14-hydroxy-4,14-retro-retinol and anhydroretinol, both of which are produced by lymphocytes from retinol, act back on these cells to regulate homeostasis in opposing directions.

Unlike many retinol dehydrogenases, the enzyme that catalyzes the conversion of retinol to anhydroretinol, retinol dehydratase, does not use CRBP-retinol as a substrate; rather, it uses free retinol (49–51). However, the enzyme responsible for biosynthesis of 14-hydroxy-4,14-retro-retinol has not yet been isolated.

3.11 Non-Receptor, RA Binding Proteins

Retinoic acids, particularly *trans*-RA, bind with high affinity to cellular retinoic acid binding proteins I and II (CRABP I and CRABP II). Like the CRBPs, CRABPs are widely expressed in the embryo and adult and are found in essentially all RA target tissues. Three functions have been ascribed to the CRABPs: (1)it is generally believed that the CRABPs serve to buffer intracellular concentration of RA by binding free RA, *(2)*CRABP may facilitate metabolism of RA in that CRABP-RA complexes are better substrates for oxidative enzymes than is free RA (14), and (3)CRABPs may also function to transport RA to the nucleus, the loci of action of the nuclear receptors RARs and RXRs (18).Functions 1 and 2 would serve to reduce the cell's sensitivity to RA, and this is consistent with overexpression studies in F9 embryonal carcinoma cells (52). However, both CRABP I- (53, 54) and II- (55, 56) null animals are remarkably normal, as are the double knock-out (CRABP $I^{-/-}$/CRABP $II^{-/-}$) animals (55). Moreover, none of these animals are more sensitive to RA-induced toxicities than animals expressing wild-type CRABP I and II (55). If CRABP I or II play a role in "buffering" intracellular concentrations of RA or in the delivery of RA to the nucleus as hypothesized, it is unclear why knock-out animals do not display more of a phenotype. Thus, the biological role(s) of the CRABPs remains an open question.

4 RETINOID METABOLISM

Tissue-specific catabolism of RA is extremely important in terminating the retinoid signal, and ultimately, in dictating the tissue responsiveness to RA. Thus, the metabolism of RA and related compounds has generated a large amount of interest for both clinicians and basic scientists. Consistent with other aspects of retinoid biology, metabolism of RA and RA precursors is a complex area. First, one of the major pathways of RA metabolism is glucuronidation at the carboxyl group (Fig. 7.12). This reaction is catalyzed by the liver enzyme UDP-glucuronyl transferase and the product of the reaction, retinoyl-β-glucuronide, participates in a large degree of enterohepatic circulation. Napoli has suggested that retinoyl-β-glucuronide may serve as a water-soluble, reclaimable pool of RA to prevent retinoid deficiency (14).

Second, oxidative metabolism (hydroxylation) of RA occurs at both the C4- and C18-

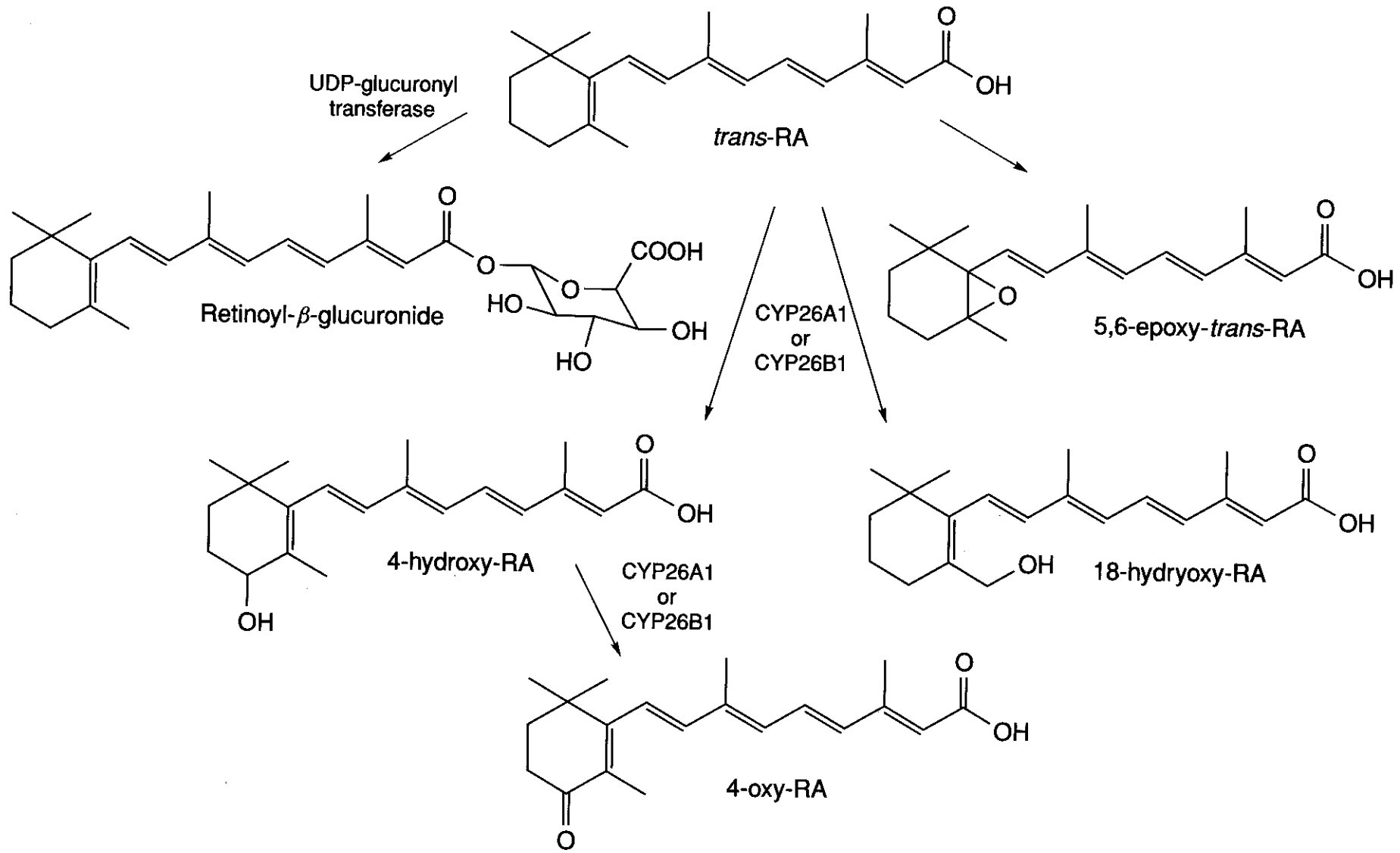


Figure 7.12. Retinoic acid metabolites.

positions of the β-ionone ring yielding 4- and 18-hydroxy RA, respectively, and this hydroxylation has been shown to be induced by RA (57, 58). The relative importance of the two pathways of oxidative metabolism are unknown, but oxidation at the 4-position has been studied more extensively. 4-Hydroxy RA is further metabolized to give 4-oxo RA that then undergoes isomerization to yield 4-oxo-13-cis-RA (59–61). Together, the retinoyl-β-glucuronide and the C4-oxidation products account for most of the steady-state metabolites of RA (59–61). Minor metabolic products include the 5,6-epoxy-*trans*-RA (61) (see Fig. 7.12).

C4-oxidation of RA is catalyzed by cytochrome P450 as suggested by the auto-induction of RA metabolism at this position (see above) and the inhibition of the hydroxylation reaction by ketoconazole and related compounds that are non-specific inhibitors of many members of the cytochrome P450 family (62). Indeed, Petkovich and colleagues recently identified a cytochrome P450 family member that hydroxylates RA at both the C4- and C18-positions in a NADPH-dependent manner, and furthermore, oxidizes 4-hydroxy RA to 4-oxo RA (63). The expression of this enzyme, Cyp26A1, is induced by RA in several cell lines and tissues, and animals lacking the enzyme display many of the features of hypervitaminosis A, particularly teratogenesis (64), suggesting that Cyp26A1 plays an important role in RA metabolism in the developing embryo. Recently, a second member of the Cyp26 family was described, Cyp26B1 (65–67), that displays a pattern of expression different from that of Cyp26A1, suggesting that each enzyme may play a distinct role in RA metabolism. Both enzymes exhibit similar substrate specificity and kinetic parameters (66). Interestingly, the 4-oxo metabolites of RA are better substrates for UDP-glucuronyl transferase-mediated glucuronidation than *trans*-RA (68), suggesting that hydroxylation of RA at the C4-position may facilitate conjugation and elimination.

The induction of Cyp26A1 expression by RA is of great clinical relevance because increased expression of the enzyme potentially reduces the intracellular pool of endogenous or pharmacologically administered trans-RA that is available to activate the retinoic acid receptor subtypes. Because the receptors likely mediate the beneficial effects of trans-RA in a therapeutic sense, this renders *trans*-RA treatment less efficacious in the application for which it is being used. This may be a particular concern for the use of trans-RA in some types of breast cancer as Petkovich and colleagues have shown that Cyp26A1 is strongly up-regulated by both trans-RA and 9-*cis*-RA in MCF-7 breast cancer cells (69). The Petkovich group and others have also shown that *trans*-RA induces Cyp26A1 expression and its own metabolism in many other cell types including human leukemic cells and cervical cancer cells, suggesting that the problem of auto-induction of *trans*-RA metabolism may generalize to other types of cancer cells. Clinically, it may be possible to circumvent this problem using at least two strategies: (*1*) use of retinoid analogs that are not Cyp26A1 or Cyp26B1 substrates or (2) concomitant use of trans-RA and an inhibitor of Cyp26A1 and/or Cyp26B1 that would result in prolongation of the half-life of *trans*-RA in the cancer cell. The synthesis of specific Cyp26A1/Cyp26B1 inhibitors capable of augmenting RA action in cancer cells represents a potentially exciting new area of drug development.

5 ROLE OF RETINOIDS IN VISUAL SIGNAL TRANSDUCTION

Metabolites of retinol play a crucial role as visual pigments in the visual systems of all vertebrate and invertebrate animals. Humans and higher vertebrates use 11-cis-retinal as the primary visual pigment (22), whereas other species use 3,4-didehyrdoretinal (fish, amphibians, and some reptiles), (3*R*) and (3*S*)-3-hydroxyretinal (insects), and (4*R*)-4-hydroxyretinal (cephalopods). In all cases, the role of the retinoid metabolite is similar: photons induce isomerization of a visual pigment which then initiates a cascade of nervous impulses that are transmitted to the regions of the brain that process visual signals (22, 23).

The role of 11-cis-retinal in the mammalian visual cycle is very well understood at the molecular level. This cycle is composed of three components: (1)light detection, (2) initiation

of signal transduction, and (3) regeneration of rhodopsin, which functions as a "receptor" for photons.

5.1 Photon Detection

Detection of photons in the mammalian visual system is accomplished by the light-induced isomerization of 11-cis-retinal to all-*trans*-retinal. The process is similar in both rods, which serve essentially as light detectors, and cones, which are capable of color recognition (22). However, the detection kinetics, regeneration time, and signal and wavelength sensitivities vary between the two types of vision-sensing cells. The photon "receptor" in all cases is rhodopsin, which consists of a protein (opsin) in complex with 11-cis-retinal through the formation of a Schiff base (23). The light-induced isomerization of 11-cis-retinal induces a conformational change in the associated protein moiety (opsin) that underlies activation of the protein (22, 23).

5.2 Initiation of a Visual Signal

Rhodopsin is a member of the seven transmembrane family of guanine nucleotide binding protein-coupled receptors (70, 71). This family contains a very large number of proteins, including receptors for neurotransmitters, peptides, odorants, and gustants (compounds that generate a taste sensation) (70, 71). All of these receptors signal through guanine nucleotide binding (G)-proteins. However, rhodopsin is unique in two respects. First, rhodopsin has a bipartite structure composed of a protein component (opsin) and 11-cis-retinal in a protonated Schiff base linkage with the ϵ-amino group of lysine^{296} (bovine opsin) (22, 23, 70, 71). Second, in contrast to other G-protein–coupled receptors, the "agonist" that activates rhodopsin is a photon of light rather than a chemical, peptide, or hormone (71). As described above, a single photon of light induces the isomerization of 11-*cis*-retinal to all-trans retinal (Fig. 7.13). The structural alteration associated with light-induced isomerization of 11-cis-retinal is transduced through the Schiff base to the opsin component of rhodopsin, resulting in the formation of the active conformation of rhodopsin (metarhodopsin II, hereafter referred to as R*; see Fig. 7.13). R* interacts with another membrane protein, transducin (Gt), the G-protein of this signaling cascade. Gt, like other G-proteins, is a heterotrimeric complex composed of a, β, and γ subunits. In the inactive state, the a subunit of transducin (Gt_α) is bound by GDP. However, interaction with activated R* promotes rapid GTP-GDP exchange on Gt, with concomitant dissociation of holo-Gt into Gt, (that is now bound by GTP) and $Gt_{\beta\gamma}$ subunits. Although this discussion focuses on the role of Gt_α-GTP, an important role for $Gt_{\beta\gamma}$ is not ruled out (71). Gt_α-GTP is known to be an important activator of a membrane-associated, cGMP phosphodiesterase (PDE), and this occurs through the direct interaction of Gt_α-GTP with PDE and subsequent displacement an inhibitory subunit of PDE (22, 23, 70, 71). In the dark state, high intracellular levels of cGMP activate a cGMP-gated cation channel that conducts primarily Na^+ and Ca^+ (22, 23, 70, 71). The current through this channel, which is known as the "dark current," determines the resting potential of the dark-adapted rod. However, activated PDE rapidly cleaves cGMP, lowering intracellular levels of cGMP and resulting in the shutting off the dark current (22). This results in a rapid hyperpolarization of the rod. On one side, the rod cell detects photons throughout the above-described mechanism. However, the opposing side of the rod is in synaptic contact with other retinal cells such as both horizontal and bipolar cells and these synapses use glutamate, an excitatory neurotransmitter (22, 23, 70, 71). Hyperpolarization of the rod reduces its excitability, which results in a decrease in the amount of glutamate released by the cell onto horizontal and bipolar cells (22). This signal is then transduced through the layers of the retina and ultimately to the optic nerve that projects to, among other areas, the visual cortex. The same signal also inhibits signaling by neighboring cells and this "surround inhibition" provides a mechanism for the precise localization of stirnulatory input, i.e., light, into the visual system (22, 70, 71).

5.3 Shutting Off R*

The a subunit of transducin, like other G-proteins, harbors an intrinsic GTPase activity

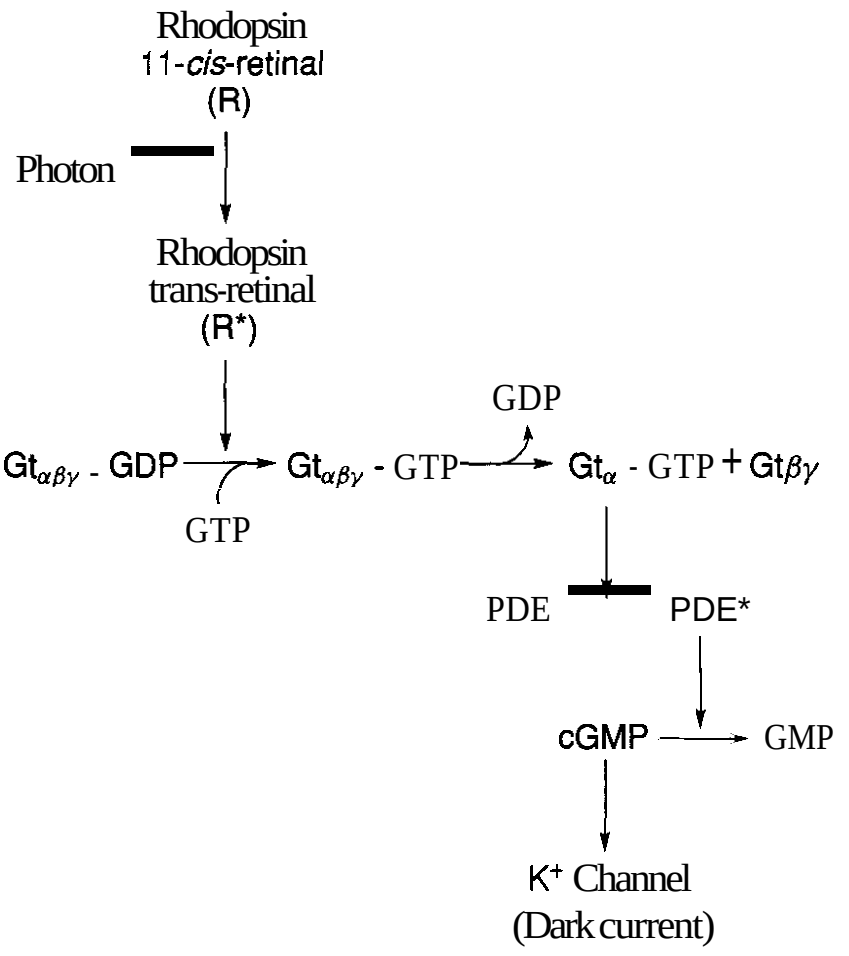


Figure 7.13. Visual system signaling **and** the role of 11-cis-retinal.

that hydrolyzes bound GTP to GDP, thereby terminating signaling by Gt_{α}-GTP (71). The Gt, GTPase activity is greatly stimulated by a GTPase accelerating protein (GAP) complex composed of the regulator of G-protein signaling (RGS)9 protein and a G-protein β subunit (Gβ5) (22). The GAP complex rapidly catalyzes inactivation of Gt_{α}-GTP to Gt_{α}-GDP, the latter of which does not interact with or activate PDE. Thus, the inhibitory and catalytic subunits of PDE rebind with a concomitant decrease in intracellular PDE activity and a rise in cGMP levels that results in reactivation of the dark current (23).

An interesting form of self-regulation also occurs at the level of activated rhodopsin. R* is the substrate for a kinase known as rhodopsin kinase that phosphorylates the protein at multiple serine and threonine residues in the carboxyl terminus of the protein (70). Although this phosphorylation somewhat compromises the ability of R* to interact with and activate transducin, complete inactivation of R* requires both rhodopsin kinase-mediated phosphorylation of R* and the binding of another protein, arrestin, to phosphorylated rhodopsin (71). The phosphorylated rhodopsin is subsequently dephosphorylated by a protein phosphatase 2A isoform to generate native opsin, which can then recombine with 11-*cis*-retinal to reform rhodopsin (see below) (22).

5.4 The Retinoid Cycle in Visual Processes

The retinal pool of 11-cis-retinal is clearly critical for photon detection and visual signal transduction. There are two key aspects regarding this that are important to consider: the precursor(s) from which 11-cis-retinal derived, and, bioconversion of isomerized *trans*-retinal back to 11-cis-retinal that can recombine with opsin to re-form rhodopsin. The latter aspect is obligatory for restoration of the dark state, regenerating a photosensitive receptor capable of undergoing another cycle of photon detection and signal transduction. Considered together, these events represent the retinoid cycle in the visual process (22). The entire process of re-isomerization and formation of a new rhodopsin molecule occurs in two different retinal tissues and involves several enzymatic steps that are described below (see Fig. 7.14).

Dietary sources of vitamin A also provide the metabolites that are necessary for vision. The ingestion and processing of β-carotene or retinyl esters to retinol and the transport of RBP-retinol complexes to target tissues was discussed earlier in this chapter, and the ret-

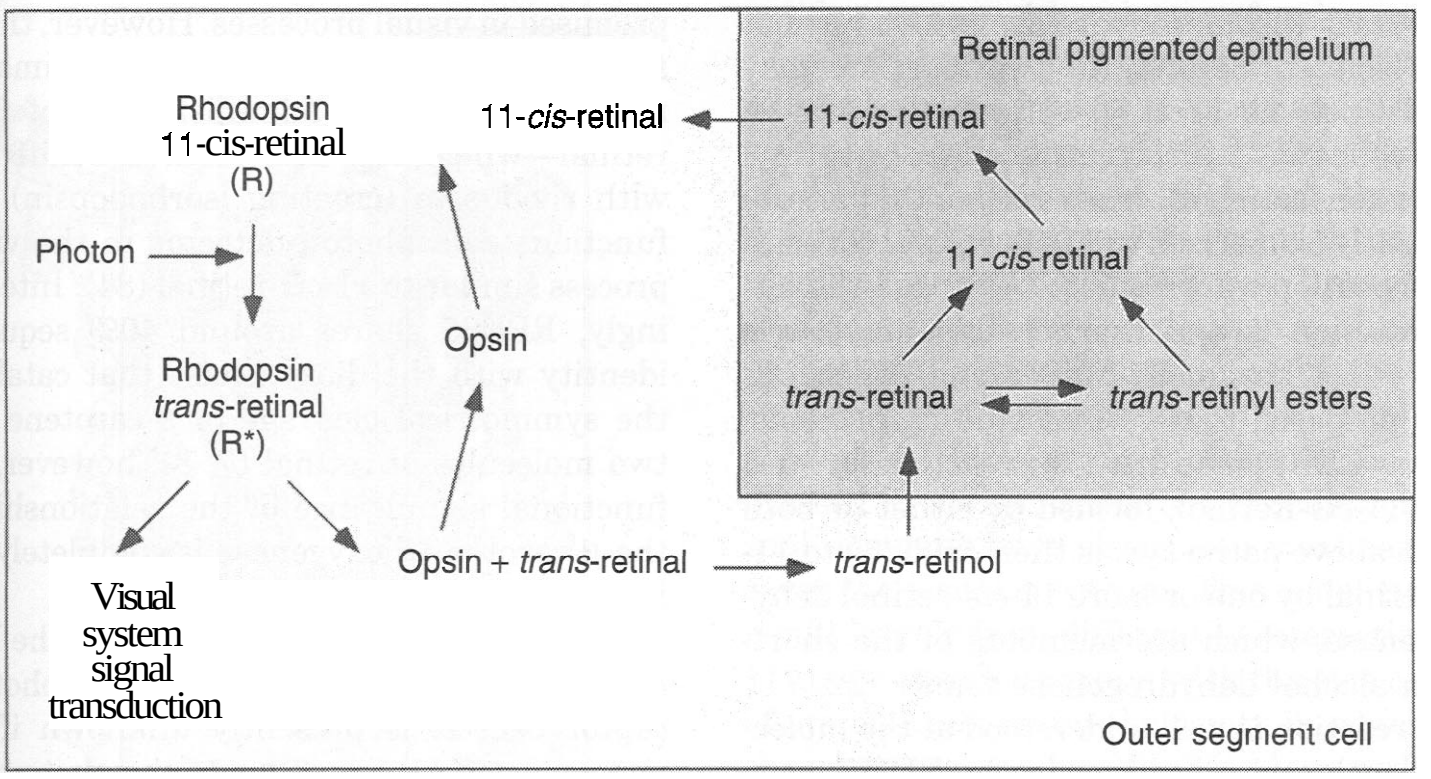


Figure 7.14. Retinoid cycle in vision.

ina should be simply viewed as a target tissue at this level. However, the retinal production of 11-cis-retinal from retinol and retinol esters, which is reasonably specific to the eye, is described below.

As described above, photon detection at the level of rhodopsin occurs in the rod (black and white vision) or cone (color vision) outer segments. Thus, this is the locus of the first step of the so-called "retinoid cycle." After photoisomerization, all-trans-retinal remains bound to R* but eventually diffuses out of the retinal binding pocket of opsin in a process that is not completely understood (22). Passive diffusion is probably the most important determinant of trans-retinal dissociation from the protein, but the existence of an ATP-dependent transporter that may facilitate this process has been demonstrated in some systems (22). Once dissociated from opsin, *trans*-retinal is reversibly reduced to all-trans retinol by a member of the membrane-associated short-chain alcohol deydrogenase family that is known as all-trans-retinol dehydrogenase (RDH). The all-trans-retinal → all-*trans*-retinol conversion occurs in the outer segments of both rods and cones, is dependent on NADPH, and may be the rate limiting step in the regeneration of 11-cis-retinal (22). Several forms of RDH capable of reducing all-trans retinal to retinol exist in the retina and the enzymes responsible for this reaction in rods and cones seem to be distinct.

All-trans retinol then diffuses out of the outer segments (rod or cone) and into the retinal pigmented epithelium (RPE). The diffusion of all-trans-retinol out of the outer segments and into RPE cells is facilitated by interstitial retinoid binding protein (IRBP), which is localized in the extracellular matrix of the retina (22). IRBP binds all-trans-retinol as it diffuses out of the outer segment cells and is believed to facilitate transport of all-*trans*-retinol to the RPE cell. Additionally, RPE cells directly acquire retinol from serum as described earlier. In both cases, retinol in the RPE cell is rapidly esterified by LRAT in a lecithin-dependent mechanism (22). Retinyl esters provide both a mechanism of storage for the RPE cell and/or provide a substrate for the isomerization reaction (22).

The isomerization reaction to 11-*cis*-retinol likely occurs through one of two possible mechanisms. First, the all-trans-retinyl ester may be a substrate for an as yet identified "isomerohydrolase" that uses the free energy of retinyl ester hydrolysis to drive the isomerization reaction (22). In this case, the product of the reaction, 11-*cis*-retinol, would be generated in essentially one step by a single enzyme,

the putative isomerohydrolase, which has not been isolated. Second, the two reactions may be distinct, i.e., retinyl ester hydrolases in RPE cells may hydrolyze the all-trans-retinyl esters generating all-trans-retinol that is subsequently isomerized to 11-*cis*-retinol through a carbocation intermediate. Although the latter reaction is endothermically unfavorable (AG = +4 kcal/mol), McBee and colleagues have proposed that retinol binding proteins, such as CRBP, may drive the reaction forward (72). 11-*cis*-Retinol, formed by either or both of the above pathways, is then oxidized to 11-cis-retinal by one or more 11-*cis*-retinol dehydrogenase, which are members of the short-chain alcohol dehydrogenase family (22, 71). This reaction is well understood at the molecular level, and mutations in the RDH5 gene, which encodes an 11-*cis*-retinol dehydrogenase, is associated with fundus albipunctatus, a human disease in which there is delayed dark adaptation in both cones and rods because of the impaired regeneration of 11-*cis*-retinal (73–78). Cellular retinaldehyde bindingprotein (CRALBP)has been proposed to be important in both the isomerization (all-trans-retinol → 11-*cis*-retinol) and the oxidation (11-*cis*-retinol → 11-cis-retinal) reactions (22); however, the underlying molecular mechanisms have not been identified. Nonetheless, mutations in the CRALBP gene are associated with compromised retinal disease such as retinitis punctata albescens (79–82), suggesting that CRALBP may play a role in the retinoid cycle in the retina. Additionally, CRBP I is expressed in RPE and has been suggested to play a role in chaperoning retinol within the cell and, like CRALBP, possibly has a role in the isomerization and/or oxidation reactions (22). Mice devoid of CRBPI have been generated and exhibit a large reduction in retinyl ester accumulation in liver (83); however, it is unknown if these mice have defects in the retinal retinoid cycle.

Finally, an abundant protein in the RPE, RPE65, seems to play a role in the retinoid cycle, but the exact role remains a mystery. Mice lacking this protein accumulate all-trans-retinyl esters that concentrate within lipid droplets in RPE cells (84). These mice are completely devoid of 11-cis-retinal and other cis-retinoid metabolites and are severely compromised in visual processes. However, the defective vision phenotype of these animals is reversed by dietary administration of 9-cis-retinal—which can also form a Schiff base with rhodopsin (creating isorhodopsin) that functions as a photon detector in the visual process similar to 11-cis-retinal (84). Interestingly, RPE65 shares around 40% sequence identity with the dioxygenase that catalyzes the symmetrical cleavage of p-carotene into two molecules of retinal (7, 8); however, the functional significance of the relationship to the β-carotene dioxygenase is completely unknown.

11-cis-retinal then diffuses out of the RPE cell and back into the outer segment photoreceptor cell. It is presently unknown if the movement of 11-cis-retinal to the photoreceptor cell requires interaction with IRBP, but this is clearly a possibility. Once in the photoreceptor cell, 11-cis-retinal reacts with opsin through formation of a Schiff base as described above (Fig. 7.14).

5.5 Retinoids and the Basis of Color Vision

The absorption maxima of free 11-cis-retinal is in the near-ultraviolet range (360–380 nm). However, the absorption maxima of the 11-cis-retinylidene moiety in the context of a protonated Schiff base linkage with rod opsins is shifted to approximately 500 nm, squarely in the middle of the visual range. Two factors contribute to this dramatic red shift in the absorption maxima of 11-*cis*-retinylidene group relative to that of free 11-cis-retinal: (1)protonation of the Schiff base and (2) the existence of intramolecular negative charges contributed by the opsin component of rhodopsin (22). One of these negative charges is derived from Glu^{113}, a residue that is conserved among all known vertebrate visual pigments. Glu^{113} is located in the highly hydrophobic environment of opsin transmembrane domain 2 and provides the counterion for the positively charged Schiff base (22). The existence of this counterion in such a hydrophobic environment shifts the p*K*a of the protonated Schiff base by 7 log units, effectively preventing spontaneous hydrolysis of the 11-*cis*-retinylidene group.

The recent elucidation of the crystal structure of rhodopsin bound by 11-cis-retinal at

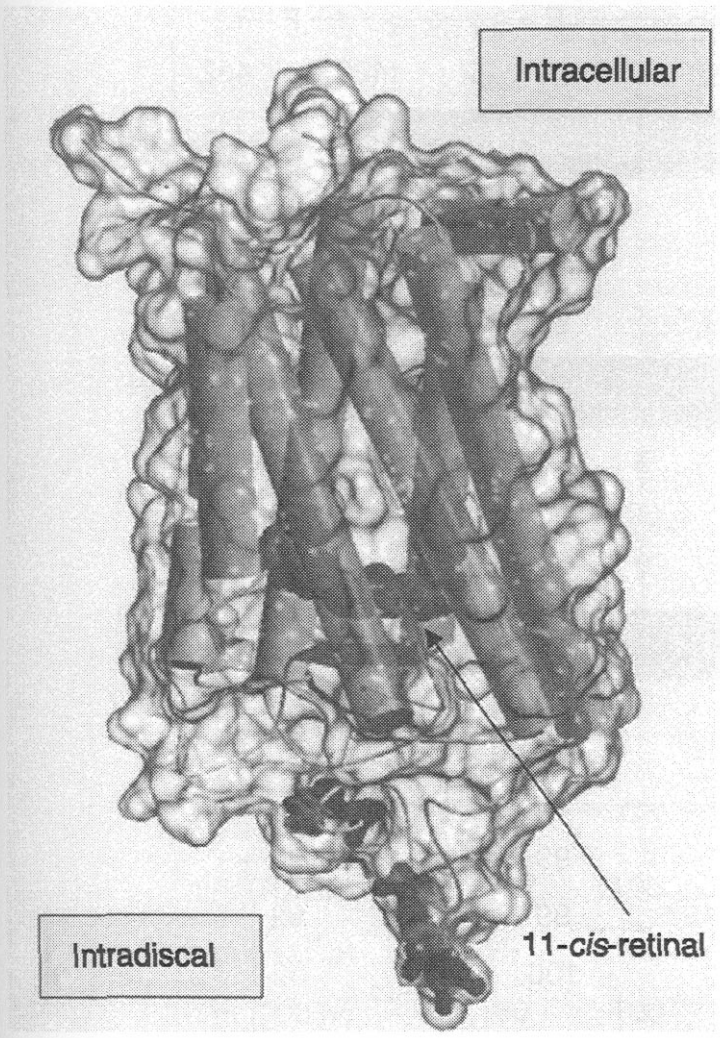


Figure 7.15. Rhodopsin crystal structure. Three-dimensional structure of rhodopsin based on X-ray crystallography (186). Note that all-trans-retinal is protected from the intradiscal side by multiple structural elements, including several β strands. Carbohydrates are in blue, 11-cis-retinal is in green, and helices are in gray. Red shadows are added for esthetic reasons. This figure was generated by C. Behnke (University of Washington) and is reprinted with permission from Prog. *Retin.* Eye Res., 20, 469–521 (2001). See color insert.

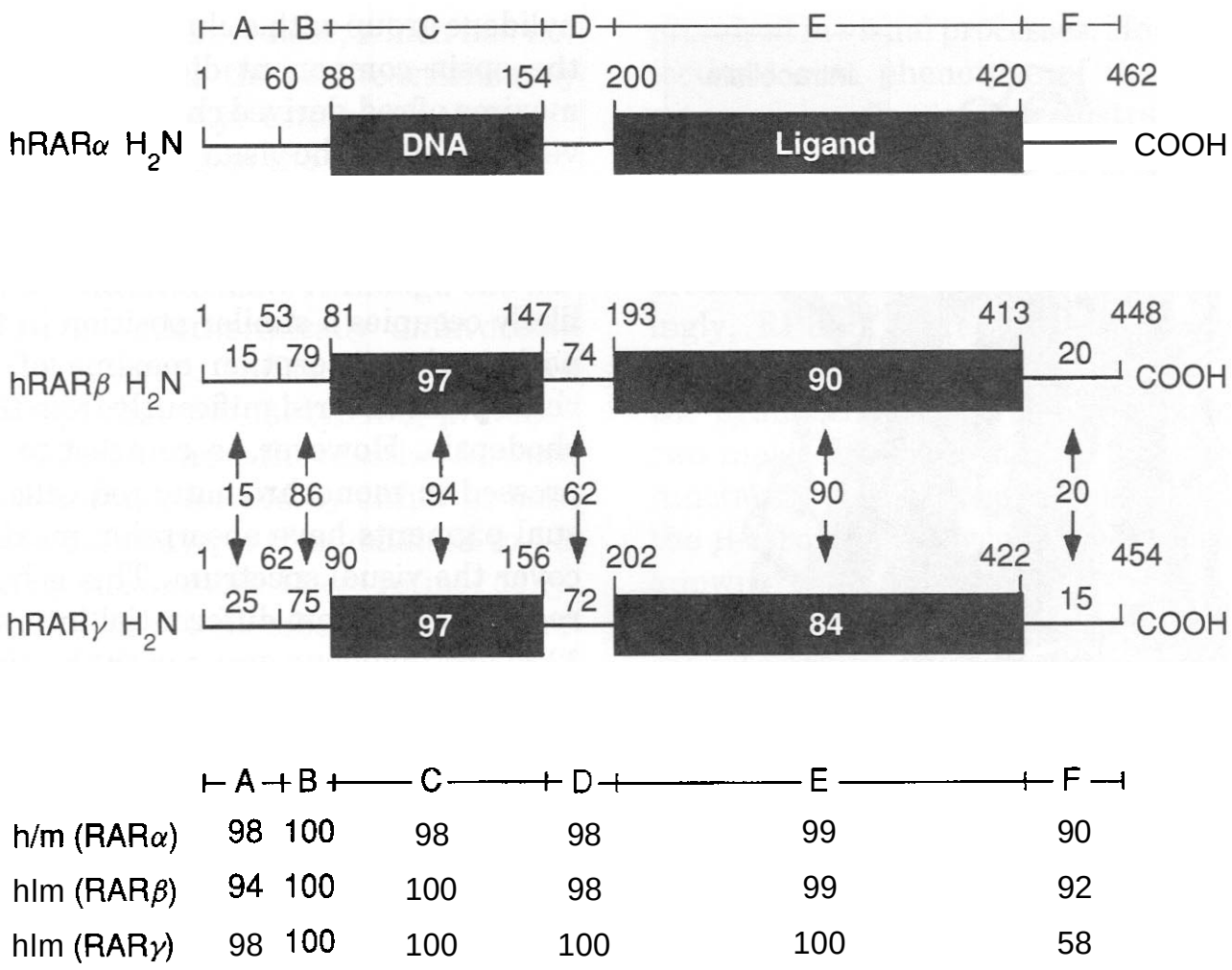
2.8Å allows direct visualization (Fig. 7.15) of the spatial arrangements of the transmembrane domains of rhodopsin and the location of 11-cis-retinal in the pocket (85). These investigators found that 11-cis-retinal contributes to the inactive conformation of rhodopsin by holding the transmembrane regions of the protein together. Although the structure of bleached rhodopsin, i.e., that containing 11-*trans*-retinal, was not reported, one can easily image the magnitude of the rhodopsin conformational change that must accompany light-induced isomerization of the chromophore, 11-cis-retinal.

The above-described structural studies confirmed that interactions of the 11-*cis*-retinylidene group with a cluster of amino acids of the opsin component dictate the absorption maxima of rod-derived rhodopsin and also provide a basis for the visual perception of color in cone cells of the retina. Although the chromophore is attached to cone opsins through an identical, protonated Schiff base linkage and likely occupies a similar position in the opsin pocket, the absorption maxima of the cone rhodopsins differ significantly from that of rod rhodopsin. However, in contrast to those expressed in monochromatic rod cells, cone visual pigments have absorption maximas that cover the visual spectrum. This is believed to result largely from differential interactions of 11-*cis*-retinylidene group with the side chains of amino acids lining the cavity in which the chromophore resides (22, 85). As a result of these differential interactions, visual pigments present in cones are activated by light of varying wavelengths giving rise to the perception of color.

6 RETINOIC ACID SIGNALING PATHWAYS

6.1 Historical Perspective

Naturally occurring retinoic acids and synthetic derivatives exert cellular effects by binding to and activating two families of nuclear receptors (NRs), retinoic acid receptors (RARs; Fig. 7.16) and retinoid X receptors (RXRs; Fig. 7.17). Both classes of retinoid receptors are comprised of three subtypes, a, p, and y, all of which belong to the steroid and thyroid hormone receptor superfamily of ligand-dependent transcription factors (1). RARα was the first member of either retinoid receptor family to be identified simultaneously and independently by the groups of Chambon (86) and Evans (87), both of which isolated RARα using a low-stringency, cDNA library screening strategy. Subsequently, the Chambon (88) and Pfahl (89) groups isolated RARβ and made the realization that the locus of this gene was a site of viral integration causing hepatocellular carcinoma. The Chambon group then cloned cDNA encoding RARγ and demonstrated that this receptor was highly expressed in skin (90, 91), long known as a primary retinoic acid target tissue. The Cham-



	A	B	C	D	E	F
h/m (RARα)	98	100	98	98	99	90
hlm (RARβ)	94	100	100	98	99	92
hlm (RARγ)	98	100	100	100	100	58

Figure 7.16. Retinoic acid receptor subtypes.

bon group also discovered the existence of multiple isoforms of RARα (92), RARβ (93), and RARy (94), all of which were generated by alternative splicing and differential promoter usage (Fig. 7.18). RXR isoforms, particularly of RXRβ (see below), have also been described (95). Finally, the Chambon group demonstrated that the retinoic acid receptors and all of the retinoid binding proteins exhibit spatially and temporally distinct expression patterns in the developing fetus and adult animals (96–99), suggesting that each may play a specific role in the retinoic acid signaling pathways. This flurry of activity in the time of 3–4 years, driven entirely by molecular biology expertise of a few groups, completely revitalized vitamin A research throughout the world. For the first time, nutritional and metabolic biochemists, toxicologists, and cancer, cell, and molecular biologists could begin to address the molecular basis of vitamin A action in health and disease.

6.2 Discovery of RXRs

As all of the above action was occurring, the group of Evans continued to isolate cDNAs encoding novel retinoic acid receptor-like proteins and in, the process, identified RXRα (100). This receptor was so-named because trans-RA activated the receptor in transient transfection experiments, however, only at concentrations much higher than those required for activation of the RARs. This finding led Mangelsdorf and colleagues to propose that the ligand for RXRα was a metabolite, the "X" metabolite, of trans-RA (100). This speculation was later proven by both the groups of Levin (32) and Evans (35), both of which identified 9-cis-RA as the RA "metabolite" responsible for activation of RXR in cultured cells (see above). However, the parent compound-metabolite relationship has never been directly demonstrated for trans- and 9-*cis*-RA and it is possible, as described above, that the two isomers of RA are interconverted in a nonenzymatic reaction as suggested by the group of Rando (36, 37). Nonetheless, both the groups of Levin and Evans made a fundamentally important discovery in identifying a ligand, 9-cis-RA, that bound directly to and activated both families of retinoid receptors, whereas trans-RA only bound and activated

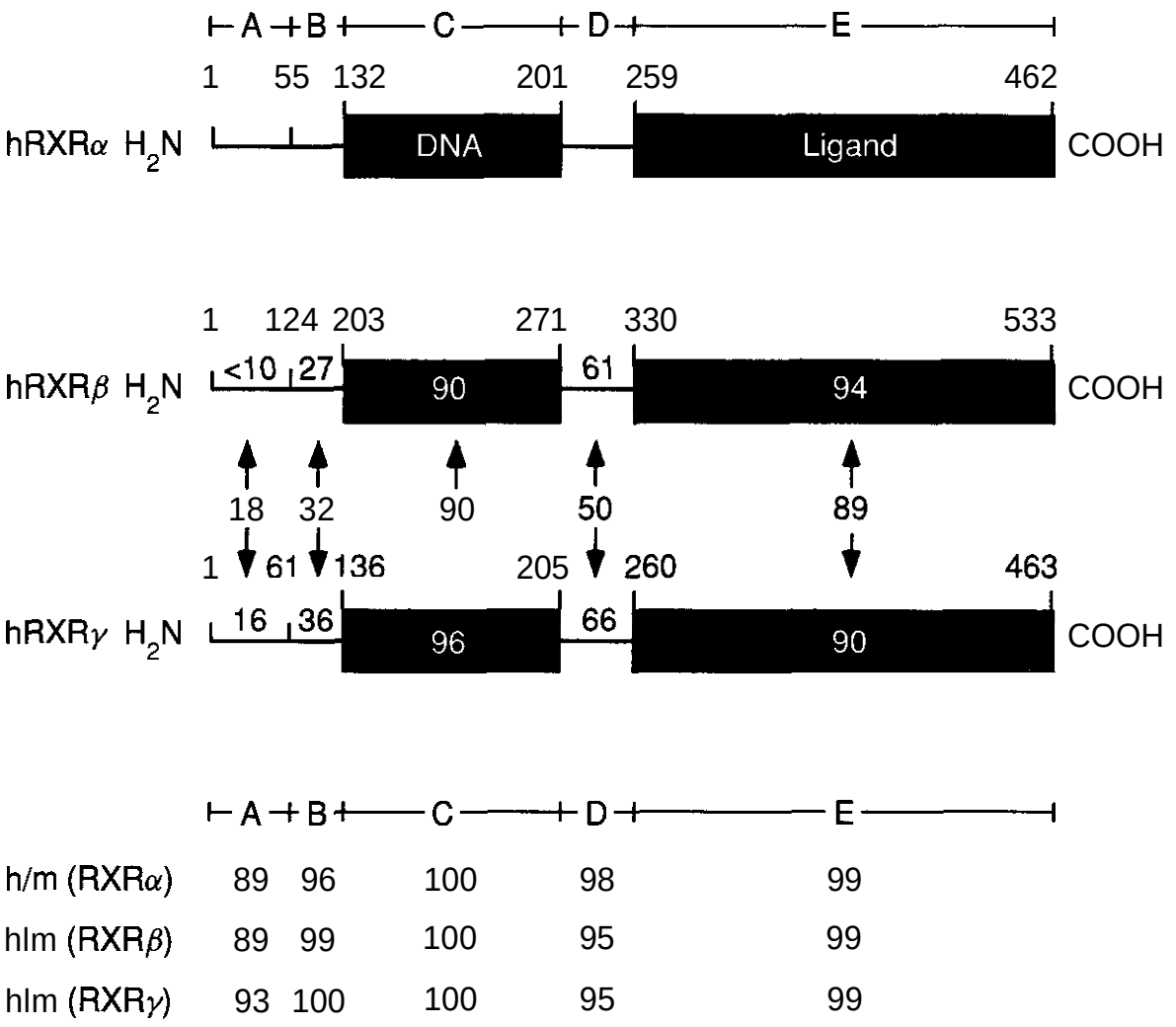


Figure 7.17. Retinoid X receptor subtypes.

RAR family members. This finding was the basis for the future development of receptor-selective retinoids.

6.3 Identification of Retinoic Acid Target Genes

Clearly, RARs and RXRs bound ligand, and this was demonstrated directly as described above, but these proteins also belong to the NR superfamily of sequence-specific DNA binding transcription factors. Thus, the next major question that required an answer was "what is the nature of the DNA response element to which the RARs and RXRs bind?" This was an extremely important question because the answer would provide clues regarding the nature of *RA* target genes. At the time, some response elements for steroid and thyroid hormones were known and these were generally corresponded to inverted repeats of a hexanucleotide sequence, for example AGGTCAxxxTGACCT for estrogen receptors or AGAACAxxxTGTTCT for glucocorticoid receptors (where x corresponds to any nucleotide). In both cases, the hexanucleotide sequence for each half-site is repeated on the opposite strand, also in the 5' → 3' orientation. Importantly in the search for retinoic acid-responsive genes, de The and colleagues had previously identified RARβ as a ***trans-RA***-induced gene in hepatoma cells (101) and subsequently identified the DNA response element conferring this inducibility as a direct repeat (DR) of the sequence GTTCACxxxxxGTTCAC (102). This crucial finding was unusual because, although the ***sequence*** of this retinoic acid response element (RARE) hexanucleotide was similar to that of estrogen and glucocorticoid receptors, the ***relative orientation*** of the two half-sites was not. In contrast to inverted or palindromic repeats of steroid hormone response elements, the head to tail orientation of the direct repeats generates an asymmetrical binding site that is typical of many non-steroid hormone receptors. Using a different approach, the group of Evans came to a similar conclusion, defining what was referred to as the "3-4-5 rule" of response element recognition in which directly repeated response elements spaced by 3,

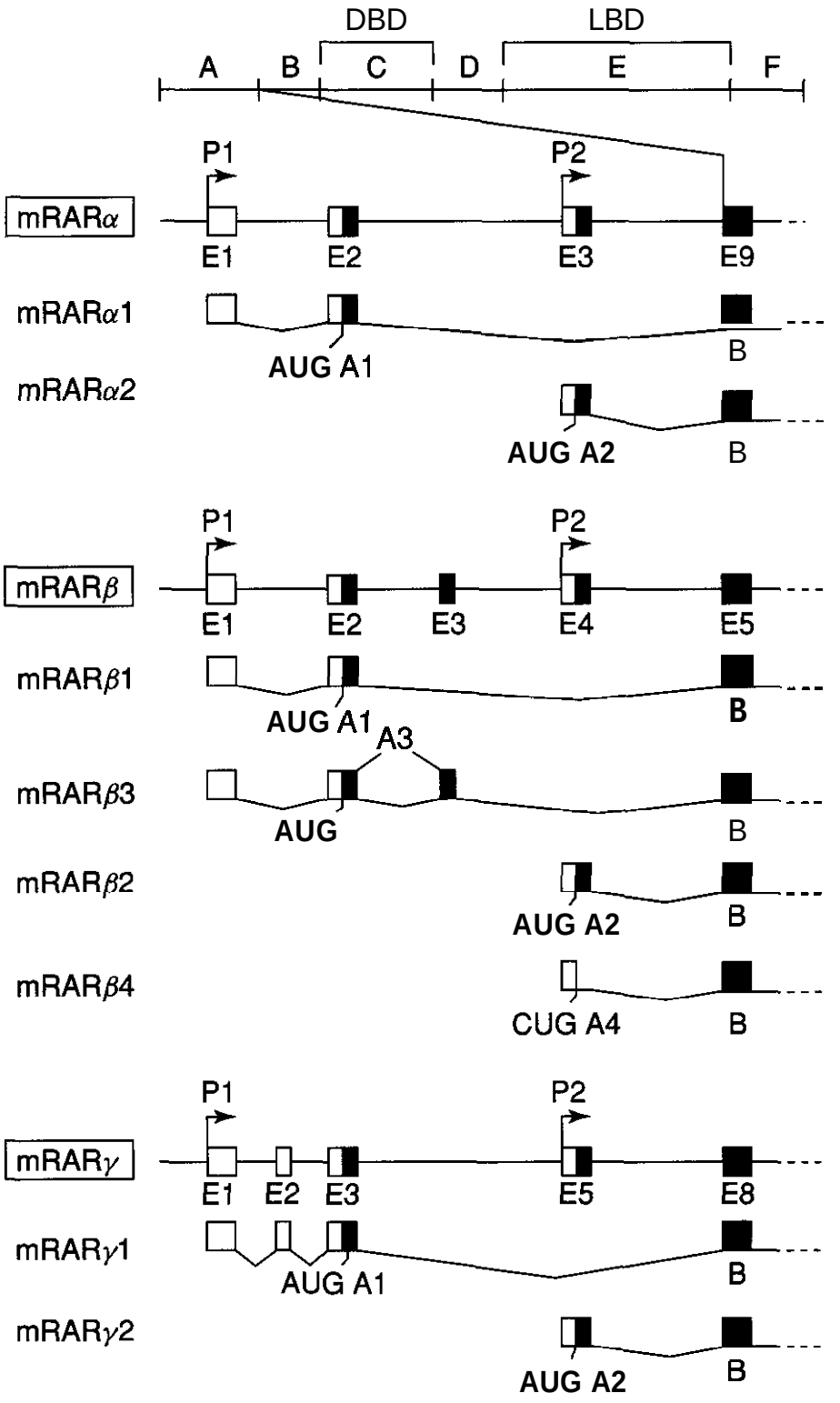


Figure 7.18. Schematic organization of 5′ region of mouse RAR genes and major isoforms. There are two major isoforms of RARα and RARγ, which in both cases, arise from differential usage of two promoters, P1 and P2. RARβ1 and RARβ3 are transcribed from the P1 promoter, but differ as a result of alternative splicing, whereas RARβ2 and RARβ4, which are both transcribed from the downstream *P2* promoter, differ in that the β4 isoform uses a CUG initiation codon and is alternatively spliced such that is it is virtually devoid of an *A* region. Exons are indicated by boxes and numbers *(E1–E5,* E8, and E9). Black and white boxes represent translated A region sequences and the 5′-UTR, respectively. For a given RAR type (a, β, or γ), A1–A4 and *B* represent the isoform-specific A region and common B regions, respectively. Reprinted with permission from ***Trends Biochem. Sci., 17, 427–433 (1992).***

4, and 5 bp functioned specifically as vitamin D, thyroid hormone, and RA response elements, respectively (103). Chambon's group then extended this rule by demonstrating that the RA inducibility of the CRBPII gene was conferred by a directly-repeated RARE spaced by 2 bp (104), while Mangelsdorf and colleagues demonstrated that the complex, directly repeated response element spaced by 1 bp (DR1) of the rat CRBPII promoter conferred RXR-mediated inducibility, thus identifying the first RXR response element or RXRE (105). Durand and colleagues then demonstrated that the mouse CRABP II promoter harbored both DR1 (RXRE) and DR2 (RARE) elements, indicating that both families of retinoid receptors may be involved in the up-regulation of this gene by retinoic acids (106). Although numerous groups have shown that DR1 elements are promiscuous and confer regulation by several other nuclear receptors, the molecular basis for response element recognition and, therefore, target gene regulation, by RARs and RXRs was well-defined by the above-described body of work. Thus, the 3-4-5 rule was modified to account for DR1 (RXR and many other orphan receptors), and DR2 (RA) response elements and became commonly known as the 1-5 rule (Fig. 7.19). Today, many more retinoid-responsive genes

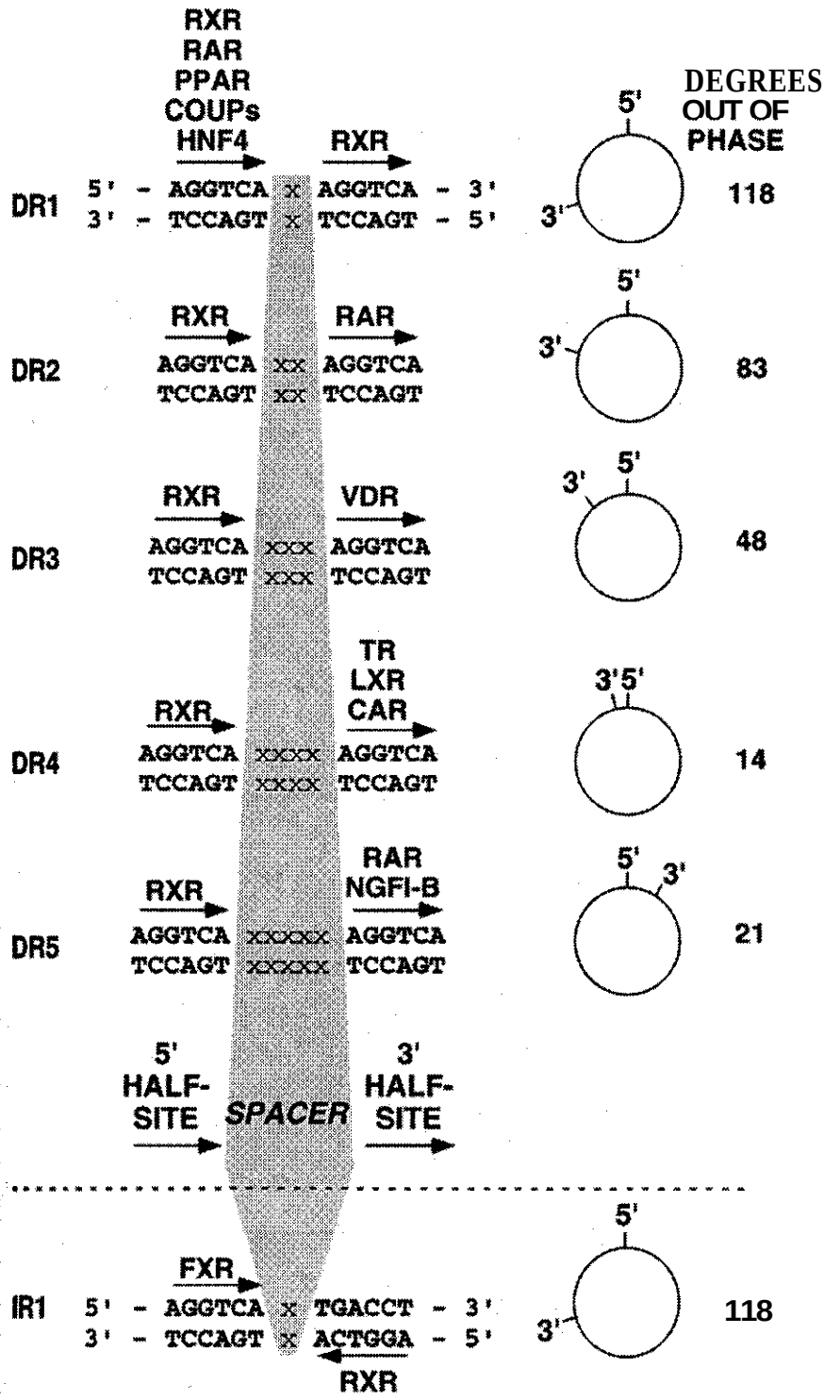


Figure 7.19. Directly repeated response elements for members of the retinoid receptor family (RXR and RAR). The sequence of each half-site is similar, but the spacing between the two half-sites is distinctive. Increasing the length of the intra-repeat spacer alters the relative orientation of the two half-sites as indicated on the right side of the figure. For example, the centers of each half-site are on different sides of the DNA helix in the context of a DR1 response element (118" out of phase), but only 14° and 21" out of phase in the context of DR4 and DR5 response elements, respectively.

have been isolated, and retinoid signals are known to induce the expression of a wide array of target genes implicated in mediating the pleiotropic effects of RAs on cellular function. These genes encode transcription factors (107–113), metabolic enzymes (114), growth factor receptors (115), extracellular matrix proteins (116, 117), secreted proteins that are postulated to convey positional information in the developing embryo (118–120), and genes encoding pro-apoptotic proteins such as TRAIL (121).

6.4 RARs and RXRs Bind Response Elements as a Heterodimeric Complex

Several groups working independently simultaneously discovered that RAR family members heterodimerize with RXR family members and bind all known RAREs as a RAR·RXR heterodimeric complex (122–126). During the course of this work, two additional members of the RXR family, RXRβ, which was originally identified as H-2RBPII by the group of Ozato (127), and RXRγ, were identified (123, 128), both of which were also shown to heterodimerize with RAR family members (123). Finally, the Chambon group used genetic means to demonstrate that RAR·RXR complexes are the functional units of both retinoid signaling pathways during mammalian development (129). Considered together, these remarkable findings indicate that the two families of retinoid receptors, RARs and RXRs, which arose independently during evolution and harbor

Table 7.3 Comparison of Amino Acid Sequence Conservation among RAR and RXR Ligand Binding Domains

	RARα	RARβ	RARβ	ERα
RXRα	28	32	30	30
RXRβ	29	30	30	31
	30	33	30	29
ERα	23	24	24	—

Percent identities between the ligand binding domains of RARs, RXRs, and estrogen receptor (ERα).

little sequence identity outside the DNA binding domain (see below and Table 7.3), physically interact in the context of a heterodimeric complex to regulate the transcription of most retinoic acid target genes. RXR was subsequently shown to heterodimerize with several other nuclear receptors (Table 7.4), a finding that implies activators of RXR may potentially affect a great number of other signaling pathways (130,131). Thus, the development of selective, RXR agonists is particularly attractive to medicinal chemists (see FUTURE PERSPECTIVES).

RAR·RXR heterodimeric complexes bind to a directly repeated, hexanucleotide motif of the consensus sequence 5'-PuGGTCA-3' (where Pu represents a purine, see above). Directly repeated response elements, in contrast to inverted repeats, are not symmetrical, and this renders the microenvironment of each half-site distinct (38). This situation implies that the orientation of RAR·RXR complexes RAREs may be ordered and occur in a nonrandom manner. Indeed, this is the case: Perlmann and colleagues used biochemical techniques to demonstrate that RXR and RAR bind to the 5' and 3' half-sites, respectively, of a DR5 response element (132). The group of Chambon published similar findings and also demonstrated that RXR occupies the 5' half-site of a DR2 element (133), and this general polarity was confirmed in crystallography studies in which RXR·thyroid hormone receptor complexes were shown to bind with a similar orientation to a DR4 thyroid response element (134). Interestingly, however, Kurokawa and co-workers (135) discovered that this binding order was reversed on a DR1 element with RAR and RXR binding to the 5' and 3' half-sites, respectively, resulting in a transcriptionally inactive complex that responds to neither ***trans*-RA** nor ***9-cis*-RA** (see below).

7 FUNCTIONAL DOMAINS OF RARs AND RXRs

The RARs and RXRs, like other NRs, are comprised of a highly modular domain structure that includes autonomous DNA and ligand binding domains and as well as regions of the proteins that function in both transcriptional activation and silencing (Fig. 7.20). These functional domains are described in detail in the sections that follow.

7.1 RAR and RXR DNA Binding Domains

The DNA binding domains (DBDs) of members of the NR family are, in general, highly conserved, displaying approximately 60% identity at the amino acid level across the en-

Table 7.4 RXR Heterodimerization Partners

Receptors with Known Ligands	Orphan Receptors
Retinoic acid receptors (RARα, β, γ)	Chicken ovalbumin upstream promoter-transcription factors (COUP-TFI, II, III)
Vitamin D receptor (VDR)	Hepatic nuclear factor-4 (HNF-4)
Thyroid hormone receptors (TRα, β)	NGFI-B and related proteins
Perooxisome proliferator-activated receptors (PPARα, β, γ)	
Liver X receptors (LXRα, β)	
Farnesoid X receptor (FXR)	
Constitutive androstane receptor (CAR)	
Benzoate X receptor (BXR)	
Steroid xenobiotic/pregnane X receptor (SXR, PXR)	

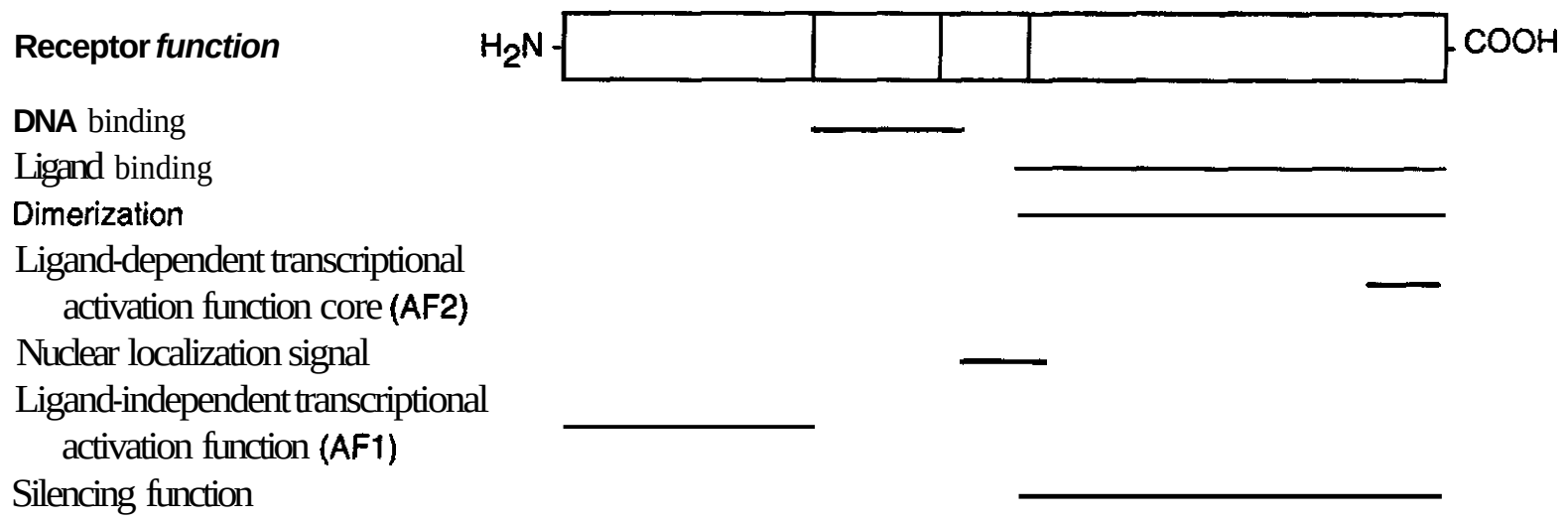


Figure 7.20. Nuclear receptor domain structure.

tire family. The DBD of both RARs and RXRs, and of all NRs, is composed of approximately 66–80 amino acids that are organized into two C_2C_2 zinc finger motifs (see Figs. 7.16 and 7.17), each of which tetrahedrally coordinates one zinc ion (134, 136). These two zinc fingers fold into a single structural motif comprised of two a helices, the recognition and support helices that are located on the knuckle on the carboxyl side of each zinc finger (134, 136). The two a-helices play very different roles in the function of the receptors: the recognition helii sits in the major groove of DNA and makes specific contacts with discriminating nucleotides within the response element; the support helix is oriented perpendicularly to the recognition helix and does not contact DNA at all (134,136). Rather, the support helii makes specific protein-protein contacts with and effectively buttresses the recognition helix into the major groove. This concept is illustrated schematically in Fig. 7.21. Biochemical (137,138) and crystallographic (134, 136) studies have revealed that three amino acids within the recognition helix are crucial for response element discrimination by most NRs.

7.2 Ligand Binding Domain

Although the ligand binding domains (LBDs) of both RARs and RXRs bind 9-*cis*-RA with high affinity, the amino acid sequence identity between the two proteins within the LBDs is surprisingly very low (Table 7.3). Indeed, the RAR LBD is as related in amino acid sequence identity to that of RXR as the receptor is to estrogen receptor a (approximately 30%; see Table 7.3). The LBDs of RAR and RXR family members have also been studied extensively by biochemical means and by crystallography (139–148). The picture that has emerged from this large body of work suggests that the overall structural organization of the LBD of RARs and RXRs is highly conserved LBD (142, 149), even if the primary amino acid sequence is not (see above). The structurally unique NR LBD is comprised of 12 a-helical bundles (H1-H12; Fig. 7.22) that sandwich a short β-turn (149). This domain is arranged into three layers forming what has been called an anti-parallel "a-helical sandwich" (142). In addition to conferring ligand binding, the ~220– to 240–amino acid LBDs of all RAR and RXR family members also harbor a major dimerization interface (homodimerization and heterodimerization) that is comprised of amino acids from H7, H9-H11, and portions of the intervening loops (140,141). The RAR and RXR LBDs also confer all of the ligand-dependent transcriptional activation potential of the corresponding receptors, which has been referred to as the AF2 activity (150). However, AF2 cannot be described simply in linear sequence, but rather is formed by the agonist-induced juxtaposition of several helices within the LBD (see below).

7.3 Apo-Receptors Interact with Transcriptional Corepressor Complexes

Essentially, the basis of retinoid receptor signaling involves differential interaction of the apo- and holo- forms of the receptors with several, specific pools of nuclear proteins. For example, in the absence of an agonist, apo-

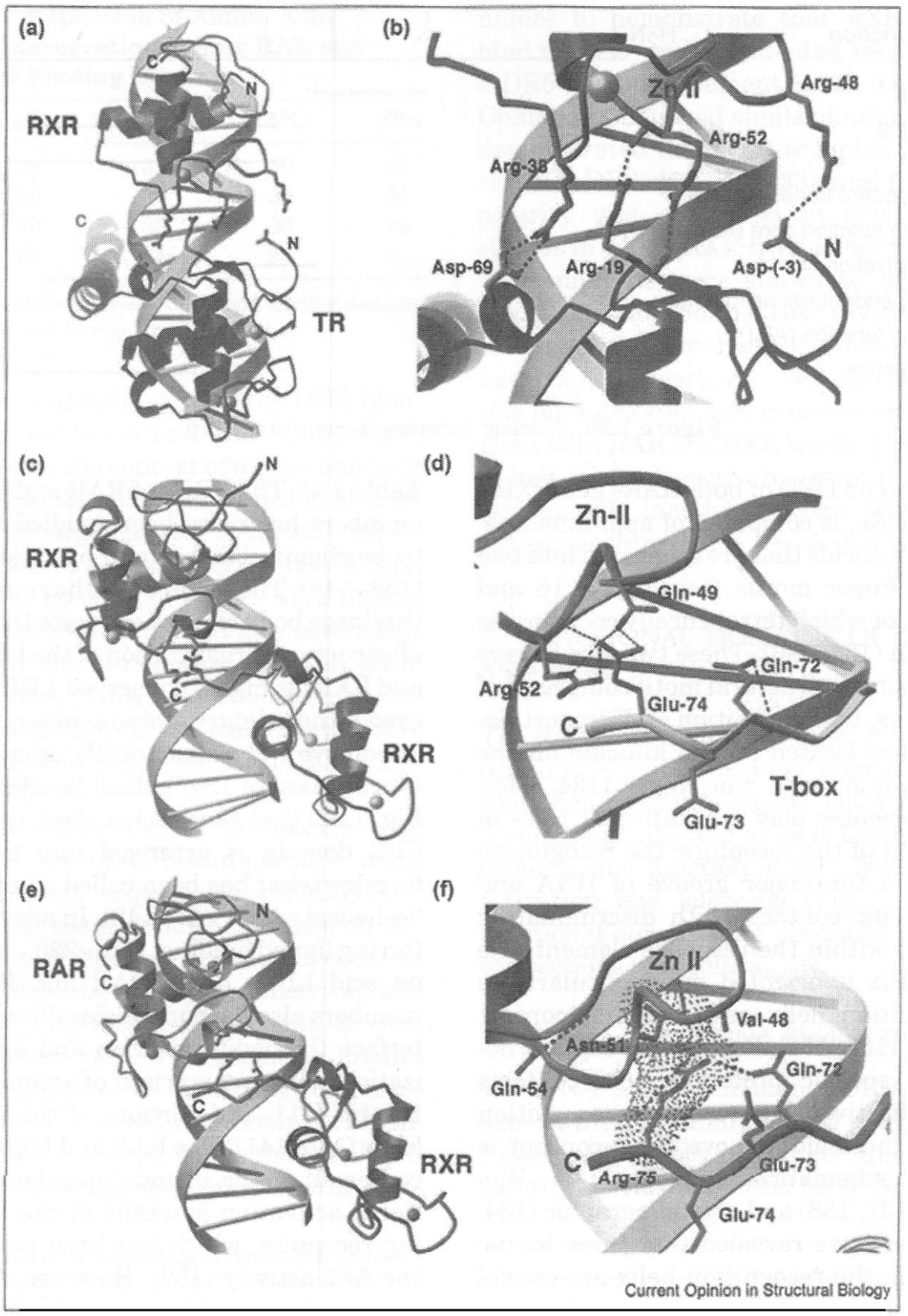


Figure 7.21. Schematic representation of DNA binding domains. Structures of DNA-binding complexes involving RXR and their dimer interfaces. The overall structures are shown on the left, with close-up views of the protein–protein interactions shown on the right. Dotted blue lines indicate hydrogen bonds between proteins or between proteins and the DNA spacing. The dotted surface indicates complementary van der Waals interactions. DNA sequences (cyan) are shown with their 5' ends pointing up, with base pairs belonging to the spacing element of the DRs shown schematically in red. In each case, protein–protein contacts are formed directly over the minor groove of the spacing, with several protein–DNA phosphate contacts stabilizing the assembly. The interacting amino acid side-chains are shown in green, with nitrogen atoms indicated in blue and oxygen atoms indicated in red. (a and b) The RXR–TR DBD heterodimeric complex with DR4; (c and d) the RXR DBD homodimeric complex with DR1, and (e and f) the RXR–RAR DBD heterodimeric complex with DR1. Reprinted with permission from ***Curr.*** Opin. ***Struct.*** Biol., ***11,3338*** (2001). See color insert.

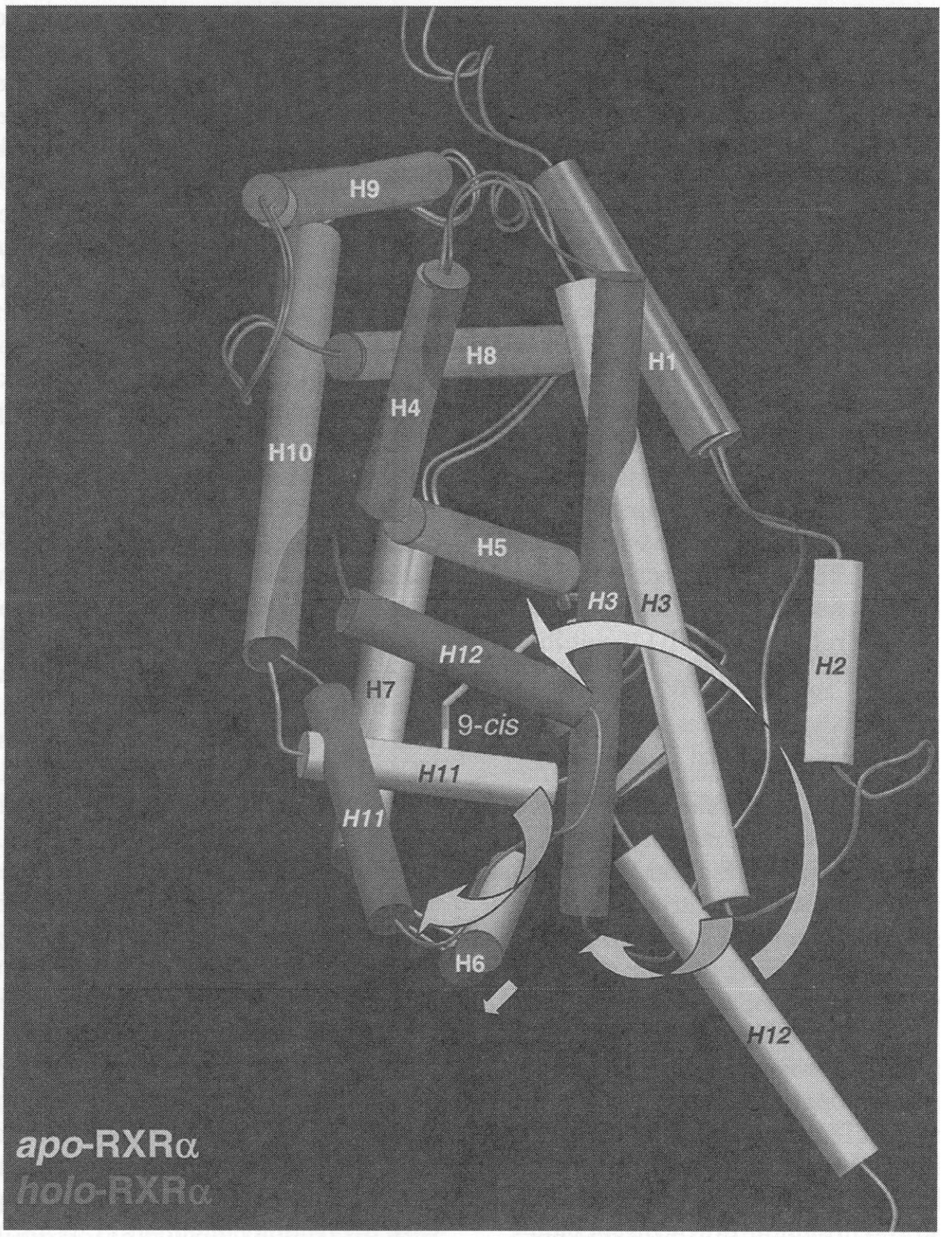


Figure 7.22. Schematic representation of apo- and holo-RXR ligand binding domain. Helices 1–12 (H1–H12) are indicated. Helices indicated in yellow and red represent the apo- and holo-forms of the receptor, respectively, whereas helices indicated in blue and green are positioned similarly in both forms of the receptor. The arrows represent movement of the helices to accommodate **9-cis-RA** in the binding pocket (indicated). This figure was kindly supplied by Dr. Pascal Egea. See color insert.

RAR·RXR complexes are known to bind to DNA response elements loosely and recruit an ATP-dependent, chromatin remodeling complex known as ISWI to the template (151). ISWI-mediated remodeling of chromatin seems to allow apo-RAR·RXR complexes to bind to the DNA response element much more tightly (151). Presumably, apo-RAR·RXR complexes can then interact with and recruit transcriptional corepressor proteins, such as nuclear receptor corepressor (NCoR) and silencing mediator of retinoid and thyroid receptors (SMRT) to the template (152, 153). NCoR and SMRT, in turn, recruit histone deacetylase complexes (HDACs) to the apo-RAR·RXR heterodimeric complexes bound to

a response element in the promoter region of a RA target gene (154). HDAC-mediated deacetylation of specific lysine residues found in multiple histones is believed to increase the affinity of the particular histone for the DNA template and, thus, favor the formation of more densely packed chromatin that is generally less likely to be transcibed (155). Ten HDACs encoded by different genes have been isolated to date, and genetic evidence suggests that there may be additional members of the HDAC family that have not been described (156–162). Thus, apo-RAR·RXR complexes can repress the expression of at least some target genes in a HDAC-dependent manner, and this inhibition can be relieved by inhibitors of HDACs such as trichostatin A or valproic acid (163). This has clinical significance because some leukemias respond more favorably to a combination of a retinoid and HDAC inhibitor than to either drug alone (163, 164).

7.4 Holo-RAR·RXR Complexes Interact with Two Classes of Transcriptional Coactivators

Agonist binding to RAR and/or RXR induces a rather substantial conformational change in the LBD of the receptors (Fig. 7.22). This conformational change is characterized by the following: (1) a large rearrangement involving H11 and H12; (2) bending of helix 3; and (3) rotation of the omega loop separating helices 1 and 2/3. The resultant conformation of the holo-receytor is not favorable for interaction with transcriptional corepressors such as NCoR or SMRT; thus, agonist binding-induced conformational change promotes dissociation of the entire corepressor complex assembled on the promoter of RA target genes.

The repositioned helix 12 is generally believed to form the "lid" of the ligand-binding pocket (143, 144, 165). The agonist binding-induced rearrangement of helix 12 also juxtaposes the core of AF2, which is found in helix 12, with amino acids in helices 3, 4, and 5 (143, 144), creating a shallow hydrophobic groove competent for interaction with a second class of proteins known as transcriptional coactivators. Interaction of a RAR·RXR complex with a transcriptional coactivator requires the agonist-induced juxtaposition of helices 3, 4, 5, and 12 of the receptor with a complementary α-helical structure in the coactivator protein comprised of the consensus sequence LXXLL, where L corresponds to a leucine residue and X can be any non-helix breaking amino acid. The canonical LXXLL-containing a-helix of transcriptional coactivators binds directly to the shallow hydrophobic groove in the receptor formed by the agonist induced conformational change (166–170). Thus, agonist binding-induced RAR and/or RXR conformational change serves two purposes: (1) to induce dissociation of the corepressor complex resulting in a relief of transcriptional repression and (2) to promote interaction of the receptor with transcriptional coactivator proteins resulting in transcriptional activation.

Transcriptional coactivator proteins that interact preferentially with activated nuclear receptors can be divided into two general families, each of which contain multiple subfamilies. Transcriptional coactivators harboring histone acetyl transferase (HAT) activity include p300/CBP (171–174), P/CAF (175–177), and the p160 family of proteins: SRC-1/NCoA-1 (167, 173, 178), TIF2/GRIP1/NCoA-2/SRC-2 (167, 179, 180), and RAC3/AIB1/PCIP/ACTRISRC-3 (167, 181–183). By covalent modification (acetylation) and subsequent decondensation of chromatin, transcriptional coactivators possessing HAT activity are believed to enhance the access of the RNA polymerase II (Pol II) complex to the template (184). In addition to interacting with activated nuclear receptors, p160 and p300/CBP proteins interact with each other and the latter interact with P/CAF (175–177). Recently, a family of histone arginine methyltransferases has been identified that interact specifically with p160 family members (185, 1861, suggesting that the multi-protein, chromatin-modifying machinery assembled on the promoter of target genes is large and complex.

Interaction of the HAT coactivator complex with the ligand-activated receptors is apparently transient as dissociation is believed to occur as a result of acetylation of one or more coactivators on lysine residues adjacent to the signature LXXLL motif (187). Subsequently, the activated receptor presumably recruits a second class of multi-protein, transcriptional coactivator complexes to the template, and this latter complex, referred to as the thyroid hormone receptor-associated protein (TRAP)

complex, seems to be similar to the SRB- and MED-containing cofactor (SMCC), vitamin D receptor-interacting protein (DRIP) and the yeast Mediator (188, 189) complexes. Because the TRAP or TRAP-like complexes harbor components of the Pol II machinery and/or recruit the Pol II complex to the template, formation of the transcriptional preinitiation complex and subsequent transcription are facilitated (190, 191). It should be noted that these later steps of RAR·RXR-mediated transcriptional activation appear to require the recruitment of another protein complex to the template, the SWI/SNF complex, which seems to be facilitated by the presence of activated receptors, either by direct interaction or interaction of the SWI/SNF complex with acetylated histones (151, 192). SWI/SNF, which also harbors an ATP-dependent chromatin remodeling activity, seems to function by displacing histones in the promoter region of the target gene, thus, facilitating tight binding of the basal transcription factors to the template—a prerequisite for formation of the preinitiation complex and transcriptoinal initiation (192).

In summary, transcriptional activation mediated by RAR·RXR complexes is a complex, multistep process that is not entirely understood at the molecular level. However, it is clear that agonist-induced, RAR·RXR conformational change, which is crucial for dissociation of the corepressor complex and induction of a receptor conformation that facilitates interaction of the receptor with two distinct classes of coactivator proteins, the HAT and TRAP proteins, plays a central role in the signaling pathways of RAR·RXR complexes leading to transcriptional activation.

7.5 "Ligand-Independent" Transcriptional Activation, AF1, of RARs and RXRs

The amino terminal of RARs and RXRs harbor what is known as a ligand-independent transcriptional activation function (Fig. 7.20). AF1 cooperates and synergizes with AF2, the ligand-dependent transcriptional activation function formed by the juxtaposition of helices within the LBD (see above) (106, 150, 193). However, AF1 of retinoid receptors was defined in artificial systems (by fusing this region to a heterologous DNA binding domains) and may not represent real life. Clearly, AF1s contribute to the global activation potential of retinoid receptor heterodimeric complexes, yet the transcriptional activation activity of these complexes is dependent on ligand, rendering the "ligand-independent" activity of AF1 entirely dependent on ligand in *vivo* (142), as has been described for other nuclear receptors (194).

8 FUTURE PERSPECTIVES

The present and future efforts of medicinal chemists in the development of novel retinoid compounds are discussed in this section. This body of work should ultimately lead to receptor and receptor subtype–selective agonists and antagonists and novel compounds that block proliferative signaling processes.

8.1 Receptor- and Receptor Subtype–Selective Retinoids

As discussed in a previous section, the systemic and topical toxicity, as well as the teratogenicity of retinoids, limits the clinical usefulness of these compounds. The general opinion of both basic scientists and clinicians is that these toxicities may be mitigated by the development of receptor subtype–selective retinoids. Although this may ultimately be proven to be true, there is currently little factual basis for this opinion, at least with respect to the RAR subtypes, because it has been quite difficult to separate clinical efficacy from toxicity. Nonetheless, this remains a noble goal in the continued development of receptor subtype–selective retinoids.

Of the seven retinoids currently approved by the FDA for clinical use, all are agonists, and most (adapalene, acitretin, isotretinoin, tazarotene, and tretinoin; see Table 7.1) are selective RAR agonists. Of these compounds, adapalene and tazarotenic acid (the active form of the pro-drug tazarotene) are selective agonists for RARβ and RARγ, (195–197), whereas the other compounds activate all RAR subtypes with roughly similar potencies. Alitretinoin (9-cis-retinoic acid) is a pan-agonist that activates all RAR and RXR subtypes and bexarotene is a selective, RXR agonist that harbors some RAR agonist activity (198–200).

Table 7.5 Discriminatory Amino Acids in the Ligand Binding Pockets of RAR Family Members

Receptor	LBD Helix 3	LBD Helix 5	LBD Helix 11
RARα	Ser^{232}	Ile^{270}	Val^{395}
RARβ	Ala^{225}	Ile^{263}	Val^{398}
RARγ	Ala^{234}	Met^{272}	Ala^{397}

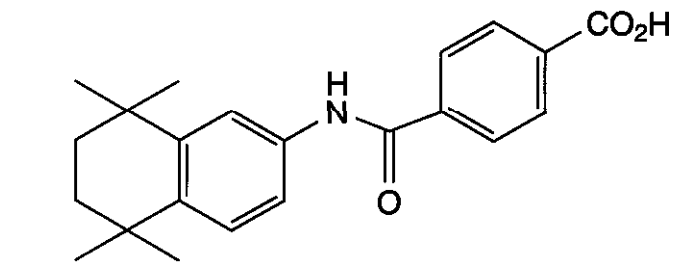
These amino acids, which are positioned within the ligand binding pocket of the indicated RARs, likely confer ligand specificity.

The LBDs of RARα, β, and γ are highly related (Fig. 7.16). Moreover, there are only three divergent amino acid residues in the ligand binding pockets of the RAR subtypes (Table 7.5) (201), rendering the development of RAR subtype-selective ligands a challenging endeavor. Nonetheless, substantial progress has been made by several groups in the design of RAR subtype-selective agonists (Fig. 7.23). Until recently, these efforts were made largely by empiricism, and in some cases, based on molecular modeling algorithms. However, medicinal chemists may now use the knowledge of the three-dimensional structures of the RAR and RXR ligand binding pockets in the development of receptor subtype–selective retinoids, and this will forever transform rational, retinoid drug design.

Many of the known RAR subtype–selective ligands described below contact one or more of the three discriminatory amino acids that line the ligand binding pocket of the corresponding receptor. For example, Moras and colleagues found that Met^{272} of RARy adopts different ligand-induced conformations depending on the presence of a hydrogen bond between its sulfur atom and the corresponding ligand (146). Similar discriminatory mechanisms seem likely for other RARs rendering the future development of RAR subtype-selective agonists possible.

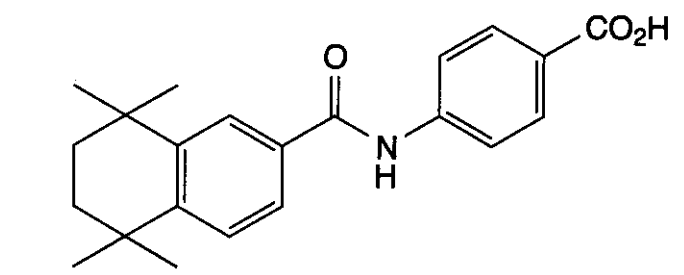
AM80 and AM580 (Fig. 7.23) are prototypic compounds that activate RARα at concentrations roughly 10- to 100-fold lower than those required for activation of either RARβ or RARy (202). In general, however, it has been more difficult to separate RARβ from RARy agonism even though these receptors differ in two of the three discriminatory amino acids that line their respective ligand binding pockets (Table 7.5). Two somewhat RARβ-selective compounds have been reported, CD417 and CD2019 (203), but the degree of RARβ selectivity reported for these compounds is not overwhelming. As RARy is highly expressed in skin and is thought to mediate many of the therapeutic effects of topical retinoids in the treatment of dermatological disorders, such as psoriasis and acne, several RARγ-selective agonists have been developed including SR11254 (204), CD437 (203,2051, BMS184394 (203, 206, 207), and CD666 (203). The structures of all of these receptor subtype–selective compounds are shown in Fig. 7.23.

Antagonists of RARs have been developed, and the prototype of these compounds is the RARα-selective Ro41-5253 (Fig. 7.24) (208). In addition, other novel RAR antagonists have been developed (209,210). The clinical usefulness of these compounds is presently unknown, but RAR antagonists have proven to be very useful as tools for the dissection of the retinoic acid signaling pathways in both cells (201) and in animals (211). One may envision that these receptors may work in one of two mutually exclusive mechanisms. Firstly, the antagonists may simply occupy the ligand binding pocket and thereby compete with endogenous agonists (trans- or 9-cis-RA, for example). In this case, the antagonist would effectively lock the receptor in the apo-form that is associated with transcriptional corepressor proteins such as NCoR and SMRT (see above). Alternatively, RAR antagonists may induce repositioning of receptor helix 12 away from the surface of the protein generating a novel conformation of the receptor. In this case, helix 12 would be unable to contribute to formation of the shallow hydrophobic groove that is required for interaction with the LXXLL motif of transcriptional coactivators (see above). This mode of action would be consistent with crystallographic studies that revealed that the binding of the retinoid antagonist BMS614 to

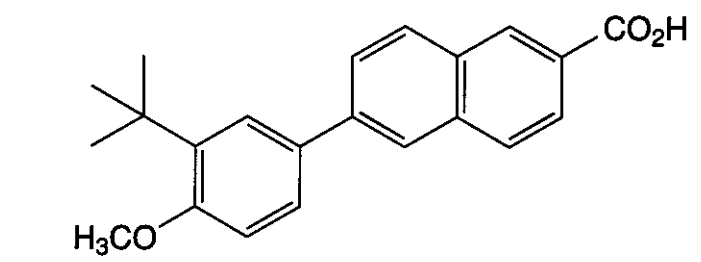


Compound: AM80

Receptor selectivity: RARα

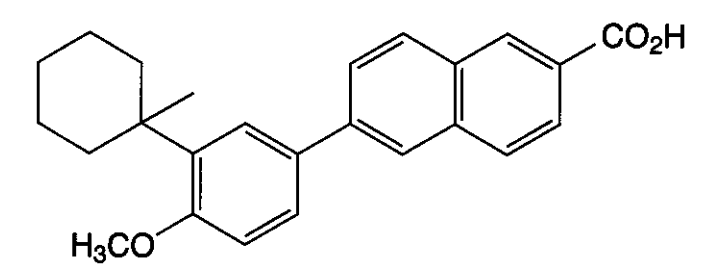


Compound: AM580

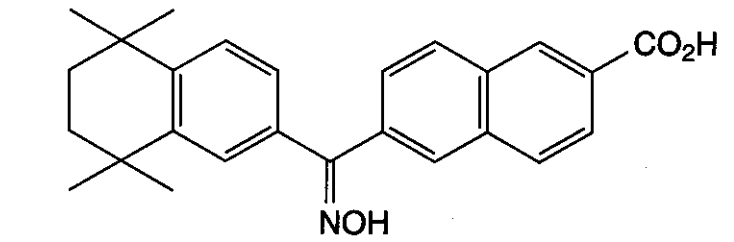


Compound: CD417

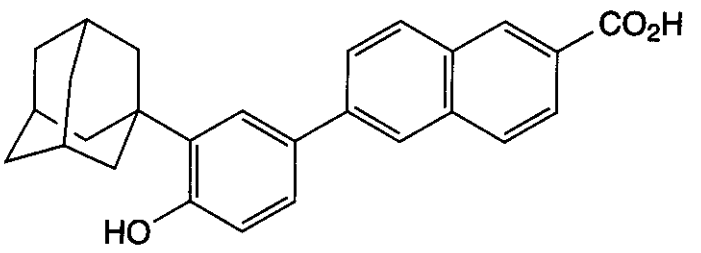
Receptor selectivity: RARβ



Compound: CD2019

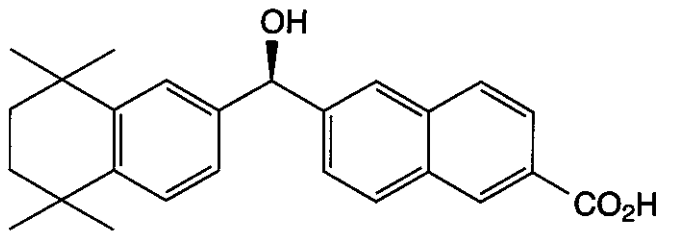


Compound: SR11254

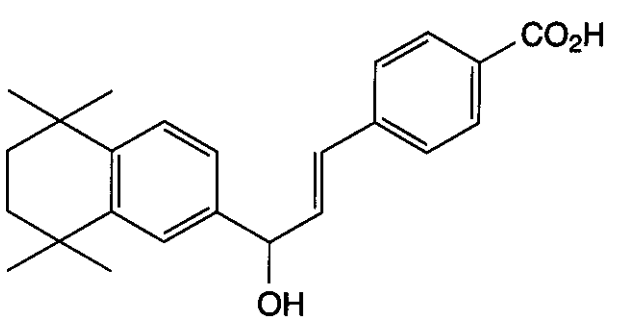


Compound: CD437

Receptor selectivity: RARγ



Compound: BMS184394-*R*



Compound: CD666

Figure 7.23. RAR subtype-selective ligands.

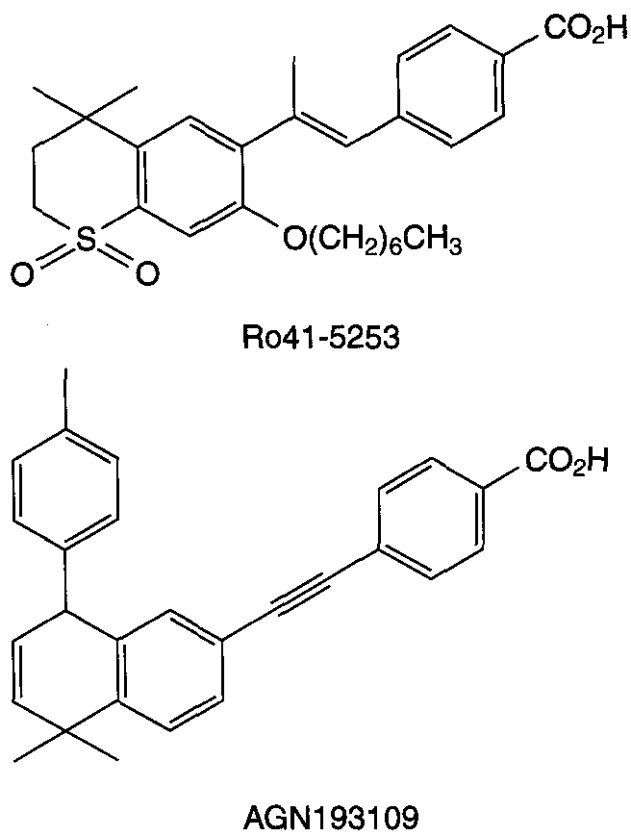


Figure 7.24. RAR antagonist **and** inverse agonist.

the RARα LBD results in the repositioning of helix 12 into the "antagonist" conformation (140) similar to that of estrogen receptor (ER)-β bound by the antiestrogen raloxifene (212) or the partial agoinst genistein (213) and ERα bound by the antiestrogen tamoxifen (214). In these examples, binding of antagonists (or the partial agonist genistein) causes juxtapositioning and subsequent interaction of helix 12 with structural motifs in helices 3 and 4 that contribute to the coactivator interaction interface. In contrast to the agonist-bound form of the receptors, however, the antagonist-induced repositioning of helix 12 does not enhance interaction of the receptors with any of the known transcriptional coactivator proteins possessing LXXLL motifs.

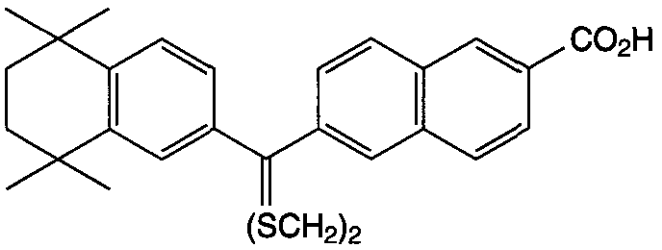
Chandraratna and colleagues at Allergan have developed a novel class of compounds possessing pharmacological properties that are distinct from both agonists and antagonists. The prototypic compound in this class is AGN193109 (215,216) (Fig. 7.24). In contrast to classical agonists that induce dissociation of RAR·NCoR complexes, AGN193109 increases the affinity of the receptor for this corepressor (215, 216), and as such, is known ***as*** an ***inverse agonist.*** Although AGN193109 is selective for RARγ, the property of inverse agonism, and therefore, the development of inverse agonists, is conceptually possible for other receptor subtypes. As is the case for antagonists, however, the clinical usefulness of such compounds is presently unknown. Nonetheless, compounds such as inverse agonists and antagonists are great tools that allow retinoid biologists to dissect the retinoid signaling pathways in various contexts.

Whereas it has been relatively straightforward to develop compounds that discriminate between RARs and RXRs using, for example, ***trans-RA*** as the prototypic pharmacophore that is specific for RAR subtypes, the development of RXR-selective compounds has also progressed as a result of the synthetic efforts of a number of medicinal chemists. RXR-selective retinoids (Fig. 7.25) include bexarotene (198, 199), SR11237 (217), SR11246 (200, 204), LGD100268 (199), and SR11345 (M. Dawson, X.-K. Zhang, and M. Leid, unpublished data, 2000). The degree of RXR subtype-selectivity of these compounds is unknown.

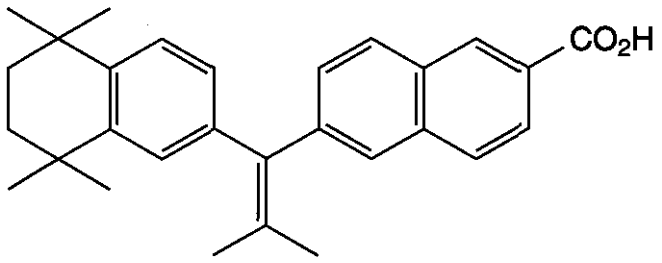
One major obstacle for the development of RXR subtype-selective compounds is that the amino acid residues lining the ligand binding pockets of RXRα, β, and γ are identical (143). However, these RXR subtypes do harbor differences in so-called "second layer residues," those amino acids that do not contact the ligand directly, but nonetheless must undergo side chain conformational alterations to accommodate ligand in the binding pocket (143, 146). Thus, it is conceivable that substitutions in second layer residues of RXR subtypes may be exploited in the development of RXR subtype–selective ligands.

8.2 "Gene-Specific" Retinoids

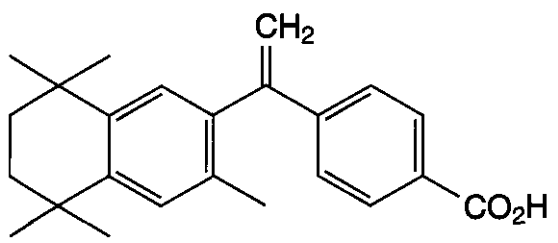
Heterodimeric RAR·RXR complexes bind to and regulate target gene transcription from DR1-, DR2-, and DR5-type response elements (see previous sections). Thus, it is conceivable that retinoids could be developed that would selectively activate a RAR·RXR complex bound to a DR5 RARE, for example, but not the same complex bound to either DR1 or DR2 RAREs, or other, unknown response elements. It has been suggested that such a phenomenon may be caused by the existence of alternative conformations of the RAR·RXR heterodimeric complexes bound to these dif-



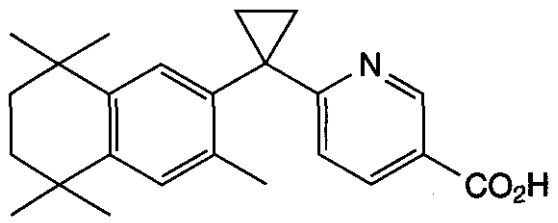
SR11237



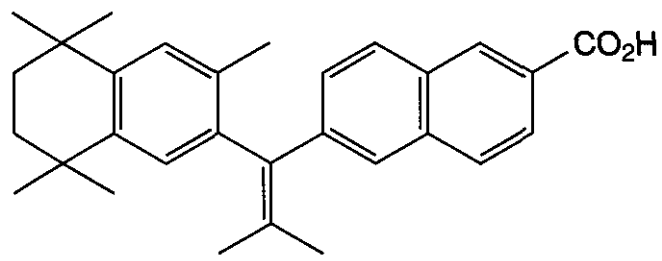
SR11246



Bexarotene



LG100268



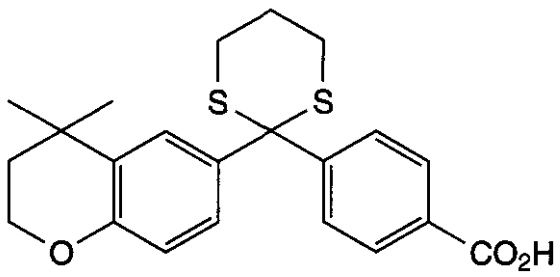
SR11345

Figure 7.25. RXR-selective agonists.

ferently spaced response elements, differing promoter contexts, or both (218). Although this is an appealing idea in principle and some evidence derived from artificial systems has been provided to support this concept, convincing data are lacking. Nonetheless, as more information becomes available regarding the structure of heterodimeric complexes bound to differently spaced response elements and the mechanisms underlying RAR·RXR-mediated transcriptional activation, it may be conceivable that so-call "gene-specific" retinoids could be developed.

8.3 Anti-AP1 Activity of Retinoids

Ligand-activated RAR·RXR complexes antagonize transcriptional activation mediated by the proto-oncogene transcription factor complex known as AP1, and the converse is also true. AP1 antagonizes RAR·RXR-mediated transcriptional activation (173, 219). Activation of RARs by classical retinoid receptor agonists, such as *trans*-RA, results in two events, activation of transcription mediated by RAR·RXR complexes and antagonism of AP1-mediated transcriptional activation that may be mediated by RAR·RXR or RAR alone (220–223). As AP1 is classically associated with cellular proliferation and inflammatory responses, retinoid receptor-mediated antagonism of AP1-mediated transcriptional activation could be exploited therapeutically in the treatment of proliferative diseases and inflammatory diseases such as arthritis. The Dawson group was the first to develop so-called anti-AP1 retinoids, the prototypes of which are SR11238 (Fig. 7.26) and SR11302 (219), which do not modulate the transcriptional activation function of any of the known RARs or RXRs. The molecular basis of action of compounds such as SR11238 is unknown, but two mechanisms have been proposed to account for this form of transcriptional interference: (1) competition between retinoid receptors and AP1 for a common transcriptional coactivator, such as CBP (173), and (2) retinoid receptor-mediated inhibition of AP1 assembly (223). Clearly, development of useful and selective retinoids that induce the receptors to inhibit AP1-mediated transcriptional activation without affecting the transcriptional activation



SR11238

Figure 7.26. Anti-AP1 retinoid, SR11238.

function of the receptors remains an important goal in the retinoid field.

8.4 CD437: An Atypical Retinoid

CD437, 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalenecarboxylicacid (AHPN; see Fig. 7.23), was originally described as a selective, RARγ agonist that may be useful in the treatment of retinoid-sensitive dermatological conditions (203, 205). However, more recently, Fontana and colleagues have discovered that this retinoid induces cell cycle arrest and subsequent apoptosis in a number of cancer cell lines (224–232) through a mechanism that does not seem to involve any of the known RAR or RXR subtypes (229). CD437/AHPN even induces growth arrest and apoptosis in cancer cell lines that are resistant to growth inhibition induced by classical retinoids such as *trans*-RA (229). Neither the putative receptor for CD437 nor the pro-apoptotic mechanism have been identified.

Again, however, the toxicity of CD437/AHPN is a problem in a clinical sense, and this toxicity seems likely to result from activation of RARs. Thus, substantial efforts have been devoted to the development of CD437/AHPN-like compounds that maintain pro-apoptotic activity with reduced ability to activate classical retinoid receptors. The first such compound is MM11453, the 3-chloro analogue of AHPN (232). MM11453 was found to inhibit the growth of both retinoid-resistant and retinoid-sensitive cancer cell lines with equal potency (232). It is highly likely the additional compounds like MM11453 will be developed for the treatment of proliferative disorders.

8.5 Novel Uses for RXR-Active Compounds

As described above, RXR heterodimerizes with several other members of the nuclear receptors family. This implies that RXR, through these diverse, heterodimeric interactions, may regulate the expression of a very large number of genes. However, RXR does not seem to be competent to active transcription in the context of VDR·RXR, TR·RXR, or RAR·RXR complexes bound to DR3-, DR4-, or DR5-type response elements, respectively (135,233). In the case of RAR·RXR complexes (234, 235) and probably also TR·RXR complexes (236), RXR can activate transcription if the other subunit of the heterodimer is bound by either agonists (235, 236) or an antagonist (234). This finding suggests that the RXR heterodimeric partner allosterically regulates the function of the RXR LBD and is known as the "RXR subordination" model (210) in that RXR is dependent on (subordinate to) activation of the its heterodimer partner for its own activation.

In other heterodimeric contexts, known as "permissive heterodirners," RXR is able to activate transcription. The most important of these in a clinical sense are probably RXR·PPAR (237–239) and RXR·LXR (240) heterodimers. The ability of RXR agonists to activate RXR·PPARγ heterodimers is extremely important in the treatment of type II diabetes (non-insulin dependent diabetes), a disease of epidemic proportions in the United States and worldwide. For example, thiazolidinedione antidiabetic drugs are becoming increasingly used in the treatment of type II diabetes. These drugs are PPARγ agonists that activate RXR·PPARγ heterodimeric complexes that, in turn, regulate the expression of genes encoding proteins that improve glucose tolerance by enhancing the cellular sensitivity to insulin. Administration of RXR agonists to diabetic and obese mice also improves glucose tolerance, and the co-administration of RXR agonists with a thiazolidinedione results in synergistic improvements in several diabetic parameters (241, 242). Interestingly, subsets of highly insulin-resistant patients harbor a mutation(s) in the PPARy locus that renders these patients resistant to thiazolidinedione therapy, yet these patients remain sensitive to the glucose-lowering effects of RXR agonists (237). Thus, the continued development of RXR-selective ligands for the treatment of type II diabetes, as monotherapy or in conjunction with thiazolidinediones, will certainly be pursued in the future.

Another interesting application for RXR-selective agents may be in the treatment of hypercholesterolemia. Mangelsdorf and colleagues have recently discovered that the RXR agonist LG100268, acting through RXR·LXR heterodimeric complexes, transcriptionally induces the expression of the gene encoding the ABC1 protein, which mediates reverse

cholesterol transport (240). In intestinal cells, this protein transports cholesterol into the lumen of the intestine, effectively reducing cholesterol uptake by the cell and ultimately lowering blood cholesterol levels. In the case of RXR agonists, this effect is amplified because RXR agonists (rexinoids) also decrease bile acid secretion through the activation of RXR·FXR heterodimeric complexes (243,244) (Table 7.4). Because bile acids are important vehicles for cholesterol dissolution and generally promote cholesterol absorption in the gut, the effect of RXR-mediated induction of ABC1 expression has an even more dramatic effect on serum cholesterol levels. In addition, the ABC1 protein mediates cholesterol efflux from macrophages that contribute to atherosclerotic processes (240). The development of additional RXR-selective ligands will clearly be at the forefront of retinoid biology in the future. However, there are problems with rexinoid therapy, including disruptions in triglyceride levels and possibly obesity, the latter of which is controlled, at least in part by RXR·PPARγ complexes (245, 246). Additionally, systemic administration of & RXR agonist has been associated with reversible, central hypothyroidism, most likely by activation of a TR·RXR heterodimeric complex that represses thyrotropin secretion (236).

8.6 Summary and Perspective

Retinoids, vitamin A derivatives, play essential roles throughout organismal life. Although the mechanisms underlying the pleiotropic effects of retinoids are not completely understood at the mechanistic or cellular level, naturally occurring retinoids and synthetic derivatives are being increasing used in clinical medicine. The past 5 years have seen a dramatic increase in the use of retinoids to treat a variety of diseases. In addition, several new uses of retinoids are presently being investigated. The next 5 years will likely see increased development of rexinoids, RXR- and RXR subtype-selective compounds to treat a variety of diseases that were not previously thought to be responsive to retinoid therapy. Now more than ever, retinoid chemistry is poised to make substantial contributions to human health through the design and synthesis of novel compounds that act at both RARs and RXRs.

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CHAPTER EIGHT

Vitamins

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Contents

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1 INTRODUCTION

Vitamins are enjoying renewed popularity with the lay public that, in some ways, mimics the attention seen in the early part of the twentieth century when they were first being discovered. Paralleling the interest by the general public is increased focus on the biochemical role of vitamins at the molecular level. Some vitamins can be considered prototypes for pharmacological agents used to treat diseases that do not appear directly related to the patient's vitamin status. Although all animals require vitamins, the discussion in this chapter focuses on humans and human biochemical pathways.

Vitamins are complex biochemically and functionally and may be classified many ways. This chapter acknowledges the traditional solubility model but focuses on functionality. Unfortunately, the loose regulation of nutritional products in the United States has led to misleading promotion of these items to the lay public, such that individuals are confused as to what can be called a vitamin. General criteria for deciding if a substance truly is a vitamin are presented. Another problem is to decide whether a patient actually has a vitamin deficiency. As noted in the next section, this can be a complex question. Finally, deciding how much of a particular vitamin should be consumed has moved from the familiar Recommended Dietary Allowance (RDA) to four different ways to evaluate vitamin requirements and consumption. These are called the Dietary Reference Intakes (DRIs).

1.1 What is a Vitamin?

In general a vitamin must be a naturally occurring, organic molecule that is a normal constituent of the diet. It should be essential and required in only minute amounts. Finally, it is required to maintain normal cellular biochemistry and tissue integrity. Let us examine these in more detail.

1.1.1 Vitamins Are Naturally Occurring. This criterion frequently is misinterpreted. Although the vitamin is a natural product, there is no difference in efficacy between ingesting the all-natural or a synthetic product as long as the natural and synthetic products are identical. This includes stereochemistry. L-Ascorbic acid is twice as active as the racemic D,L-ascorbic acid. A similar statement can be made for D-pantothenic acid and S-biotin relative to their racemic mixtures. The situation for a-tocopherol (vitamin E) with three asymmetric centers is more complex and is discussed in more detail later in this chapter.

1.1.2 Vitamins Are Essential Because They Are Not Produced by Human Biochemical Pathways. With two exceptions, this requirement is obvious. If a compound is required for a biochemical process and our pathways cannot produce it, it must be obtained from exogenous sources. The evolutionary history of the human species is such that we sought out sources in our environment that contained these essential substances, and those substances containing vitamins became our food.

One of the substances commonly treated as a vitamin is niacin, which is synthesized from the essential amino acid tryptophan. The ratio is approximately 60 mg of tryptophan being required to produce 1 mg of niacin (1). This has led to niacin requirements being expressed as niacin equivalents (NE), based on the amount of tryptophan in the diet. It must be kept in mind that tryptophan is essential and is the precursor to the neurotransmitter serotonin in addition to being part of protein structure. Therefore, niacin can be thought of as tryptophan sparing.

The other exception is vitamin D_3, or cholecalciferol. Assuming adequate sunlight, cholecalciferol is the photochemical product from ultraviolet irradiation of 7-dehydrocholesterol found in our skin. A significant proportion of the world's population produces all of the cholecalciferol it needs from this photochemical reaction. It is true that a photochemical reaction is not a biochemical pathway, but exposure to adequate sunlight does fulfill the requirement for cholecalciferol. As noted in the discussion for this particular substance, the compounds commonly referred to as vitamins D_2 and D_3 are not normal constituents of most human diets.

1.1.3 Organic. Trace elements are not properly called vitamins. Therefore, iron, zinc,

magnesium, manganese, chromium, selenium, and the other trace elements that are obviously obtained from food sources are essential, but they are not vitamins.

1.1.4 Normal Constituent of the Diet. With the exception of cholecalciferol, all of the other compounds that we treat as vitamins are normal constituents of our diets, with most found in more than one food group. The situation with cholecalciferol is such that, except for the small part of the world's population that obtains its protein from marine species, neither cholecalciferol nor ergocalciferol from irradiation of the plant sterol ergosterol, is a normal component of foods.

1.1.5 Vitamins Are Required in Minute Amounts. This is arbitrary, but ranges from 2.0 μg (10^{-6} g) for cyanocobalamin to 90 mg for ascorbic acid. Other essential nutrients including the essential amino acids and fatty acids generally are required in larger amounts. For example, the National Research Council's recommended daily intake of amino acids ranges from a low of 245 mg for tryptophan to 980 mg for phenylalanine and tyrosine (combined) and leucine (2). Note that the value for tryptophan provides only about 4 mg of niacin, assuming all 245 mg of tryptophan is converted to niacin.

1.1.6 Vitamins Are Required to Maintain Normal Biochemical Functions of the Tissues. Most vitamins function either as a hormone/chemical messenger (cholecalciferol), structural component in some metabolic process (pantothenic acid), or a coenzyme (phytonadione, thiamine, riboflavin, niacin, pyridoxine, biotin, folic acid, cyanocobalamin). At least one vitamin has more than one biochemical role. Vitamin A as an aldehyde (retinal) is a structural component of the visual pigment rhodopsin and, in its acid form (retinoic acid), is a regulator of cell differentiation. The precise biochemical functions of ascorbic acid and a-tocopherol still are not well defined.

1.2 Causes of Vitamin Deficiencies

There are a variety of reasons why a person might be experiencing a vitamin deficiency including economics, genetics, diseased intestinal tract, alcoholism, a variety of medical conditions, lifestyle, and vitamin–drug interactions. Table 8.1 contains a classification scheme for possible causes of vitamin deficiencies.

2 DIETARY REFERENCE INTAKES

2.1 Determination of a Vitamin Dose

This is not easy. Ideally, a dose-response curve could be constructed that would provide defined endpoints such as an ED_{0} or ED_{0} and doses that would cause serious toxicities, the LD_{50}. The problem is defining a biological or pharmacological endpoint. Not all vitamin deficiencies have defined syndromes. Little is known of the pharmacokinetics of individual vitamins. In contrast with most drugs, the body stores vitamins, sometimes several months' supply. This should not be surprising when one examines human history. Until fairly recent times, humans regularly experienced food plenty and food deprivation (famine) as crops failed and animals moved away from settlements. Causes ranged from weather to war. To survive, the evolutionary development of humans includes ways to store and reuse vitamins. Assuming a good diet, humans have a 6- to 9-month supply of vitamin A. Humans efficiently recycle cobalamin (vitamin B_{0}) by enterohepatic circulation. The intestinal flora produce a precursor to vitamin K.

2.2 Methods to Determine a Valid Dose of a Vitamin

Each of the methods outlined in the following sections has significant problems in determining a dose-response curve for a vitamin. Table 8.2 lists methods that are being used to determine vitamin requirements or are under development as possible procedures.

2.2.1 Extrapolate from Animal Studies. This method is dependent on the species. The vitamin requirements differ among the common laboratory animals. The classic example is ascorbic acid, which is not a vitamin in most animals. Humans do not exhibit specific defi-

ciency symptoms for a-tocopherol, biotin, and pantothenic acid, whereas many animals do.

2.2.2 Metabolic Balance Studies in Humans. This usually involves a specified number of days on a defined diet in which the urine and feces are monitored. The problem is that corrections have to be made for the vitamins that are stored. The pharmacokinetics of most vitamins have not been carefully determined.

2.2.3 Compare Nutrient Intake in Areas with and without the Deficiency Disease. First, not all vitamin deficiencies lead to a defined deficiency state. Second, rarely does one find an area deficient in only one nutrient. Most common deficiencies are attributed to inadequate diet, which means several nutrient deficiencies will result.

2.2.4 Saturation of Biochemical Function. A reliable biochemical indicator is required. For niacin, which NAD^+- or $NADP^+$-containing enzyme should be selected? Which transaminase will be the indicator for pyridoxine? Which function of vitamin A should be selected for retinol, vision in the rods or cell differentiation? As noted from Table 8.2, many of the assays for vitamin status have significant limitations to estimate reliable doses.

2.2.5 Serum Levels. This probably is the most reliable, but it does require very sensitive assay methods for those vitamins required in microgram (10^{-6}) amounts. It also requires knowledge as to how the vitamin is transported: free or bound to a plasma protein or on a specific transport protein. Examples of the latter are transport proteins for vitamin A and vitamin D,. The tocopherols will be found in the lipoproteins (VLDL, LDL, etc.).

2.3 Dietary Reference Intakes (DRIs) (3, 4)

The adult DRI values for each vitamin are found in the section for that vitamin.

2.3.1 Introduction. The last official set of reference values were the 1989 Recommended Dietary Allowances (RDAs) for the United States and 1990 Recommended Nutrient Intakes for Canada. These are being replaced by the Dietary Reference Intakes, a joint effort of the United States and Canada. The DRIs are published by the Food and Nutrition Board of the National Academy of Sciences National Research Council. The expert panels are made up of U.S. and Canadian scientists.

2.3.2 How Do the DRIs Differ from RDAs?. There is one set of reference values for both Canada and the United States and clear documentation on how reference values are selected. A goal is the promotion of nutrient function and biologic-physical well-being. Evidence concerning the prevention of disease and developmental disorders is examined in addition to the traditional "how much nutrient is needed to prevent a deficiency symptom." Data supporting food components that, up to this time, have not been considered essential nutrients will be examined. Finally, there are recommendations for future research.

2.3.3 Uses of Dietary Reference Intakes. The DRIs consist of four components. Each type of reference value is calculated from daily intakes averaged over time (usually one or more weeks). The surveys include, but are not limited to, (1) random selection of healthy individuals and asking them to either report what they have eaten or to maintain food diaries, (2) monitoring overall food production and consumption, and (3) correlating a defined population's health status with the group's food intake. Sometimes the results from the surveys are correlated with the type of assays listed in Table 8.2. The four Dietary Reference Intakes are:

2.3.3.1 Estimated Average Requirement (EAR). The EAR is the intake that meets the estimated nutrient need of 50% of the individuals in that group (i.e., infants, children, adult males, adult females, pregnant women, nursing women, the elderly, etc.). It is used to evaluate the adequacy of nutrient intakes of population groups and for planning intakes for group. It can be used in diet planning. The EAR is based on a median rather than a mean.

2.3.3.2 Recommended Dietary Allowance (RDA). The RDA is the intake that meets the nutrient need of almost all (97–98%) individu-

Table 8.1 Causes of Nutrient Deficiencies

Cause	Mechanism/Reason	Remarks
Inadequate ingestion usually from a poor diet	Economic deprivation	Inability to purchase adequate amounts and variety of food.
	Self-imposed reducing diets	A 1200 calorie diet professionally selected from the four major food groups (dairy, fruits and vegetables, grains, and meat) containing no fried food nor added sugar has been considered the least amount of food not requiring a vitamin supplement.
	Disease	This usually is attributed to loss of appetite from such conditions as cancer chemotherapy, depression, and eating disorders.
Inadequate absorption	Diseased intestinal tract	Examples include chronic inflammatory conditions such as Crohn's disease and parasites.
	Mineral oil laxatives, which may dissolve the oil-soluble vitamins	This could include retinol, cholecalciferol/ergocalciferol, a-tocopherol, vitamin K from food.
	Ion exchange resins (colestipol, colestyramine), which complex with the bile salts and can interfere with the absorption of the oil-soluble vitamins	A vitamin supplement can be taken 1 h before or 2 h after taking the resin.
	Aluminum antacids can complex with some of the vitamins and, when used chronically, most definitely can cause hypophosphatemia	Aluminium antacids are no longer commonly used.
	Cystic fibrosis, which can cause fat malabsorption (steatorrhea) attributed to inadequate production of pancreatic lipases	This could include retinol, cholecalciferol/ergocalciferol, a-tocopherol, vitamin K from food, all of which are more readily absorbed when they can be part of a normal mixed micelle process that occurs with lipid digestion and absorption

Inadequate utilization	**Genetic diseases**	**Examples include variants of maple syrup urine disease** (MSUD), which will respond **to** thiamine (vitamin B_1) supplements, and **homocystinuria,** which will respond to pyridoxine (vitamin B_6). The mutation lies with apoenzyme such that the equilibrium between the apoenzyme and the coenzyme lies to the left. To push the reaction to the right requires additional coenzyme.
	Drug–vitamin interactions	These *can* interfere with vitamin processing in the intestinal tract, tie up the vitamin preventing it from being used, or possibly promote elimination of the vitamin. Examples include: **isoniazid–pyridoxine,** phenobarbital–cholecalciferol, methotrexate–folic acid, phenytoin–folic acid.
Increased requirements above the recommended daily allowances (RDA)	Reference dietary indices are based on an average population performing average duties. Increased physical activity or medical needs make these people statistical outliers beyond the requirements of the "average" healthy population group.	Individuals performing more strenuous activities requiring additional intake of calories also will require more nutrients including vitamins. Vitamin and caloric requirements increase for patients experiencing debilitating illness, including severe burns, major surgeries, and malignancies. Nutritional assessments are becoming a more common part of medical treatment.
Chronic intake of alcohol (alcoholism)	Ethyl alcohol *can* interfere with the uptake, processing, or storage of vitamins.	The two most common vitamin deficiencies seen in patients chronically consuming alcohol are folic acid and thiamine.

Table 8.2 Methods for Estimating Vitamin Requirements

Vitamin	Determination Methodology	Remarks
Retinol (vitamin A)	Dark adaption test	Considered reasonably sensitive.
	Pupillary response test	Data do not exist relating pupillary threshold sensitivity to retinol intake.
	Plasma retinol concentration	Insensitive to liver stores.
	Relative dose response	An initial plasma concentration is determined followed by a second concentration a defined time after administration of a small dose of retinol. The ratio is proportional to the liver stores of retinol.
Calciferol (vitamins D_2 and D_3)	Serum 25(OH)D_2 or D_3	This is considered a more accurate indicator of vitamins D status as compared to serum vitamins D, or 1,25(OH)$_2$D.
a-Tocopherol (vitamin E)	Lipid peroxidation markers	They are not specific to vitamin E.
	Plasma a-Tocopherol concentration	It does not seem to correlate with daily intake, but there is a linear relationship seen in tocopherol-depleted subjects.
Vitamin K	Prothrombin time	It is used to assess patients on cournadin anticoagulant therapy, but does not appear to be a reliable measure of vitamin K status in otherwise healthy subjects.
	Factor VII	Even though factor VII only has a 6-h half-life, it does not appear to be reliable.
	Plasma or serum phylloquinone concentration	This does respond to changes in dietary intake within 24 h.
	Urinary γ-carboxyglutamyl residues	This is considered promising.
Thiamine (vitamin B_1)	Erythrocyte transketolase activity	This is considered the most accurate.
	Urinary thiamine excretion	This method also is considered good.
	Erythrocyte thiamine concentration	It is not as widely used.
Riboflavin (vitamin B_2)	Erythrocyte glutathione reductase activity	It is the most common assay.
	Erythrocyte flavin	Some question the sensitivity of this test because the difference between adequacy and inadequacy is small.
	Urinary riboflavin excretion	Care must be exercised with the size of the dose and method of administration.
Niacin/niacinamide	Urinary excretion	N^1-Methylnicotinamide and N^1-methyl-2-pyridone-5-carboxamide are the two metabolites that are considered sensitive measures of niacin status.
	Plasma concentration	N^1-Methyl-2-pyridone-5-carboxamide plasma levels provide an indication of low niacin intake by dropping below detection limits.
	Erythrocyte pyridine nucleotides	Erythrocyte NAD levels may replace measuring metabolites found in the urine.

Vitamin B_6 family	**Plasma pyridoxal phosphate**	It changes slowly in response to changes in vitamin intake.
	Erythrocyte and total blood pyridoxal phosphate	There may be racial differences because of lower kinase activity.
	Urinary pyridoxic acid	It tends to reflect recent vitamin intake rather than general vitamin status.
	Various erythrocyte transaminases	Aspartate and alanine transaminases have been studied most extensively. There is a lack of consensus regarding their usefulness as indicators of vitamin B_6 status.
	Tryptophan catabolites	Although useful, some of the reactions affected changes in steroid hormone status.
	Plasma homocysteine	See discussion in the vitamin B_6 section.
Pantothenic acid	Urinary excretion	It is strongly dependent on intake.
	Blood levels	There is poor correlation with urinary excretion values.
Biotin	Urinary excretion	There is a correlation with induced deficiencies caused by eating raw egg white.
Folic acid	Erythrocyte folate	Only the developing erythrocyte takes up folate. Therefore, this test is an indicator of long-term status and not a measure of immediate changes in folate status.
	Plasma homocysteine	There is no question that elevated homocysteine decreases with folate administration. There is not enough information to support its use for determining DRIs.
	Serum folate	A measure of dietary folate intake, although it is not considered a reliable measure of folate status.
	Urinary folate	This test may underestimate folate needs.
	Hematological status	Characteristic megaloblastic anemia develops late in folic acid deficiency.
Vitamin B_{12} (cobalamin)	Hematological response	These are the typical hemoglobin, hematocrit, and erythrocyte counts. Partial responses are of limited values.
	Serum or plasma vitamin B_{12}	A problem is that serum values may be maintained at the expense of tissue stores.
	Methylmalonic acid	Studies need to be done to see whether it will correlate with vitamin B_{12} status.
	Homocysteine	This can be elevated in folate and vitamin B_6 deficiencies.
	Holotranscobalamin II	This protein is responsible for receptor-mediated uptake of B_{12} into cells. Further work needs to be done before it is adapted for routine clinical use.
Ascorbic acid (vitamin C)	Antioxidant functions	A variety of markers have been evaluated including LDL, VLDL, malondialdehyde, hydroxynonenal, and reduced glutathione.
	Cellular DNA damage	These have not proved useful for estimating ascorbate requirements.
	Urinary markers	Oxidized DNA is nonspecific.

als in that group. The RDA are the values found on most vitamin products. They vary depending on whether the product is intended for infants, children, or adults. The RDAs can function as a guide to achieve adequate nutrient intake. By themselves, they are not generally recommended for diet planning for specific groups of individuals. Diet planning must take into account extensive physical activity, type of body build including lean vs. adipose tissue, general lifestyle, and so forth. If the sampling and endpoints are well defined, the RDA can be calculated from the EAR.

$$RDA = EAR + 2SD_{EAR}$$

where SD_{EAR} is the standard deviation above the EAR.

2.3.3.3 Adequate Intake (AI). The AI is the average observed or experimentally derived intake by a defined population or subgroup that appears to sustain a defined nutritional state, such as normal circulating nutrient values, growth, or other functional indicators of health. It is derived from mean intakes of groups (rather than individuals). The AI becomes most useful when a reliable EAR is not available. Many times the AI probably exceeds the EAR and possibly the RDA.

2.3.3.4 Tolerable Upper Intake Level (UL). The UL is the maximum intake by an individual that is unlikely to pose risks of adverse health effects in almost all (97–98%)individuals. It includes intake of a nutrient from all sources (food, fortified food, water, and supdements). Water can include fluoride and minerals depending on the source of water. "Tolerable" is used to "avoid implying any possible beneficial effect." It is the amount of vitamin that can be "tolerated" without the person's exhibiting or experiencing adverse reactions. The UL should not be considered the upper dose for those who self-dose with megadoses of vitamins.

3 VITAMINS

The order of vitamins in this chapter follows the classical classification-based solubility: fat soluble and water soluble. This classification originated before there was any understanding of the structural chemistry of the vitamins. There was fat-soluble A and water-soluble B. Thiamine was the first vitamin (B_1) whose structure was elucidated. It is an amine leading to the term vital amine and finally vitamin (without the *e*). The letter designations, for the most part, are being relegated to history. Many of the vitamins are groups of structures and, therefore, using a single letter can be misleading. Also, the structures occurring naturally in food and commercial preparations may actually be provitamins that have to be converted to the biochemically active form. Unless specified differently, the information in the following summaries is found in the Dietary Reference Intakes, published by the Institute of Medicine (5–8).

3.1 Retinol (Vitamin A) Family (9)

Early work on this vitamin was confusing because similar outcomes were seen with ingestion of "yellow" vegetables and colorless fish liver oils. It finally was shown that carotene (the yellow pigment) extracts from vegetables were converted to colorless retinal. Because the retinoids are discussed in considerably more detail elsewhere, this chapter presents only a cursory overview of their biochemical functions.

3.1.1 Chemistry. The commercial form of vitamin A is all-trans retinol, usually formulated as the acetate or palmitate ester. The active forms are the two oxidation products (Fig. 8.1, (1) retinal, which is a structural component of the visual pigment rhodopsin, and (2)retinoic acid, which is required for cell differentiation. There are specific nuclear receptors for retinoic acid. Although the vitamin is marketed in the all-trans form, retinal and retinoic acid are present, in *vivo*, in cis forms. There are also commercial forms related to the retinoic acid structure that have cis stereochemistry.

Carotenes are promoted commercially for their antioxidant activity rather than as a source of the retinol group. Nevertheless, the carotenes are the source of the vitamin in yellow vegetables. There are many carotenes, of which three are shown in Fig. 8.2. Only β-carotene is symmetrical (note the dashed line)

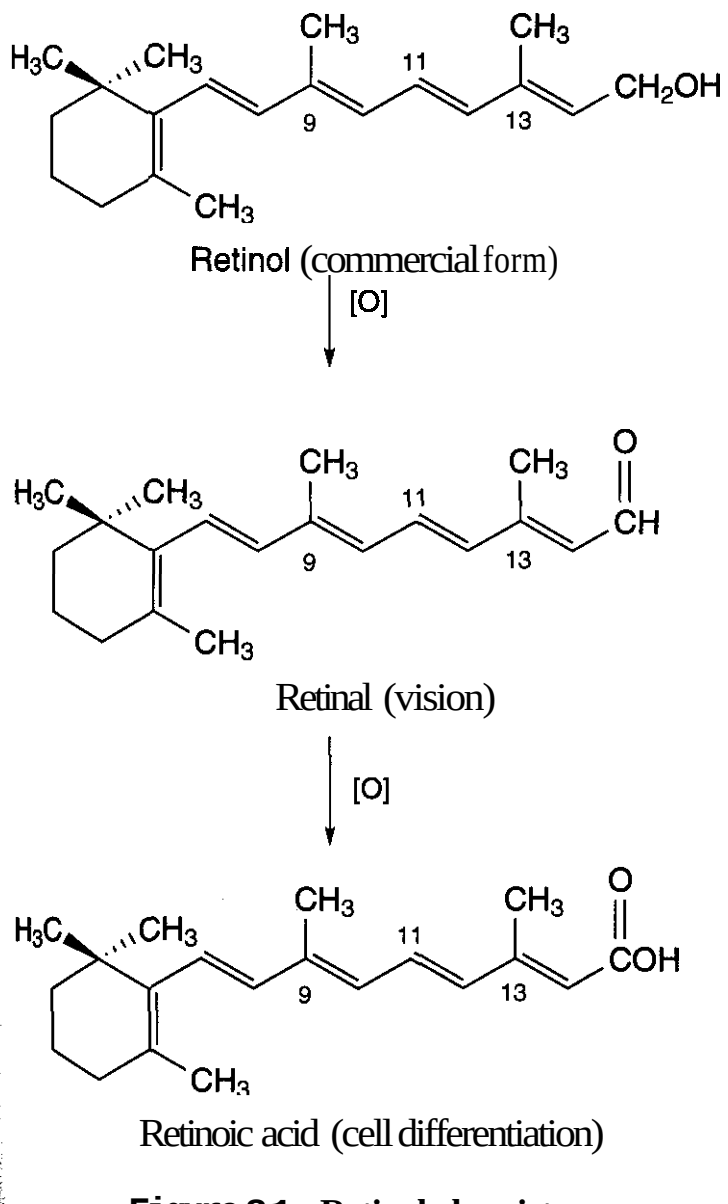


Figure 8.1. Retinol chemistry.

and theoretically will produce two equivalents of retinal after the enzyme-catalyzed oxidative cleavage. In reality, more recent studies indicate that vitamin A activity is six times the vitamin A activity derived from β-carotene. The problem is one of capacity in the intestinal mucosa cell to cleave the carotenes. Further, there appears to be regulation of the cleavage of β-carotene. As the stores in the liver reach capacity, there is less conversion of β-carotene being oxidized to retinal. This is one of the reasons that β-carotene nutritional supplements enter the body intact. Further, the bioavailability of β-carotene is significantly lower than that of retinol (10).

3.1.2 Uptake and Metabolism (Fig. 8.3). Both retinol esters, whether from animal tissues or a vitamin supplement, and β-carotene must be incorporated into mixed micelles along with other lipid material. The retinol esters are hydrolyzed by intestinal esterases. Both retinol and β-carotene are then absorbed into the mucosa cell, where β-carotene is oxidatively cleaved to retinal and then reduced to retinol. The retinol, independent of the source, follows the same steps seen with 2-monoglyceride from triglyceride digestion. The retinol is reesterified, usually with palmitic acid, and attached to the chylomicrons along with the other dietary lipids. The chylomicrons first enter the lymph and then move to the circulatory system. The triglycerides are removed from the chylomicrons and deposited in the adipose and skeletal muscle cells, leaving chylomicron remnants, which are transported to the liver where the esterified vitamin is stored (11). Transportation from the liver to tissues where required is done on specific retinol-binding proteins. Humans consuming a balanced diet store several months of retinol esters in their livers.

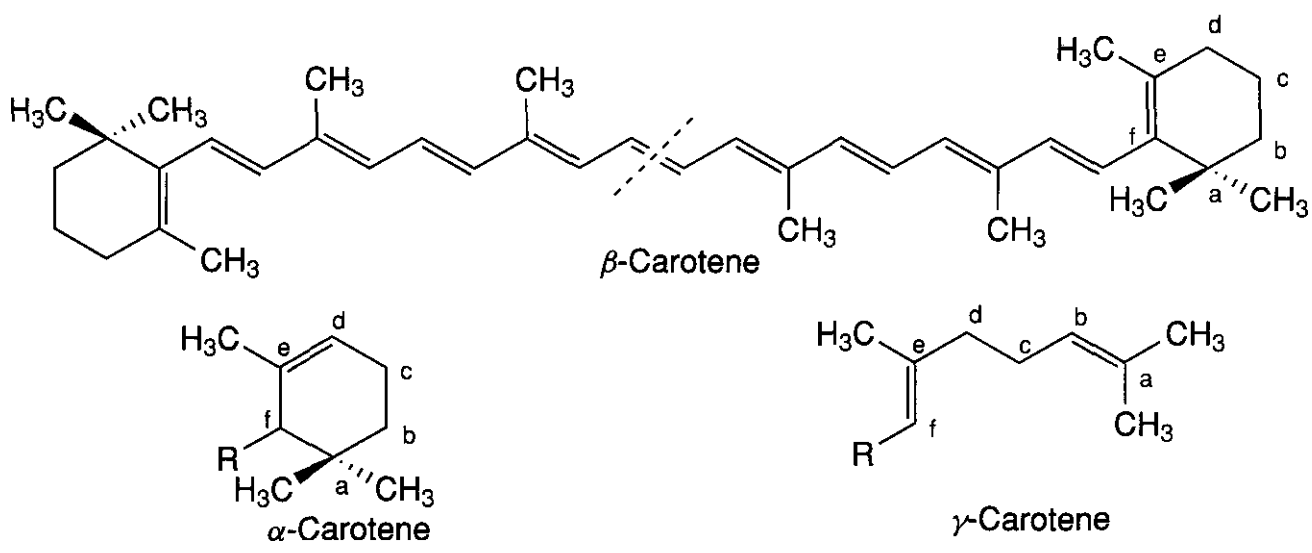


Figure 8.2. Carotene chemistry.

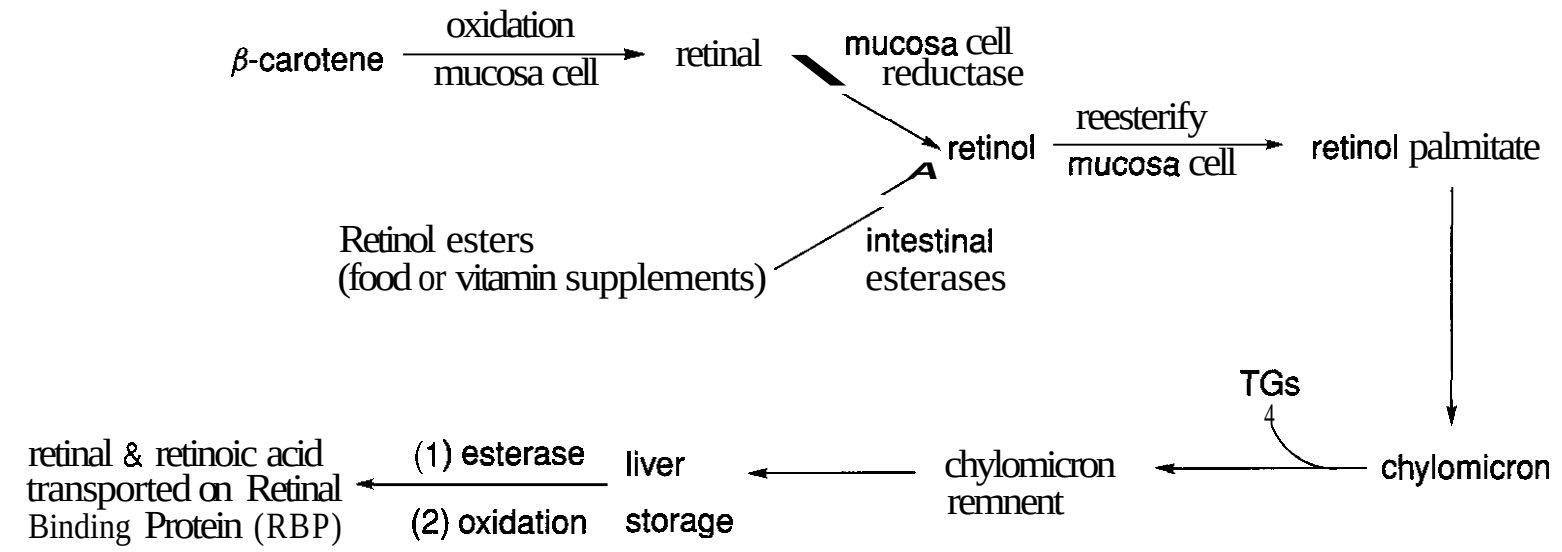


Figure 8.3. Uptake and metabolism of retinol esters and β-carotene.

3.1.3 Biochemical Functions and Deficiency. Two retinoids, retinoic acid and retinal, appear to have most of the biochemical functions attributed to vitamin A. Retinoic acid is required for cell differentiation and is the ligand for two families of nuclear receptors, $RAR_{\alpha,\beta,\gamma}$ and $RXR_{\alpha,\beta,\gamma}$. These receptors are part of a family of superreceptors that include the steroid hormones and cholecalciferol. Vitamin A deficiency can lead to a variety of symptoms depending on the age of the deficient person. The most serious syndrome is keratomalacia, which results in desiccation, ulceration, and xerophthalmia of the cornea and conjunctiva. It is one of the leading causes of blindness in infants and children.

Retinoic acid is required for the development of goblet mucous cells. A deficiency results in basal cell proliferation with increased keratinization of the epithelial structures. Mucus is one of the essential physical barriers (part of innate immunity) that prevents pathogens from entering the body. Therefore, a retinol deficiency increases the risk of infection.

The aldehyde form, retinal, is an essential component of the visual pigment found in the rods of the eye. A very brief outline of the rhodopsin cycle is shown in Fig. 8.4. Retinol is transported from the liver to the eye, where it is converted to 11-cis-retinal. In the rod, the aldehyde forms an enamine (Schiff's base) with a lysine on opsin forming rhodopsin (Fig. 8.5). In the presence of light, trans-retinal forms with cleavage of the enamine, sending a nerve impulse to the brain along the optic nerve. In the dark, 11-*cis*-retinol re-forms followed by oxidation to 11-cis-retinal and the cycle repeats.

A deficiency causes night blindness, which is considered an early symptom of retinol deficiency. Night blindness refers to decreased ability to see in very dim light because there is an inadequate amount of retinal in the eye to fully "stock" the rods with functional rhodopsin. There is some evidence that as retinol levels in the liver decrease, the equilibrium favors the movement of retinol from the eye back to the liver.

3.1.4 Dosage Forms. Retinol is unstable. It easily dehydrates (Fig. 8.6), forming a stable carbonium ion. Therefore, the two most common forms for both oral and parenteral administration are the acetate or palmitate esters. With the extensive conjugation retinol is light sensitive and subject to oxidation. Therefore, the vitamin formulator must protect the product from light and air.

3.1.5 Hypervitamininosis A. In high doses, retinol can be toxic. Acute poisoning is rare and dependent on the dosage form. Nausea and vomiting are the most common symptoms. Most rapidly absorbed are the "clear" emulsions (usually formulated with a Tween or other surfactant). Next in order are the standard emulsions, usually produced from fish liver oils. The most slowly absorbed are the dry tablet formulations or an oil solution in a capsule. Chronic hypervitaminosis is

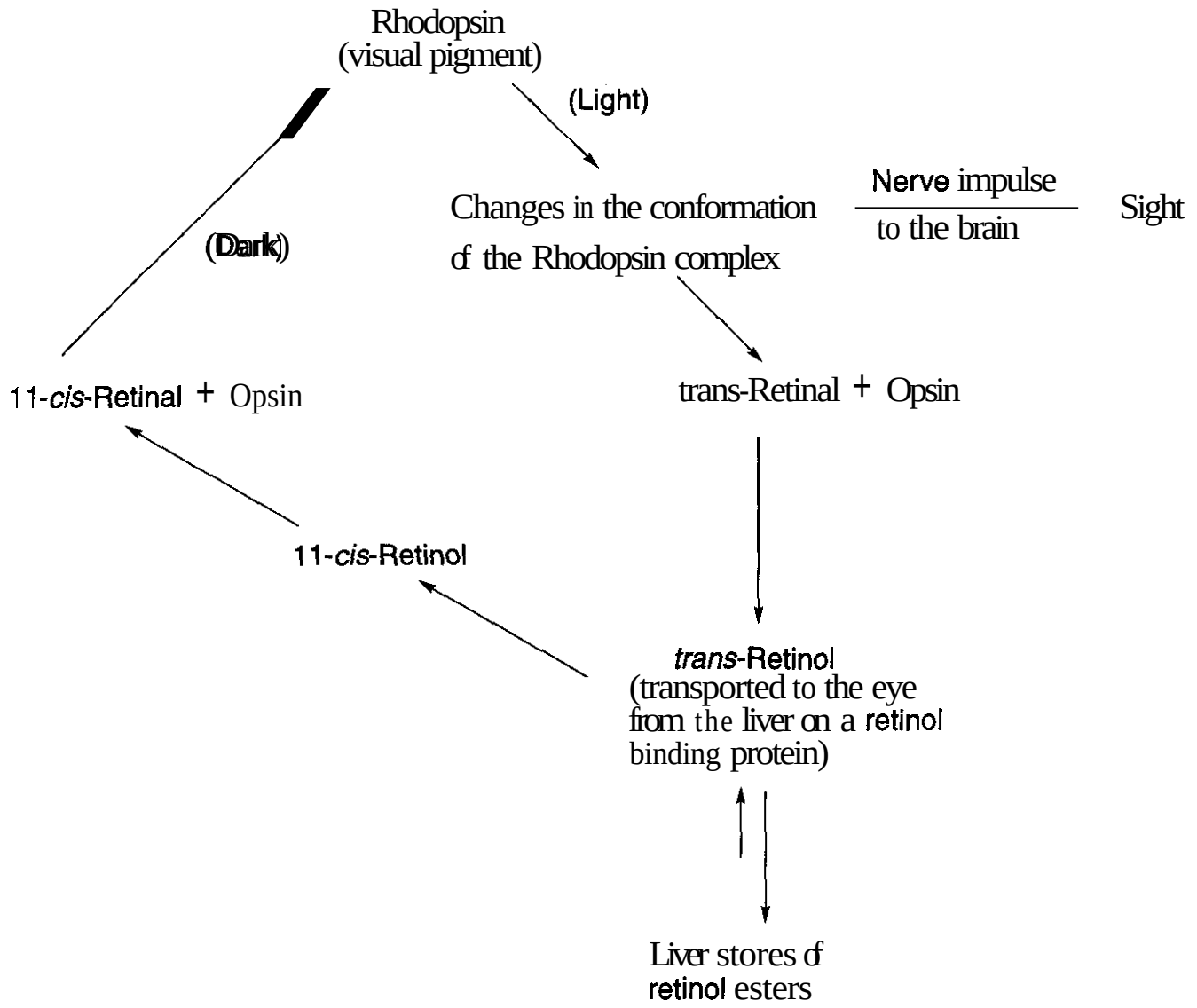


Figure 8.4. Outline of the rhodopsin cycle.

more common and is more commonly seen when people consume fish liver oil concentrates. The symptoms are nondescript and include fatigue, malaise, lethargy, abdominal discomfort, bone/joint pain, severe and throbbing headache, insomnia, restlessness, dry and scaly skin, loss of body hair, brittle nails, constipation, and irregular menses, symptoms that might make users conclude that they are vitamin A deficient. Depending on the health of person's liver, there is risk of developing cirrhosis. There is a daily Tolerable Upper Intake Level (UL) of 3000 μg for this vitamin. The UL to RDA ratio is narrow (3–5), relative to that of most vitamins. This is somewhat comparable to vitamins D.

3.1.6 Hypercarotenosis. This occurs from massive doses of carotene that exceed the capacity of the mucosa cells to cleave the molecule to retinal derivatives. The excess carotene becomes deposited in the body tissues. Except for the yellow or bronze-orange skin, there seem to be no other symptoms. The skin coloration slowly disappears when carotene intake stops. A commercial form of carotene is indicated for the photosensitivity seen in erythropoietic porphyria. Carotene capsules are also sold with the claim that a person can have a tanned appearance without the need of UV radiation. Patients who drink large amounts of carrot juice sometimes show signs of hypercarotenosis.

3.1.7 Dietary Reference Intakes (as Retinoic Acid Equivalents).

AI	
Infants (1–12 months)	400–500 μg/day
EAR	
Children (1–8 years)	210–275 μg/day
Boys (9–18 years)	445–630 μg/day
Girls (9–18 years)	420485 μg/day
Men (19–70+ years)	625 μg/day
Women (19–70-t years)	500 μg/day
Lactating	885–900 μg/day

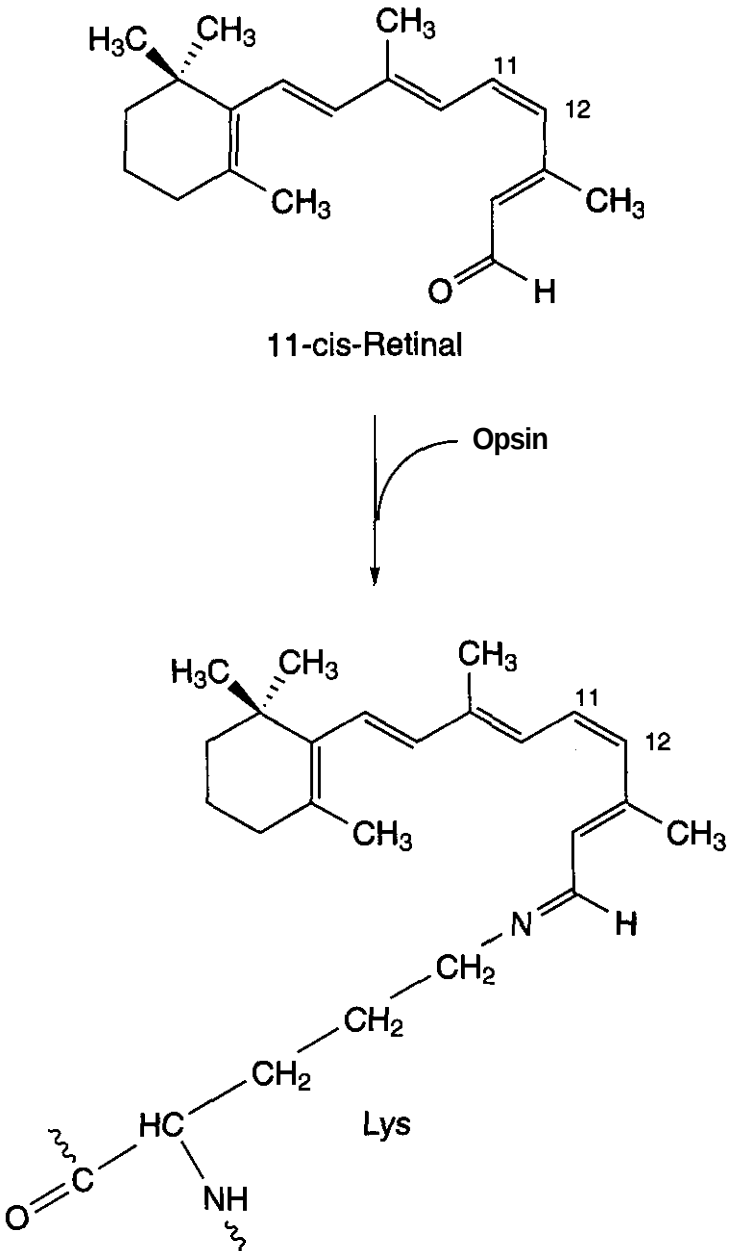


Figure 8.5. Rhodopsin.

RDA

Children (1–8 years)	300-400 μg/day
Boys (9–18 years)	600–900 μg/day
Girls (9–18 years)	600–700 μg/day
Men	900 μg/day
Women	700 μg/day
Pregnant	750–770 μg/day
Lactating	1200–1300 μg/day

UL

3000 μg/day for all adults including pregnant women. There is some concern of teratogenic effects based on the experience of the retinoids used in therapy.

3.1.8 Pharmacologically Active Retinoids. Because retinol deficiency results in keratinization of epithelial tissue, at one time retinol was recommended for skin conditions including acne. There is no clinical evidence that retinol is effective for skin conditions. Now that it is realized that the active form is retinoic acid, the focus has been on developing pharmacologically active compounds based on this structure. These are divided into treatment of three groups: (*1*) acne, (*2*) the autoimmune disease psoriasis, and (*3*) malignancies.

3.1.8.1 Retinoid and Retinoid-like Drugs Used in the Treatment of Acne (Fig. 8.7) (12). The first product introduced was tretinoin, which is a topical all-trans retinoic acid. Its effectiveness may not be related to any direct retinoid activity, but attributed by its producing a complex response related to increasing

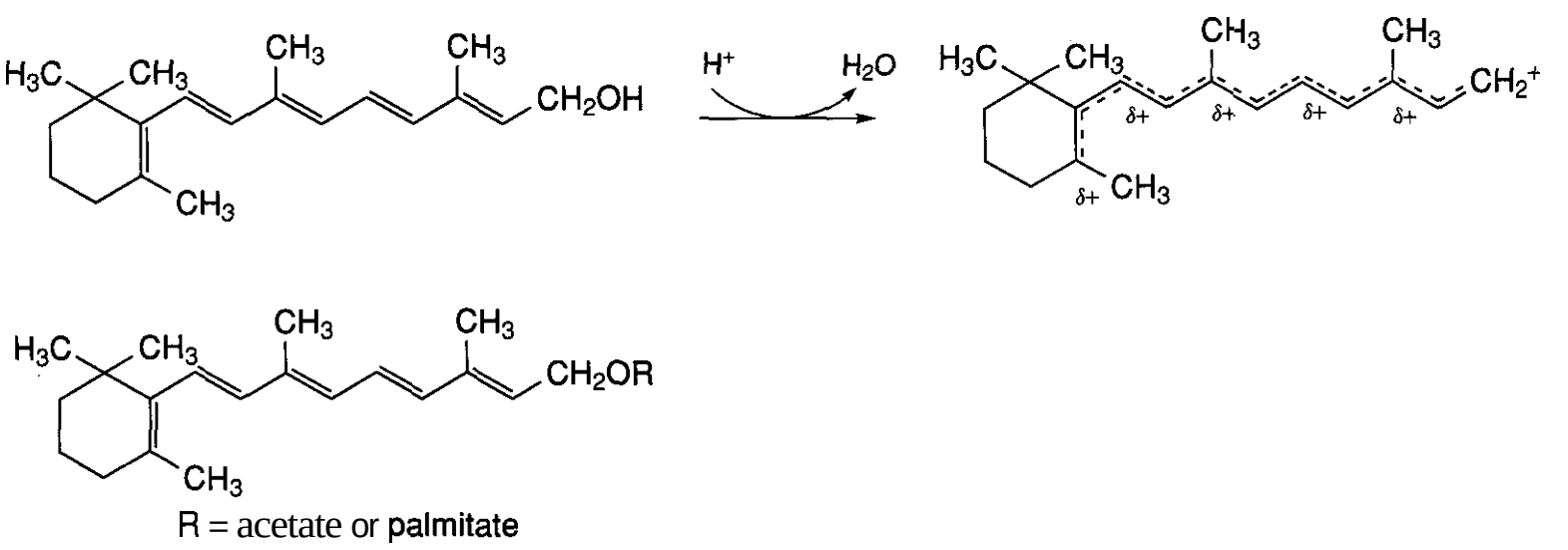
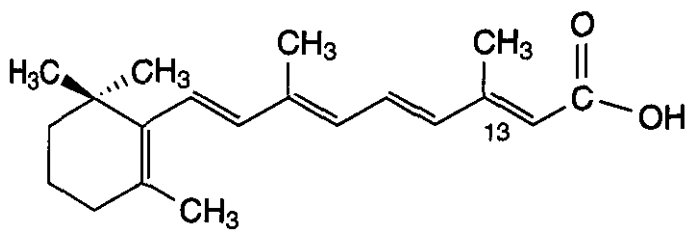
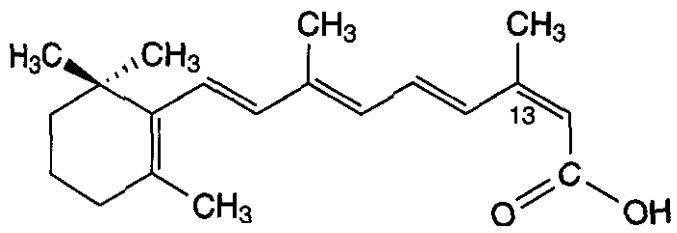


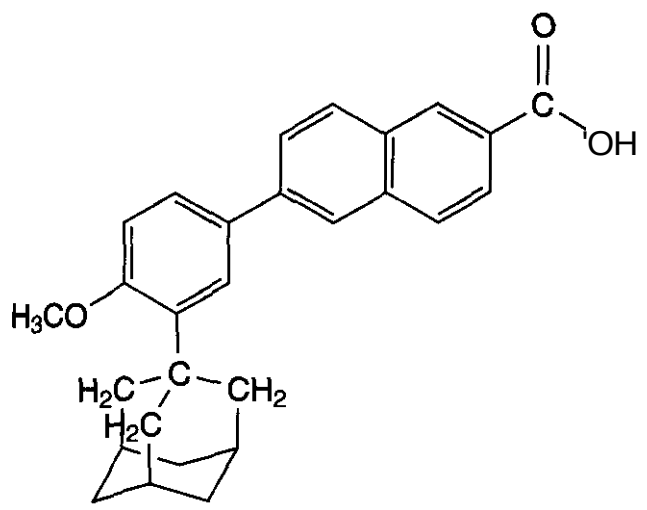
Figure 8.6. Commercial retinol esters.



Tretinoin (Retin-A)
Retinoic acid

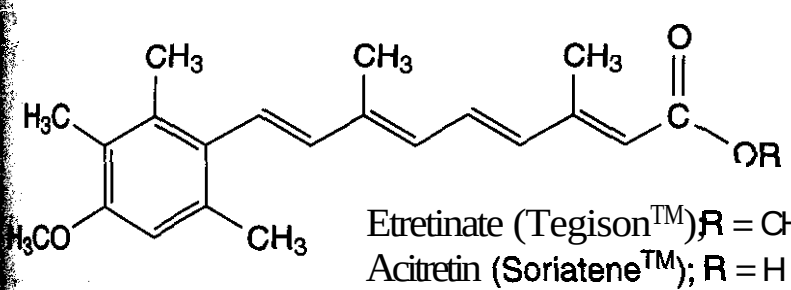


Isotretinoin (Accutane™)
13-cis-Retinoic acid

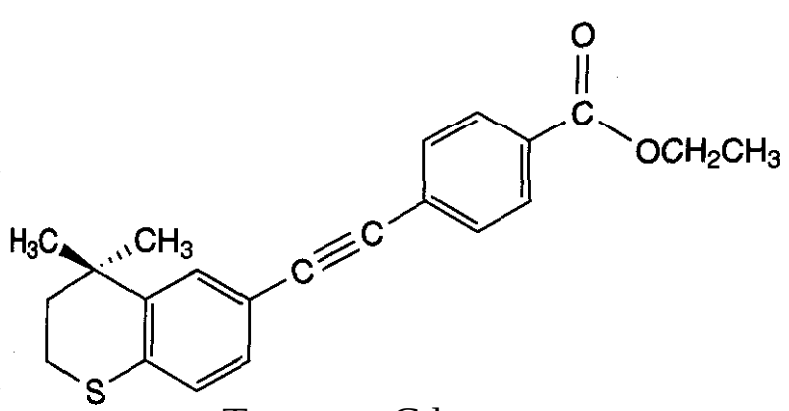


Adapalene (Dierin Gel™)

Figure 8.7, Retinoid and retinoid-like drugs used in the treatment of acne.



Etretinate (Tegison™); R = CH_2CH_3
Acitretin (Soriatene™); R = H



Tazarotene Gel

Figure 8.8. Drug based on the retinoid structure used to treat psoriasis.

the turnover of follicular epithelial cells. The result is decreased cohesiveness of follicular epithelial cells.

Tretinoin is also used as an antiwrinkle cream. There is disagreement on the mechanism and there may be more than one. Some improvement may be attributable to the irritation the drug causes. There is an increase in epidermal cell turnover, shedding older cells and thickening the skin. Also the drug may combine with epidermal retinoic acid receptors, thereby decreasing keratin production. Keratin can contribute to skin wrinkling (13).

Isotretinoin, 13-cis-retinoic acid, is very effective at treating severe forms of acne. It also is very teratogenic. There are elaborate procedures involving the prescribing physician and dispensing pharmacist before a female patient can receive the drug. There is also some concern that the sperm of men using the drug might be affected.

Although used topically, the nonretinoid, adapalene, does bind to the retinoic acid nuclear receptor and does affect cell differentiation, keratinization, and inflammatory responses.

3.1.8.2 Drug Based on the Retinoid Structure Used to Treat Psoriasis (Fig. 8.8). Etrinate is the ethyl ester of acitretin and is active after hydrolysis to the acidic drug. The "terminal" half-life after 6 months of etrinate therapy is 120 days. In contrast, the "terminal" half-life of acitretin is only 33–96 h. Both drugs are

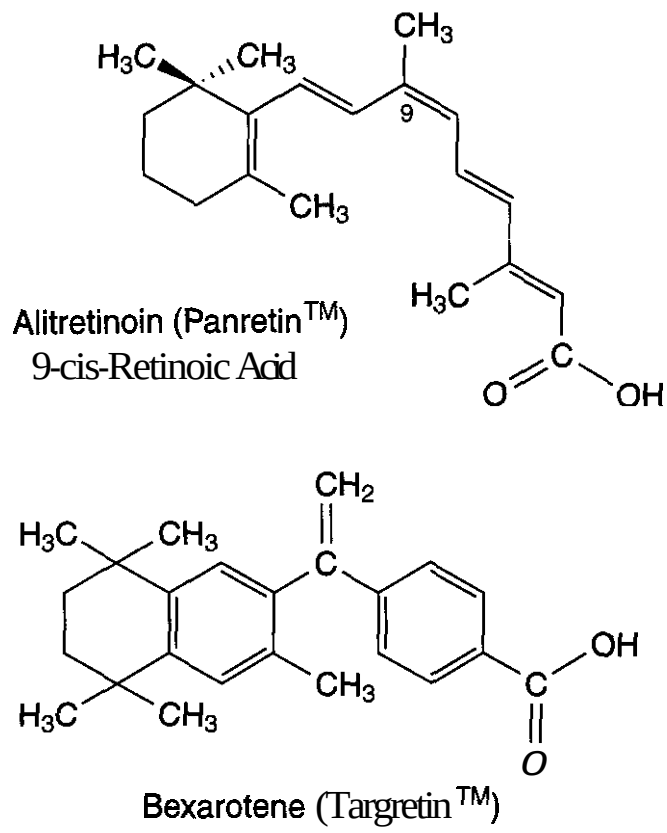


Figure 8.9. Retinoids used in the treatment of malignancies.

teratogenic and require elaborate warnings before being prescribed and dispensed. The third drug, tazarotene gel, is administered topically and is indicated for both acne and psoriasis. Although there appears to be minimal absorption if used over a limited skin area, there is some absorption, with retention by the body for up to 3 months. It also can cause fetal damage and cannot be used by pregnant women or women who may become pregnant.

3.1.8.3 Retinoids Used in the Treatment of Malignancies (Fig. 8.9). Retinoic acid has been evaluated as a possible treatment for malignancies. This is based on the fact that it is required for proper cell differentiation and products based on the **retinoid** structure are teratogenic. All-trans retinoic acid is used in combination with other chemotherapeutic agents for the treatment of acute promyelocytic leukemia (14). It does not **kill** the malignant cell, but seems to facilitate the cell's differentiation into a nonproliferating myelocyte. Alitretinoin, 9-cis-retinoic acid, is indicated for Kaposi's sarcoma and is administered topically. It binds to all six **retinoic** acid receptors.

Bexarotene is most selective for the three $RXR_{\alpha,\beta,\gamma}$ receptors. It also binds several other nuclear receptors including the RAR, vitamin D, thyroid, and **peroxisome** proliferator activator receptors, which probably explains its numerous adverse reactions. The drug is indicated for cutaneous T-cell lymphoma and is available as capsules and a topical gel.

3.2 Vitamin D Family (15)

Rickets was first reported in the mid-seventeenth century. It could be lethal, but bone deformation was more common. In the United States, rickets continued to be a problem into the 1930s until vitamin D–fortified milk became common. Older adults show bowlegs characteristic of childhood rickets. There were many attempts at giving calcium **and/or** phosphorous supplements. Finally, it was realized that rickets was not found in sunny climates, and populations whose main source of protein was fish did not have rickets. In 1924, Professor Henry Steenbock, University of Wisconsin, demonstrated that irradiation of foods, including milk, produced food that was antirachitic (16, 17).

When there is adequate sunlight, no dietary source of the vitamin is required. Indeed, an argument can be made that the calciferols are not normal components of the diet. In the United States. it is added to milk. other dairy products, and dairy substitutes. Fish is about the only natural food source. **Cholecalciferol** is produced in the body from endogenously **synthesized** 7-dehydrocholecalciferol (Fig. 8.10). Consistent with a hormone model, excess amounts of **cholecalciferol** can result in excess calcium uptake from the intestinal tract, leading to calcification of soft tissues.

3.2.1 Chemistry. There are two forms **of vitamin** D, and both are considered biologically equivalent. **Irradiation** of the major plant sterol, ergosterol, produces ergocalciferol, also known as vitamin D_2 (Fig. 8.11). Because they are photochemical reactions and in contrast to enzyme-catalyzed biochemical reactions, the formation of cholecalciferol is not clean. Exposure of human **skin** to sunlight of **295–300** nm converts 7-dehydrocholesterol to **previtamin** D_3. The **isomerization** to **cholecalciferol** (vitamin D_3) is heat catalyzed. Continuous exposure to ultraviolet radiation from the sun results in the reversible formation of lumisterol

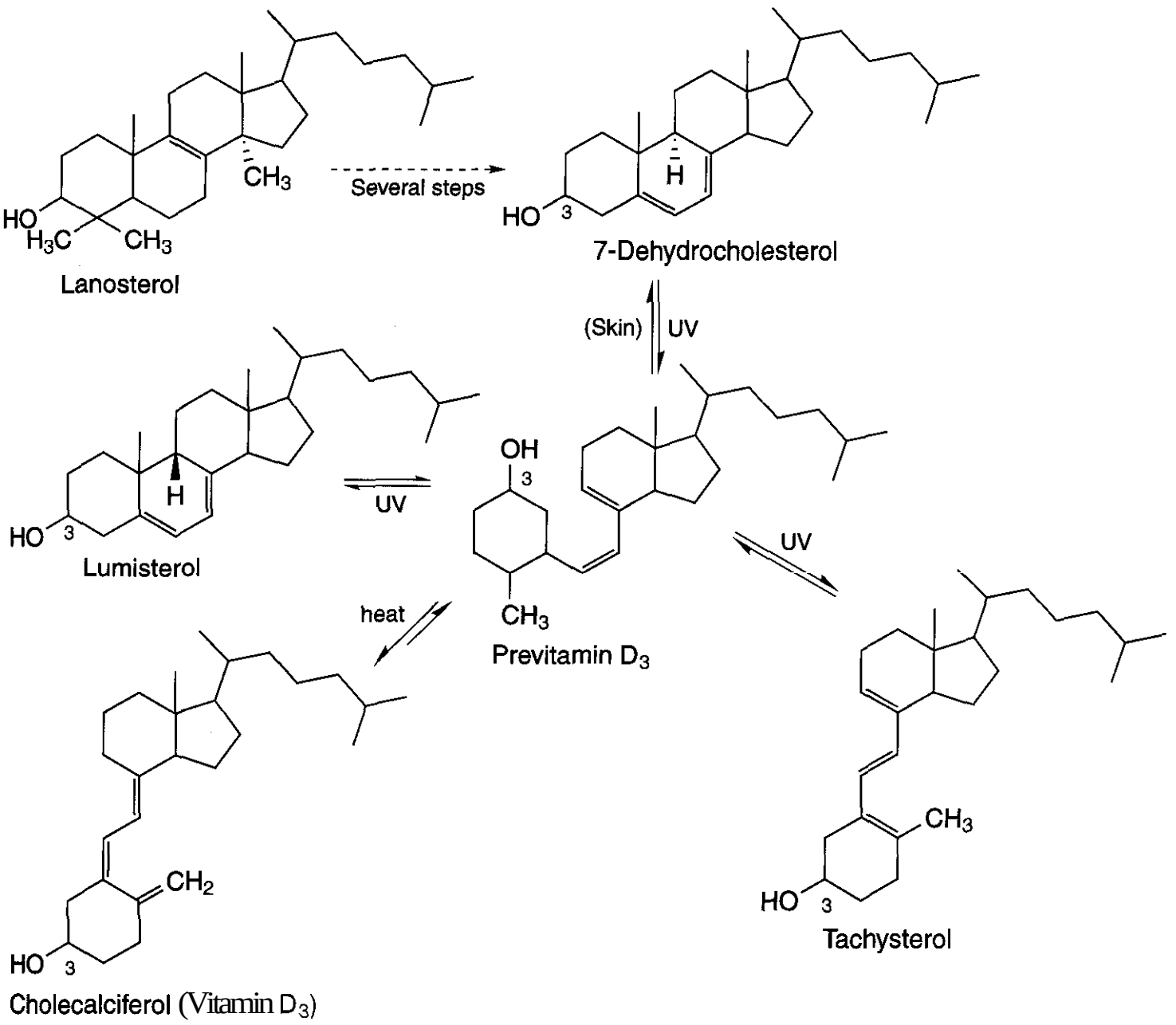


Figure 8.10. Photochemical formation of cholecalciferol (vitamin D_3).

and tachysterol (18, 19). Once the B-ring of the two steroids has been cleaved, the products should no longer be referred to as steroids. It is incorrect to call these compounds steroidal vitamins.

3.2.2 Vitamin Uptake and Metabolism. This is very complicated and is dependent on the source. From a biochemical viewpoint, it is a hormone because, in the presence of adequate sunlight, the cholecalciferol is produced from the 7-dehydrocholesterol in the skin. In this context D vitamins, when administered in supplements, could be considered replacement therapy. When photochemically synthesized in the skin, it is transported to the liver by a specific binding protein formed in the skin where the cytochrome enzyme system hydroxylates it to 25-OH-cholecalciferol (Fig. 8.12). This pulls the reversible reactions shown in Fig. 8.10 toward the desired cholecalciferol. Dependency on the binding protein for transport from the skin also provides a control to prevent overproduction of the hormone and possible hypercalcemia.

When taken orally, it follows the same route as that of other dietary lipids. It is part of the mixed micelles and ends up on the chylomicrons formed in the intestinal mucosa cells. It is then transported to the liver on the chylomicron remnants where, like the endogenously produced cholecalciferol from sunlight, it is hydroxylated to 25- OH-cholecalciferol.

The 25-hydroxylated product is then transported to the kidney where it is hydroxylated a second time by 25(OH)D-1 α-hydroxylase, forming the active 1,25-dihydroxycholecalciferol [$1,25(OH)_2D_3$]. The latter is transported to the intestinal tract, where it attaches to a nuclear receptor that signals the mucosa cell

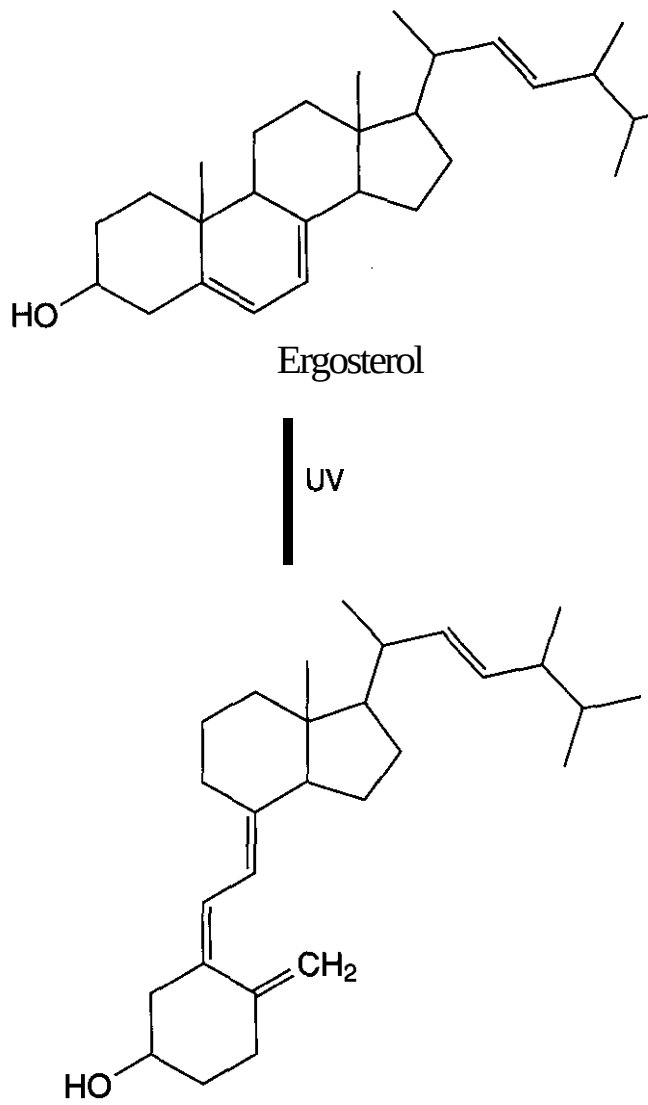


Figure 8.11. Formation of ergocalciferol from ergosterol.

to synthesize a calcium transport protein. The final $1,25(OH)_2D_3$ product can be considered a kidney hormone that regulates calcium intake. Finally, $1,25(OH)_2D_3$ is oxidized to inactive calcitroic acid that is excreted through the kidney. The $24,24(OH)_2D_3$ metabolite may be part of the degradation process for $25(OH)D_3$ that is not transported to the kidney, but it also can elevate serum calcium levels (16). Patients with kidney failure can experience vitamin D–resistant rickets. Because they cannot carry out the final hydroxylation step, $1,25(OH)_2D_3$ (calcitriol) is prescribed for their hormone replacement therapy.

3.2.3 **Biochemical Function.** Calciferol function is complex and, with the exception of calcium transport from the intestinal tract, is poorly understood. Specific vitamin D receptors (VDRs) are found in 30 different tissues including bone, intestine, prostate, hematopoietic cells, and skin (20). Like the retinoic acid receptors, the vitamin D receptor is a nuclear receptor belonging to the steroid hormone superfamily of nuclear receptors, which includes receptors for estrogen, glucocorticoids, thyroid hormones, and retinoic acid. There are at least 50 genes that respond to hydroxylated calciferols regulating calcium release and uptake and cell division.

3.2.3.1 Calcium Regulation. There are at least three hormones that regulate calcium metabolism, parathyroid (PTH), calcitonin, and $1,25(OH)_2$. Bone is the principal calcium reservoir and is a dynamic tissue, with calcium being released and deposited. New calcium comes from our diet, and excess serum calcium is excreted through the kidneys. In response to low serum calcium levels, PTH stimulates the hydroxylation of $25(OH)D_3$, leading to formation of calcium transport protein and activation of osteoclast cells required to release calcium from bone. PTH also inhibits its calcium excretion by the kidney. In contrast, calcitonin (produced in the thyroid gland) acts when serum calcium levels are high. It promotes the deposition of calcium into bone by osteoblast cells and excretion of calcium by the kidney.

Rickets in infants and children and osteomalacia, and possibly osteoporosis, in adults is caused by a deficiency of D vitamins. Today most deficiencies are caused by a lack of sunlight or restricted diet. The lack of sunlight may be caused by living in northern latitudes. The latter also can be affected by the amount of skin pigmentation (21–23). A strict vegetarian diet lacking cholecalciferol-fortified dairy products or fatty fish, particularly in children, may also result in rachitic lesions in the bone (24,25). Mechanistically, rickets and osteomalacia are similar and are characterized by bone softening. Normal bone growth and maintenance require that the osteoblast cells lay calcium hydroxyapatite onto a cartilage matrix. A deficiency of D vitamins results in *a* lack of mixed calcium salt available to the osteoblast cells. In infants and children, the cartilage continues to grow. Cartilage, being soft, cannot support the child's weight, leading to the typical bowlegs seen in a rachitic child. An

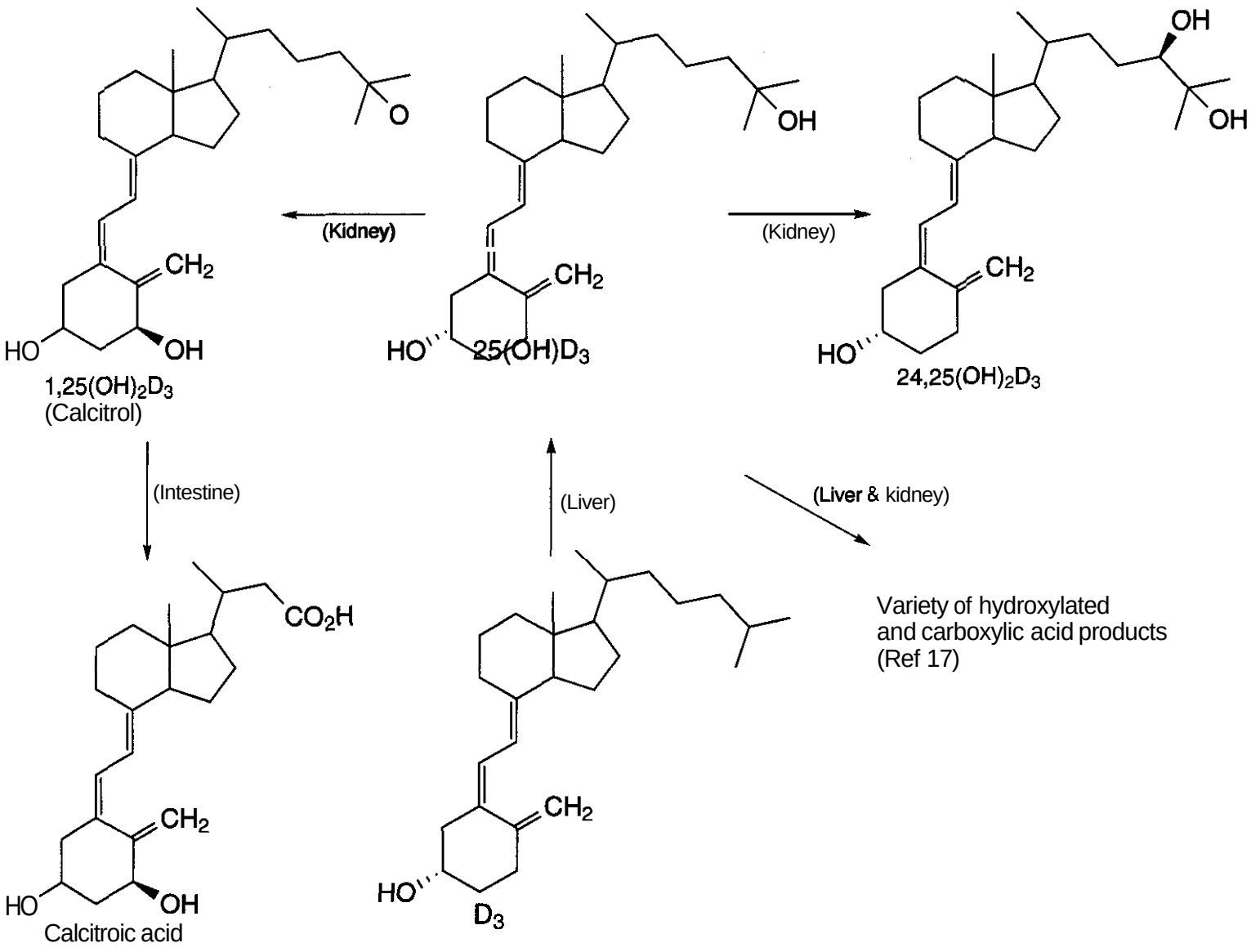


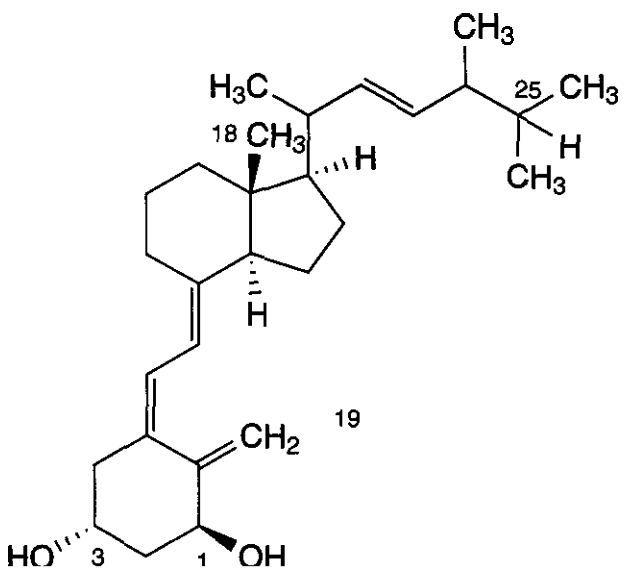
Figure 8.12. Metabolism of D vitamins.

adult also will have bone deformations, particularly in the pelvic area, because the bones cannot support the heavy upper torso.

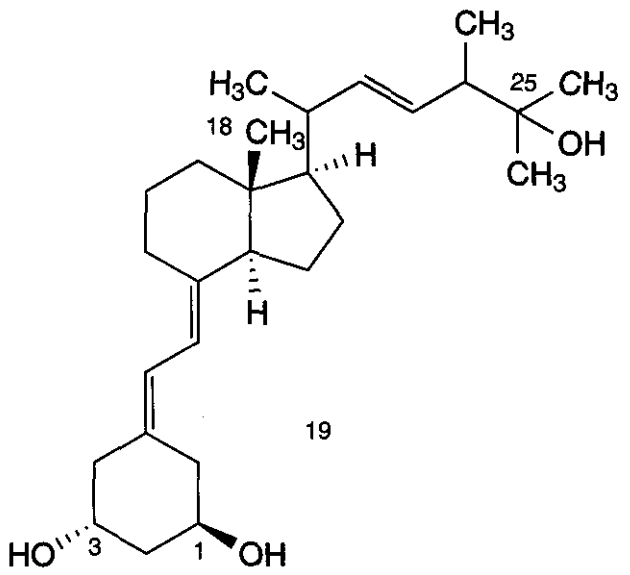
Osteoporosis is a different disease. It *can* be thought of as osteoclast cells removing calcium more quickly that osteoblast cells *can* lay calcium down. The result is porous, brittle bones that break easily. At one time calcitonin sometimes was prescribed to decrease the release of calcium from bone by osteoclast cells. In addition to the bisphosphonates and impact exercise, calcium with D vitamins is currently recommended to replace calcium being released from bone and excreted through the kidney.

3.2.3.2 Vitamin D Analogs Used in Chronic Renal Failure. Because $1.25(OH)_2D_3$ is produced in the kidney, renal failure leads to a deficiency of this hormone and vitamin D–resistant rickets. The solution is prescribing synthetic $1,25(OH)_2D_3$ (generic name: calcitriol). Nevertheless, these patients can experience hyperparathyroidism caused by the parathyroid gland attempting to compensate for the lack of $1,25(OH)_2D_3$. The result is increased osteoclast activity, leading to loss of calcium from the patient's bones, further resulting in hypercalcemia and either osteomalacia or osteoporosis. To overcome these complications, two synthetic ergocalciferol analogs (Fig. 8.13) are indicated for secondary hyperparathyroidism associated with chronic renal failure. Note that both compounds contain the hydroxy group at position 1, the position at which the kidney carries out its hydroxylation to produce the 1,3,25-trihydroxy product. Doxercalciferol is 1,3-dihydroxy-ergocalciferol and, in the liver, is hydroxylated to active $1,25(OH)_2D_2$ (the ergocalciferol analog of calcitriol). It is not clear why doxercalciferol is more selective than calcitriol. In contrast, paricalcitol is the 19-normethyl analog of $1,3,25(OH)_3D_2$ produced from ergocalciferol and requires no further hydroxylation reactions.

3.2.3.3 Cell Division. The role of $1,25(OH)_2$ D vitamins in regulating cell division is under active investigation. There are many reports

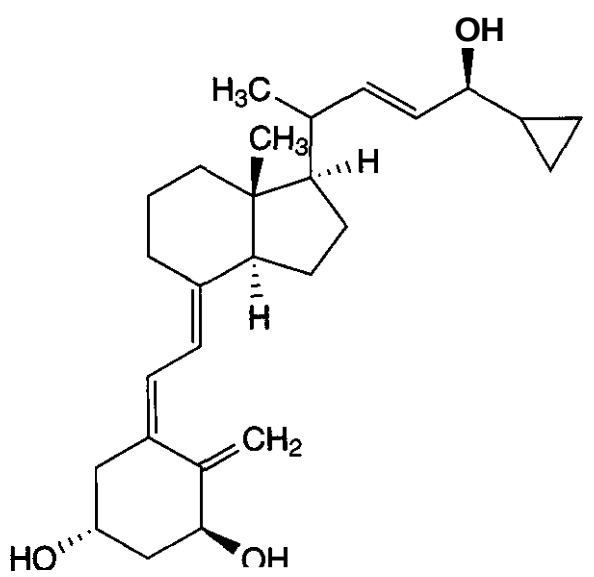


Doxercalciferol (Hectoral™)
1,3-dihydroxy-ergocalciferol



Paricalcitol (Zemplar™)
19-nor-1,3,25-trihydroxy-ergocalciferol

Figure 8.13. Ergocalciferol analogs indicated for chronic renal failure.



Calcipotriene (Dovenex™)

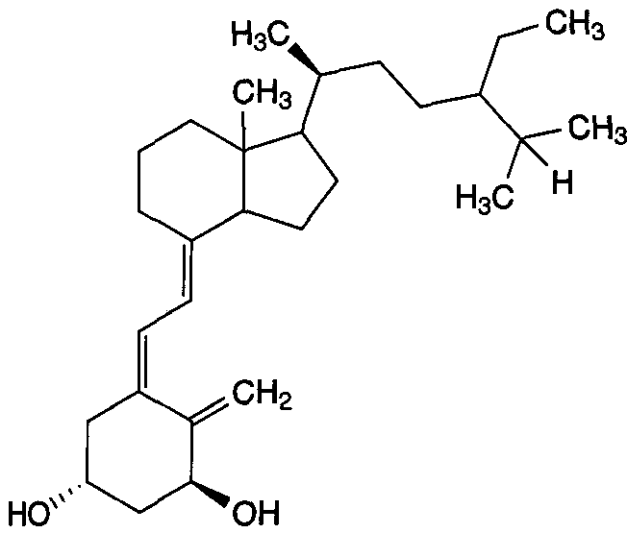
Figure 8.14. Calciferol analog indicated for psoriasis.

that indicate that vitamin D has a cancer-preventive role. Populations living in areas with higher exposure to sunlight have lower incidence of prostate, breast, and colon cancers (26).

In the intestine, the vitamin D receptor also functions as a receptor for lithocholic acid, a bile acid produced by the action of intestinal bacteria on endogenously produced chenocholic acid. Lithocholic acid is hepatotoxic and may be a carcinogen. In the intestine, vitamin D receptors, activated by vitamin D and possibly lithocholic acid, induce both liver and intestine cytochrome P450 3A oxidase (CYP 3A). This enzyme metabolizes lithocholic acid to inactive hydroxylated forms. The process of inducing CYP 3A might explain how vitamin D may exert its protective effect against cancer of the colon (27).

In contrast with other vitamins that are associated with reducing cancer risk and have minimal adverse reactions in high doses, such as a-tocopherol and ascorbic acid, taking larger doses of D vitamins can lead to clinically significant hypercalcemia. Therefore, the challenge is to develop compounds that not only are selective for receptors involved with controlling cell division but also will activate calcium transport leading to hypercalcemia. The initial analog on the market is calcipotriene, which is indicated for psoriasis, a nonmalignant proliferation of cells (Fig. 8.14). Its use is limited to topical application. When administered internally, hypercalcemia can result. Attempts have been made at pulse dosing of $1,25(OH)_2D_3$, to maximize inhibition of cell division with minimal calcemic activity (28).

Most of the calciferol analogs are based on modifications of the 17-alkyl side-chain. Modifications of the A-ring include 19-nor methylene and 3-nor hydroxy analogs with and without the 1-hydroxy moiety. The 1a-hydroxy-24-ethyl cholecalciferol analog (Fig. 8.15) was less calcemic in mice and inhibited the development of preneoplastic lesions in mammary glands (29). Unsaturation at position 16 (Fig. 8.16) provides modest antiproliferative effects on prostate cancer cells with little hypercalcemia (30). A series of 20-cyclopropyl-cholecalciferol analogs (Figs. 8.17 and 8.18) showed good activity against human prostate, breast, and myeloid leukemia cell

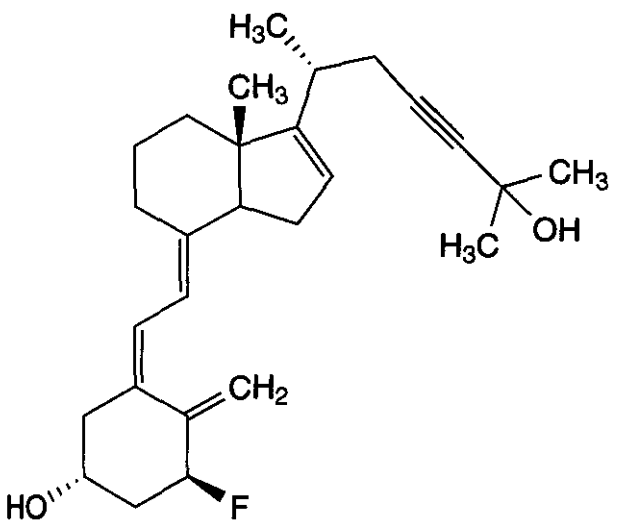


1-(OH)-24-ethyl-cholecalciferol

Figure 8.15. "1α-Hydroxyvitamin D,."

lines (31). The most potent in this series were the 19-nor methylene analogs, independent of whether the side-chain had ethylene or acetylene unsaturation. Modification of the side chain with a 25 keto or oxime (R_1 = O or NOH, respectively), with or without an additional hydrogen or methyl (R_2 = H or CH_3, respectively), produced analogs as antiproliferative *in vitro* as $1,25(OH)_2D_3$ (Fig. 8.19). The oximes were less calcemic (32).

3.2.4 **Dosage Forms.** The commercial products are produced by irradiation of 7-dehydrocholesterol or ergosterol under controlled conditions. The final yield is about 50%. Although more stable than vitamin A, D vitamins are sensitive to oxygen and tend to isomerize into inactive isomers in the presence of trace metals, which can cause problems in formulating a vitamin/mineral supple-



1,25-$(OH)_2$-16-ene

Figure 8.16. 16-ene-23-yne D_3 analogs.

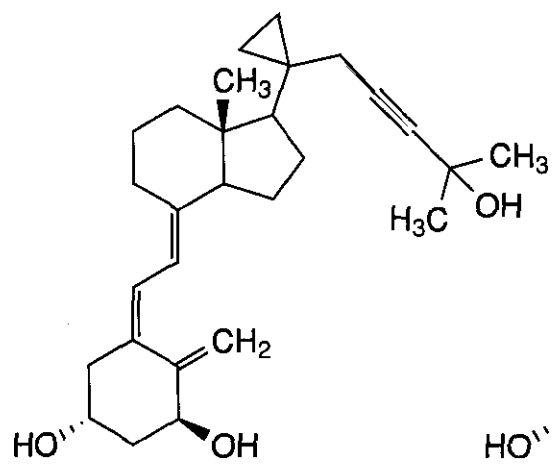
ment. It is stabilized with antioxidants and protective coatings.

3.2.5 Hypervitaminosis D. Think of a vitamin D overdose in the same way as an oversupply of any hormone. The role of the hormone is exaggerated or magnified. Hypervitaminosis D causes increased absorption of calcium and phosphorous (P follows Ca), leading to calcification of the tissues, vomiting, kidney damage, and so forth. It can be the most serious of the hypervitaminoses. The reports that D vitamins inhibit proliferation of tissue and may protect against certain cancers could cause the public to overdose with this vitamin. The calciferols have Tolerable Upper Intake Levels (UL) with UL to RDA ratios of 5 to 10.

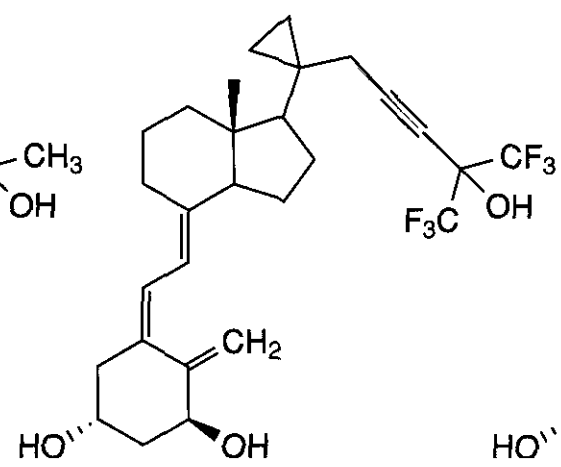
3.2.6 Dietary Reference Intakes. There is no RDA for this vitamin. It would be difficult to derive a RDA for a population because a significant percentage will receive adequate amounts of the vitamin from sunlight based on the area of the country where they live. On the other hand, the diverse population of the United States means that people of color may need more of the vitamin from fortified milk relative to those whose ancestors came from Northern Europe.

AI	
Infants (0–12 months)	5 μg (200 IU)/day
Children (1–8 years)	5 μg (200 IU)/day
Boys (9–18 years)	5 μg (200 IU)/day
Girls (9–18 years)	5 μg (200 IU)/day
Men (19–50 years)	5 μg (200 IU)/day
Women (19–50 years)	5 μg (200 IU)/day
Men (51–70 years)	10 μg (400 IU)/day
Women (51–70 years)	10 μg (400 IU)/day
Men (70+ years)	15 μg (600 IU)/day
Women (70+ years)	15 μg (600 IU)/day
Pregnancy	5 μg (200 IU)/day
Lactation	5 μg (200 IU)/day
UL	
Infants	25 μg (1000 IU)/day
Children (1–18 years)	50 μg (2000 IU)/day
Adults (over 19 years)	50 μg (2000 IU)/day
Pregnancy	50 μg (2000 IU)/day
Lactation	50 μg (2000 IU)/day

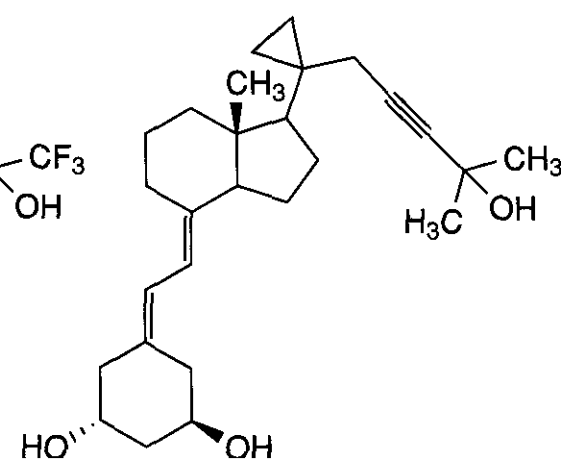
3.2.7 Drug Interactions. Phenobarbital and possibly other anticonvulsants used in epi-



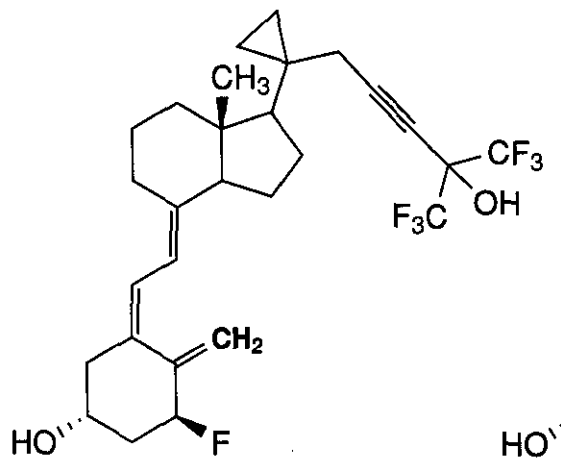
1,25-$(OH)_2$



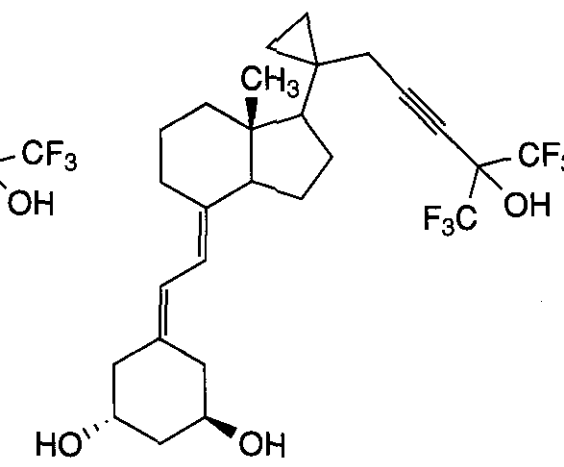
1,25-$(OH)_2$-25,27-F_6



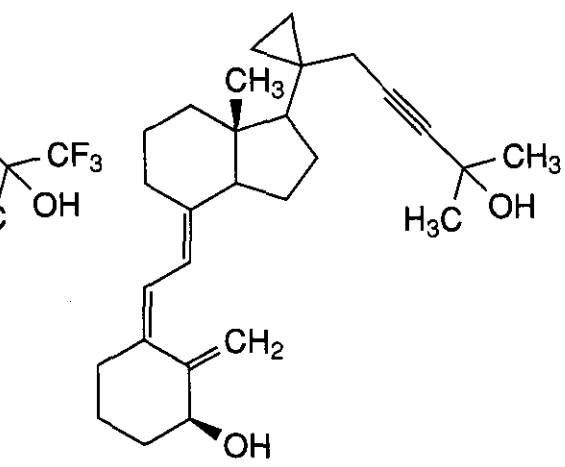
1,25-$(OH)_2$-19-nor



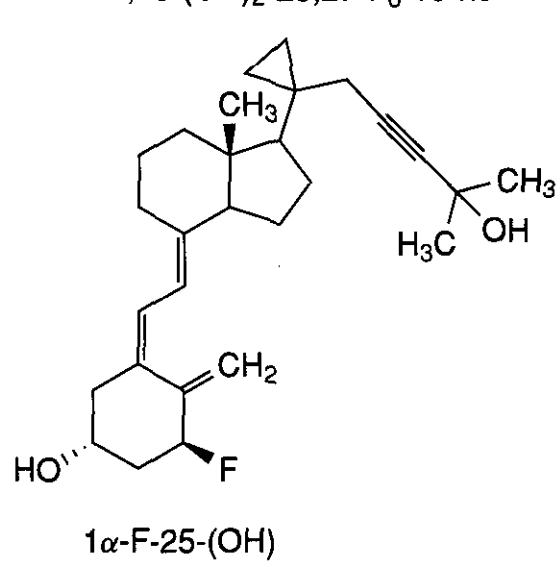
1α-F-25-(OH)-26,27-F_6



1,25-$(OH)_2$-25,27-F_6-19-nor



3-Desoxy-1,25-$(OH)_2$



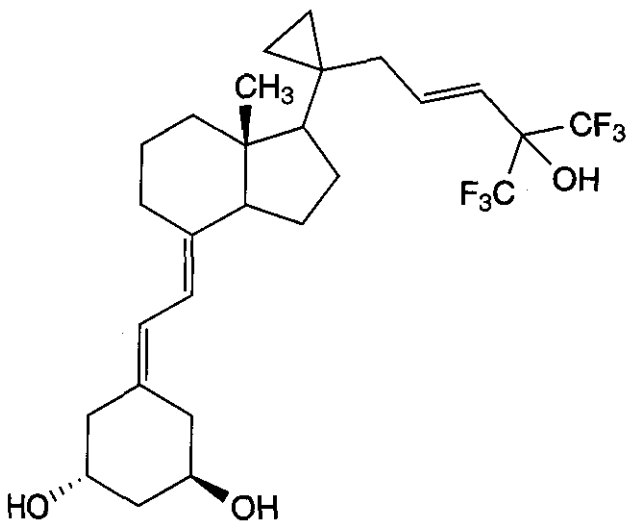
1α-F-25-(OH)

Figure 8.17. 23-yne-20-cyclopropylD_3 analogs.

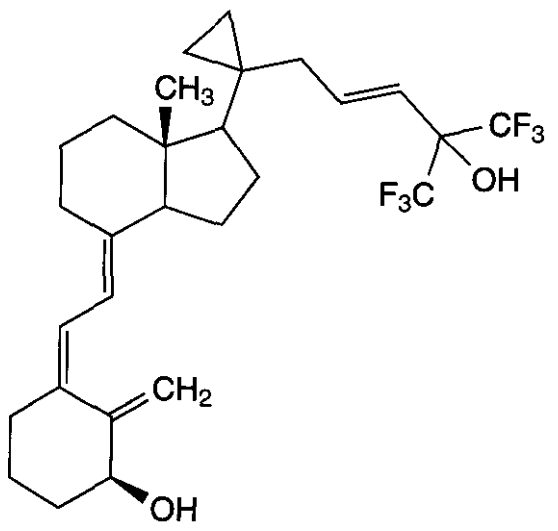
lepsy induce liver hydroxylation, leading to subsequent formation of the inactive end products. **As** long as the epileptic child receives a normal amount of fortified milk, there is no problem with this interaction.

3.3 Vitamin E Family (Tocopherols and Tocotrienols) (33)

This vitamin group was discovered in rat feeding experiments, which resulted in these animals not being able to produce offspring. These same rats seemed normal in all other respects including physical growth. The condition could be corrected by addition of lettuce, whole wheat, and cereal grains, with the best source being wheat germ followed by vegetable oils. Think of the tocopherols and tocotrienols (Table **8.3)** as nature's antioxidants. Most of the vitamin activity is found in a-tocopherol. "Tocopherol" means child-bearing alcohol.



1,25-$(OH)_2$-26,27-F_6-19-nor



3-Desoxy-1,25-$(OH)_2$-26,27-F_6

Figure 8.18. 23-E-ene-20-cyclopropyl D_3 analogs.

3.3.1 Chemistry. The vitamin E family consists of tocopherols and tocotrienols. There has been considerable debate as to whether the RRR isomer is the only biologically active form of the vitamin. The vitamin of commerce is α-tocopherol, either as a racemic synthetic

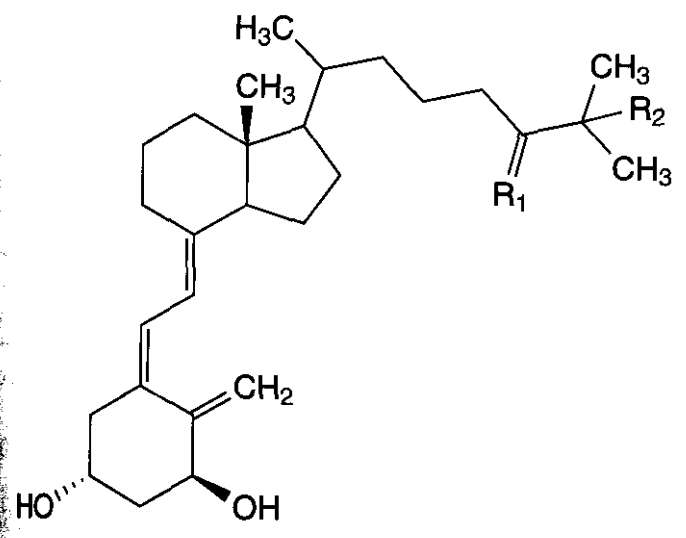


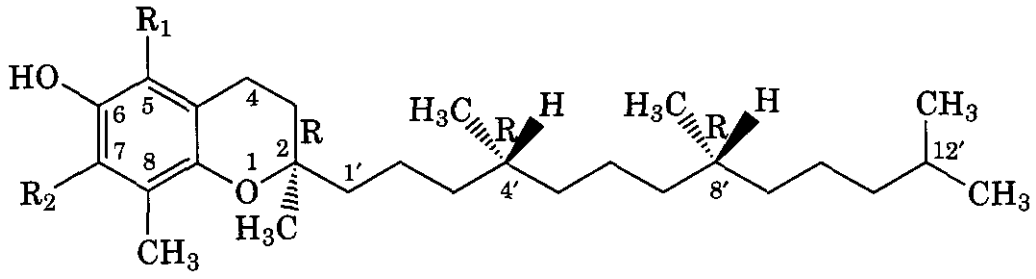
Figure 8.19. Keto and oxime cholecalciferol analogs.

mixture or the natural RRR-a-tocopherol. All of the tocopherols found in food have the R configuration at position **2.** The synthetic form of the vitamin is a mixture of all eight stereo isomers (now referred to as *rac*-α-tocopherol rather than *d,l*-α-tocopherol). Because there is both general and stereospecific antioxidant activity, the RDA tables state that the activity ratio of *RRR*-α-tocopherol to *rac*-a-tocopherol is 1 to 1.36 (Table 8.4) (34). All isomers are antioxidants and probably do provide general antioxidant protection internally or in *vivo*. On the other hand, evidence points to the RRR isomer as being specific for a variety of reductase systems, possibly involving selenium (Se) and glutathione (GSH/GSSG). The "Relative Biological Activity" in Table 8.3 is based on a rat resorption/gestation assay, as are the conversion factors in Table 8.4.

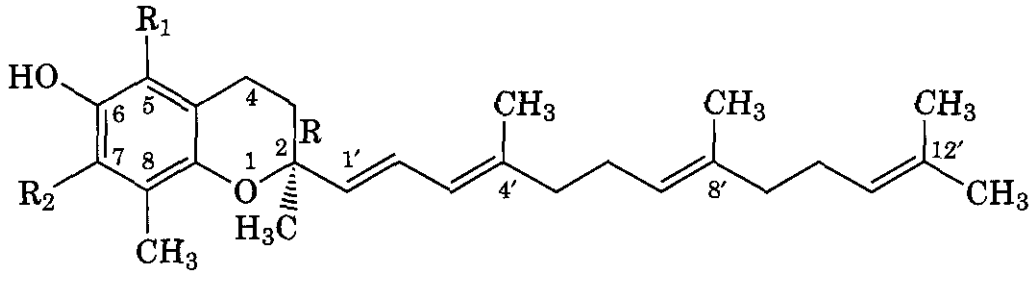
3.3.2 Uptake and Metabolism. Like the retinol esters, tocopherol, both esterified and nonesterified, requires bile salts to become part of the mixed micelles containing the dietary lipids. They are absorbed into the mucosal cell by passive diffusion. The tocopherols follow the dietary lipids onto the chylomicrons. Because the latter first enter the lymphatic system before the circulatory system, the tocopherols do not go directly to the liver. Instead, the chylomicrons are distributed throughout the body, and some of the tocopherols enter the adipose tissue with the fatty acids that were also being transported on the chylomicrons. The chylomicron remnants finally reach the liver. The remaining tocopherols leave the liver on the very low density lipoproteins (VLDL), which become the low density lipoproteins (LDL). In other words, tocopherols will be found wherever there is a significant amount of lipid material, including the high density lipoproteins (HDL). There is no specific organ where the tocopherols are stored. Consistent with antioxidant model, the RDA is based on the polyunsaturated fatty acid (PUFA) consumption. The implication is that, if increased PUF'A intake is recommended, the RDA for tocopherol should be increased.

There is increasing evidence that the RRR stereoisomer is preferentially transferred in the liver onto the lipid transport proteins, but it is not absolute. This could explain why

Table 8.3 Relative Biological Activities of Tocopherols and Tocotrienols[a]



Tocopherols	R_1	R_2	Relative biological activity[b]
α-Tocopherol	CH_3	CH_3	1
β-Tocopherol	CH_3	H	0.5
γ-Tocopherol	H	CH_3	0.1
δ-Tocopherol	H	H	0.03



Tocotrienols	R_1	R_2	Relative Biological Activity[b]
α-Tocotrienol	CH_3	CH_3	0.3
β-Tocotrienol	CH_3	H	0.05
γ-Tocotrienol	H	CH_3	Inactive
δ-Tocotrienol	H	H	Inactive

"Dietary Reference Intakes for Vitamin C, Vitamin E, Selenium and Carotenoids, Food and Nutrition Board, Institute of Medicine, National Academy Press, 2000, p. 193.

[b]Rat fetal reabsorption assay.

Table 8.4 Factors for Converting International Units of Vitamin E to a-Tocopherol (mg) to Meet Recommended Intake[a]

	USP conversion factors[b]		Molar conversion factors	a-Tocopherol conversion factors
	(IU/mg)	(mg/IU)	(μmol/IU)	(mg/IU)
Synthetic vitamin E and esters[c]				
d,l-α-Tocopherol acetate	1.00	1.00	2.12	0.45
d,l-α-Tocopherol succinate	0.89	1.12	2.12	0.45
d,l-α-Tocopherol	1.10	0.91	2.12	0.45
Natural vitamin E and esters[d]				
d-a-Tocopherol Acetate	1.36	0.74	1.56	0.67
d-a-Tocopherol Succinate	1.21	0.83	1.56	0.67
d-a-Tocopherol	1.49	0.67	1.56	0.67

"Dietary Reference Intakes for Vitamin *C,* Vitamin E, Selenium and Carotenoids, Food and Nutrition Board, Institute of Medicine, National Academy Press, 2000, p. 192.

[b]The United States Pharmacopeia (USP) has defined one IU as 1 mg of all racemic a-tocopherol acetate based on a 1940s rat fetal resorption assay.

"Synthetic vitamin E supplements labeled as *d,l*-α-tocopherol can consist of all eight possible isomers (*RRR*-, RSR-, RRS-, RSS-, SSS-, SRS-, *SRR*-, and *SSR*-).

[d]*d*-α-Tocopherol refers to the RRR-isomer, the only one found naturally in foods, and the other two R stereoisomers (*RRS*-, RSR-, and *RSS*).

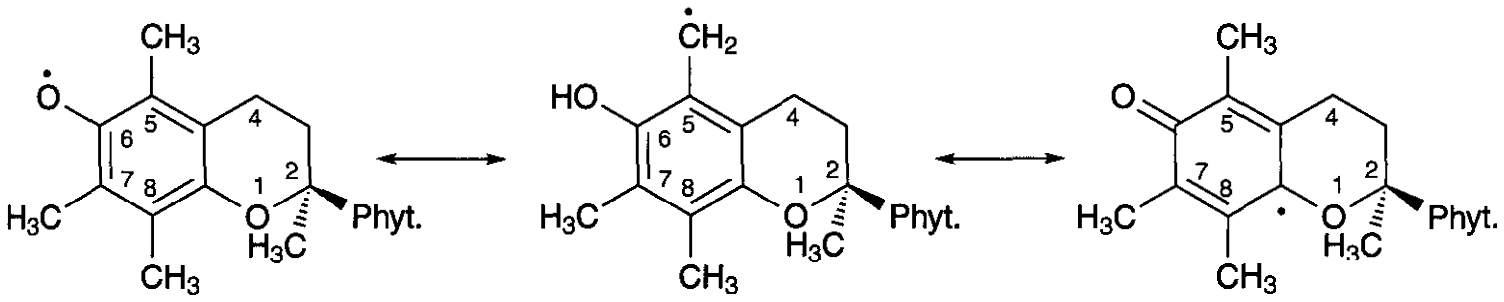


Figure **8.20.** Resonance-stabilized tocopherol radicals.

RRR-α-tocopherol is more active *in vivo*, but the other isomers are also active.

3.3.3 Biochemical Function. The best way to describe tocopherol's role is that of a lipid-soluble antioxidant. It protects unsaturated lipids from oxygen-induced peroxide formation. There is evidence for both free-radical one-electron chemistry (Fig. 8.20) and two-electron quinone-hydroquinone chemistry (Fig. 8.21) **(35).** The oxidized/reduced glutathione system may be part of the system that regenerates reduced a-tocopherol. At one time it was thought that the preference for the 2-*R* stereoisomers indicated that the vitamin was part of a biochemical oxidation/reduction system, possibly as a coenzyme. So far that role for α-tocopherol has not been found. The current evidence points to the hepatic tocopherol transfer protein's preference for the RRR stereoisomer as the explanation for the partial steric preference.

3.3.4 Deficiencies. The current model for the cause of vitamin E deficiencies points to malabsorption of lipids. Thus, there may be malabsorption of other lipid-soluble vitamins. Little is known regarding the pharmacokinetics of the tocopherols. Part of the reason for this may be attributed to lack of a specific storage organ for the vitamin.

Correlating human medical conditions with the biochemical role of the tocopherols is difficult because of the lack of correlations between deficiency diseases seen in animals relative to what is seen in humans. Deficiency diseases seen in animals include reversible re-

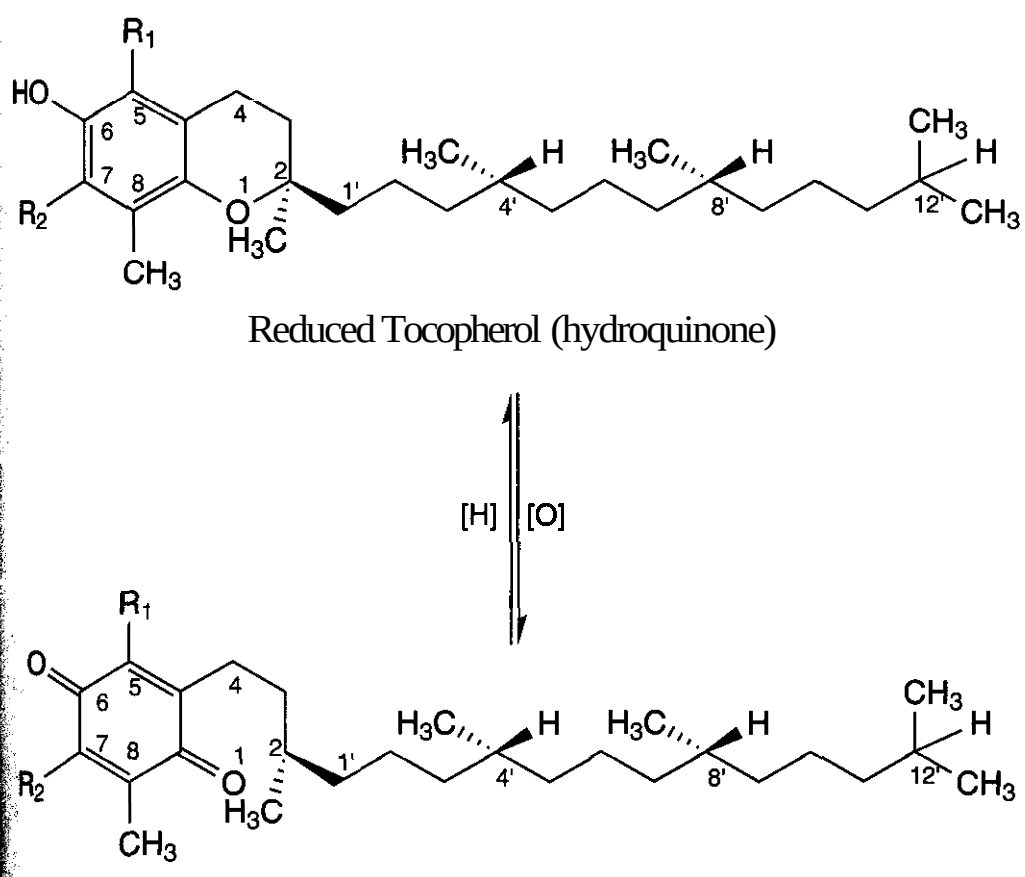
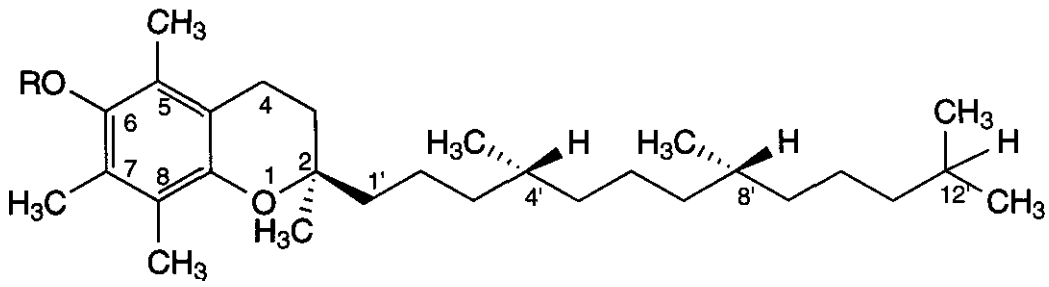


Figure 8.21. Tocopherol oxidation/reduction.



R = acetate, hemisuccinate (sodium salt)

Figure 8.22. a-Tocopherol dosage forms.

productive failure in female rats; irreversible degeneration of rat testicular tissue leading to male sterility; nutritional muscular dystrophies in monkeys, rabbits, guinea pigs, lambs, calves, turkeys, and chicks; and an anemia in monkeys. Vitamin E does not treat human muscular dystrophy nor the various causes preventing a couple from conceiving a child or inability of pregnant women to go to full term. What is seen in humans is a partially reversible set of neurological problems and hemolytic anemia in premature infants. Because of poor placental transfer, newborns have little of the vitamin. Human milk contains 2–5 mg/L; cow's milk contains less.

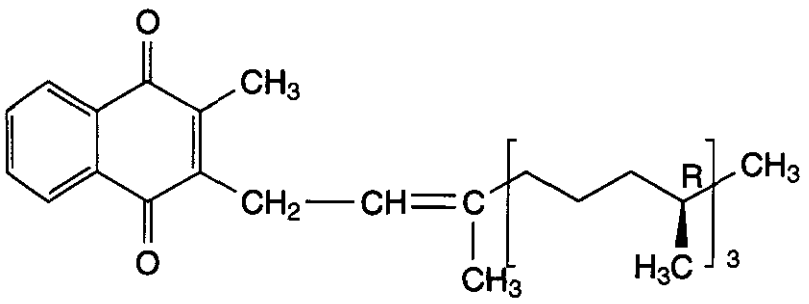
There are numerous studies evaluating the possible role of tocopherols in preventing and/or treating cardiovascular disease, malignancies, diabetes mellitus, cataracts, immune function, and Alzheimer's Disease. In all of these conditions, there is evidence of free-radical formation or general oxidation mechanisms as part of the disease process. The evidence for taking supplements in addition to proper diet is mixed (36, 37).

3.3.5 Hypervitaminosis E. This is a relatively safe vitamin. Toxicities have been reported involving chronic administration of 300–1200 mg per day. The symptoms can be very serious and include thrombophlebitis, pulmonary embolism, hypertension, breast development in men and children, severe fatigue, and nonmalignant breast tumors. Nevertheless, the UL to RDA ratio is about 66 to 1 for adults, making it a very safe vitamin.

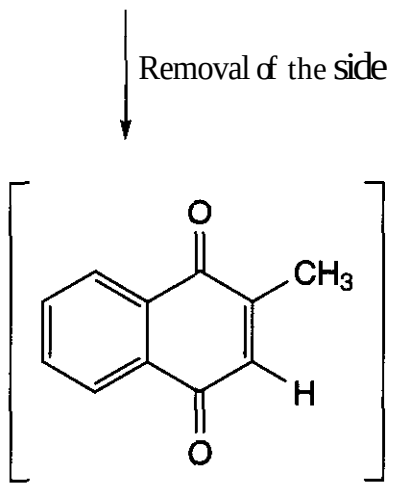
3.3.6 Dosage Forms. The tocopherols, being antioxidants, are very sensitive to oxygen. Sensitivity to UV light is another problem. There are two provitamin esters that are used commercially (Fig. 8.22), the oil-soluble acetate and water-soluble hemisuccinate. The latter is commonly found in dry dosage forms requiring a free-flowing powder. Oxidation to the quinone form is blocked by esterifying the free phenolic hydroquinone.

3.3.7 Dietary Reference Intakes (based on d-a-Tocopherol).

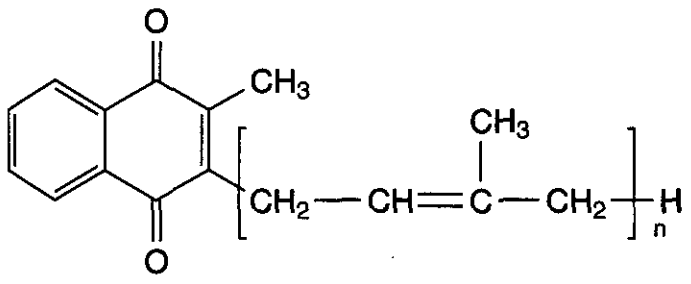
AI	
Infants (0–12 months)	0.6 mg/kg^{-1}/day^{-1}
EAR	
Children (1–8 years)	5–6 mglday
Boys (9–18 years)	9–12 mg/day
Girls (9–18 years)	9–12 mg/day
Men (19–50 years)	12 mglday
Women (19–50 years)	12 mg/day
Men (51–70+ years)	12 mg/day
Women (51–70+ years)	12 mg/day
Pregnancy	12 mglday
Lactation	16 mglday
RDA	
Children (1–8 years)	6–7 mg/day
Boys (9–18 years)	11–15 mglday
Girls (9–19 years)	11–15 mglday
Men (19–50 years)	15 mglday
Women (19–50 years)	15 mg/day
Men (51–70+ years)	15 mg/day
Women (51–70+ years)	15 mglday
Pregnancy	15 mglday
Lactation	19 mg/day
UL	
Infants	Not established (Do not give supplements; use only food and formula for sources.)
Children	200 mg/day (1–3 years) up to 600 mg/day (9–13 years)



Phytonadione (Vitamin K_1; phylloquinone)



Menadione



Vitamin K_2 (n = 4; menoquinone-4)

Figure 8.23. Formation of menoquinone form of vitamin K.

Adolescents	800 mg/day
Adults (19+ years)	1000 mg/day
Pregnancy	800–1000 mg/day
Lactation	800–1000 mg/day

3.4 Vitamin K Family (38)

Vitamin K was discovered by accident by Danish scientists who, using a special fat-free diet designed to determine whether chickens synthesize cholesterol, observed that the animals developed a hemorrhagic condition characterized by a prolonged clotting time. The condition could be cured by an organic factor found in fresh cabbage, ether extract of alfalfa, purified fish meal, cereals, or hog livers. It was named *Vitamin* K for *koagulation* vitamin. This may be the only vitamin that humans receive in significant amounts from their intestinal bacterial, and there is some question regarding this commonly held assumption. Because of this source, it has been very difficult to establish a recommended daily allowance. An estimated safe intake was established for this vitamin for the first time with the 1989 RDA tables. With the release of the Dietary Reference Intakes, there is an adequate intake, but no RDA.

3.4.1 Chemistry. There are two series for this vitamin (Fig. 8.23). The vitamin K_1 series is mostly obtained from green plants, whereas the K_2 series is the product of bacteria. The active vitamin is in the K_2 series. Menadione has sometimes been referred to as vitamin K_3.

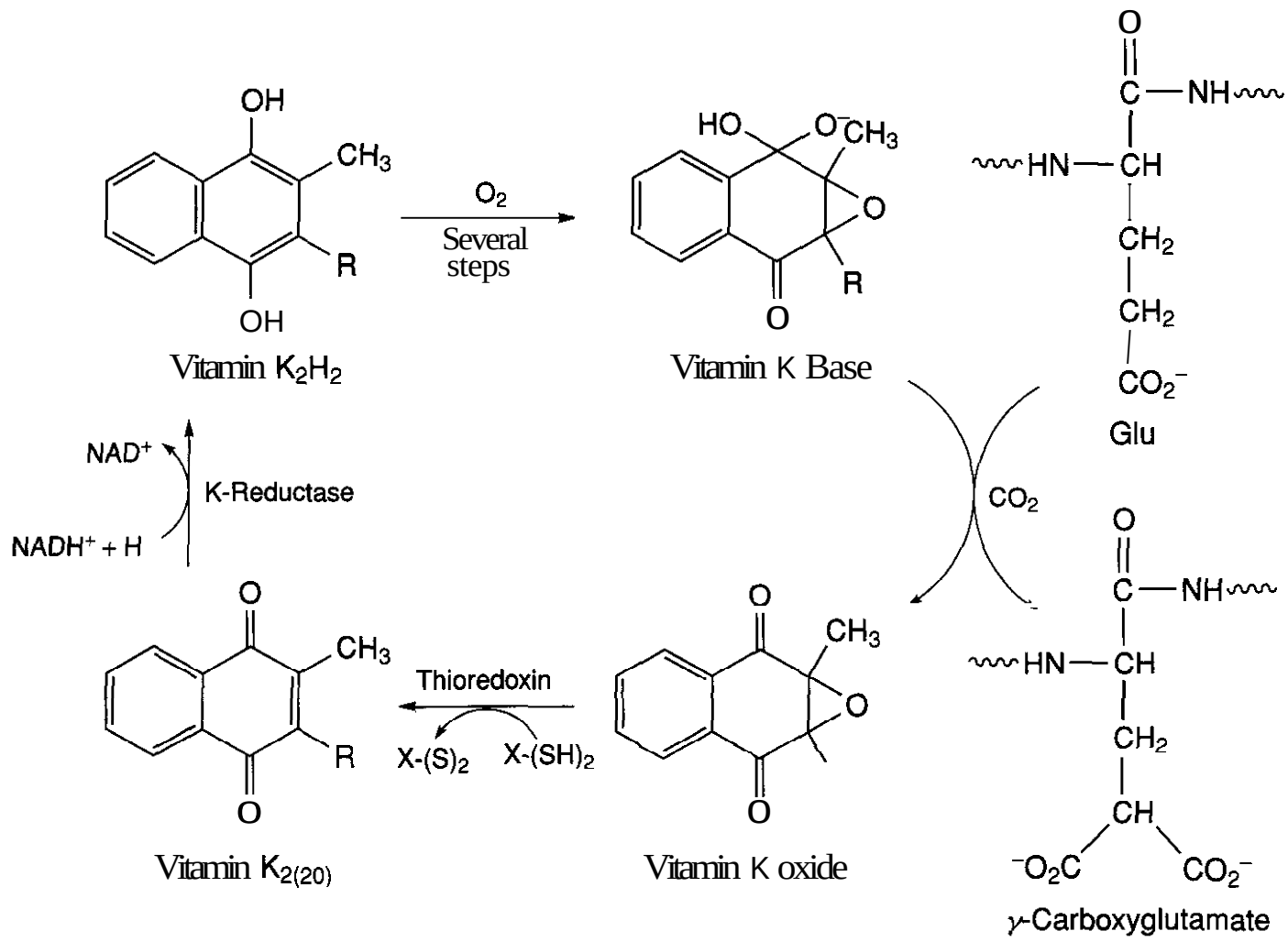


Figure 8.24. Outline **of** vitamin K in **carboxylation of** glutamic acid.

The common commercial form is called **phytonadione** in the United States Pharmacopeia and phylloquinone by Chemical Abstracts.

3.4.2 Vitamin K Uptake and Metabolism. Dietary vitamin K_1 and the pharmaceutical form, phytonadione or vitamin $K_{1(20)}$ must be converted to the K_2 series known as menoquinones. The most common of these is menoquinone-4 or $K_{2(20)}$. This conversion to the K_2 series occurs in the liver and possibly the intestinal flora. It involves removing the **phytyl** chain producing the intermediate **menadione**. Menadione sometimes is prescribed when there is impaired uptake of lipids from the intestine. There is little storage reserve in the liver, and a deficiency can result when dietary intake of vitamin K is restricted or absorption is impaired.

Dietary vitamin K and supplements are processed similarly as **with** the other **fat-soluble** vitamins. Bile salts are required for emulsification and formation of mixed micelles. They travel to the liver on chylomicrons along with vitamins **A** and E.

3.4.3 Vitamin K Biochemistry and Deficiency. Deficiencies of this vitamin lead to serious hemorrhaging. The vitamin is required for formation of proteins that complex calcium. This is done by functioning as a coenzyme in the y-carboxylation of glutamic acid (Fig. 8.24). Vitamin K_2 is reduced to the hydroquinone. After several steps, a complex oxidation occurs, resulting in the "vitamin K base" that is an integral part of the **carboxylation** step. In a key step, the **vitamin** K oxide is reduced to the original vitamin K_2. It is this final reduction that is inhibited by the **coumadin** anticoagulants widely used by patients susceptible to stroke, pulmonary embolism, phlebitis, and coronary thrombosis. This interaction between the coumadin anticoagulants and the regeneration of vitamin K_2 is the reason that patients on coumadin must monitor their vitamin K intake both from vitamin supplements and diet. This usually is done by regularly scheduled determinations of **prothrombin** time.

The carboxylation reaction is required for production of several clotting proteins **includ-**

ingprothrombin, protein C, protein S, and factors VII, IX, and X. It is also required for γ-carboxylation of osteocalcin, an important calcium-binding protein found in the matrix of bone and required for proper deposition of calcium onto bone. This latter finding has led to several studies to determine whether patients prescribed coumadin anticoagulants are at increased risk for osteoporosis and fractures (39–42). All of these studies indicate that vitamin K supplementation might be beneficial for the prevention of osteoporosis. If vitamin K helps prevent osteoporosis, then it would appear that patients on anticoagulant therapy would be at increased risk for bone fractures. There is some indication of this, but it is not conclusive. Nevertheless, some calcium supplements have both vitamins D and K added to their formulation.

3.4.4 Causes of Vitamin K Deficiency. Rarely is a vitamin K deficiency caused by insufficient diet. It more likely is attributable to a medical condition. At one time, multivitamin supplements rarely contained vitamin K. It is now routinely found in these products. Causes of vitamin K deficiency include obstructive jaundice (now uncommon), loss of intestinal flora in preparation for intestinal surgery, and hemorrhagic disease of the newborn.

Deficiencies caused by obstructive jaundice were caused by blockage of the bile duct, usually from cholelithiasis, preventing the release of bile salts into the intestinal tract for emulsifying the lipid contents. At one time, surgical removal of gallstones was delayed to see whether the problem would correct itself. Menadione would be prescribed because it does not require micelle formation, given that it is directly absorbed through the mucosa into the portal vein and flows to the liver. Alternatively, intramuscular injections of phytonadione were given.

In nonemergency surgery of the intestinal tract, the patient would undergo 1–2 weeks' antibiotic therapy to reduce the level of intestinal bacteria. Usually the patient did not eat well because of the intestinal problem. The combination of reduced intake of dietary vitamin K and vitamin from the intestinal bacteria could result in a vitamin K deficiency. Determination of prothrombin time is ordered by the surgeon to determine whether menadione or phytonadione is indicated.

The third cause is hemorrhagic disease of the newborn. Infants are born with a sterile intestinal tract. Until the flora are established, the infant will have to get along with the vitamin K they received from the mother. In the past an infant might die from hemorrhaging. Most states require that each newborn receive an injection of phytonadione. Menadione injection is not given because it can cause a hemolytic anemia in the newborn.

3.4.5 Hypervitaminosis K. Although it is possible to overdose with this vitamin, the fact that it is available only over the counter in small doses in multivitamin preparations has resulted in little knowledge of any toxicities. Toxicities do not appear in animals administered large doses. It is known that excess intake of the vitamin does not promote clot formation. There is no Tolerable Upper Intake Level.

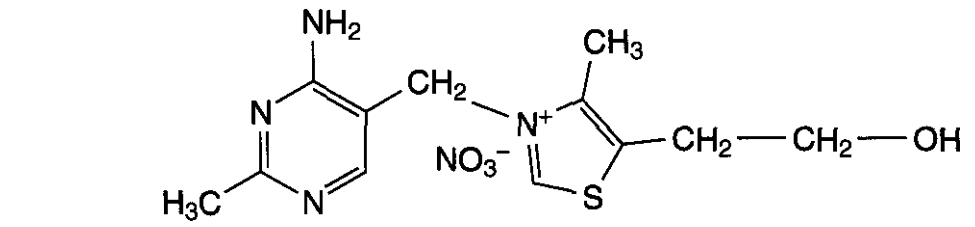
3.4.6 Dietary Reference Intakes.

AI	
Infants	2–2.5 μg/day
Children (1–8 years)	30–55 μg/day
Boys and Girls (9–18 years)	60–75 μg/day
Men	120 μg/day
Women	90 μg/day
Pregnancy	75–90 μg/day
Lactation	75–90 μg/day

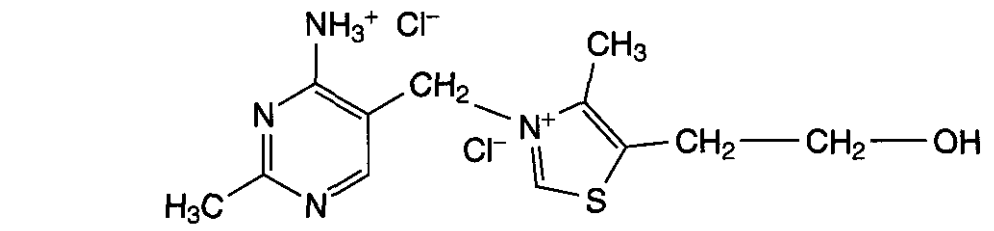
3.5 Thiamine (Vitamin B_1) (43)

> ***After 26 years of constant research, the vitamin preventative of the disease beri-beri has been isolated, its chemical constitution determined and the vitamin itself synthesized at a cost far lower than that of recovering it from bran.*** (*Scientific American,* February 1938; reprinted in **258,** 12 February 1988)

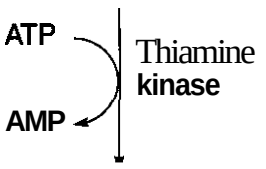
This quote summarizes the debate between those who believed that beriberi was caused by an infectious agent vs. those who promoted proper diet. Some of the early researchers oscillated between the two theories. About 1912 there were enough definitive feeding experiments in humans to conclude that beriberi's origin was dietary (44).

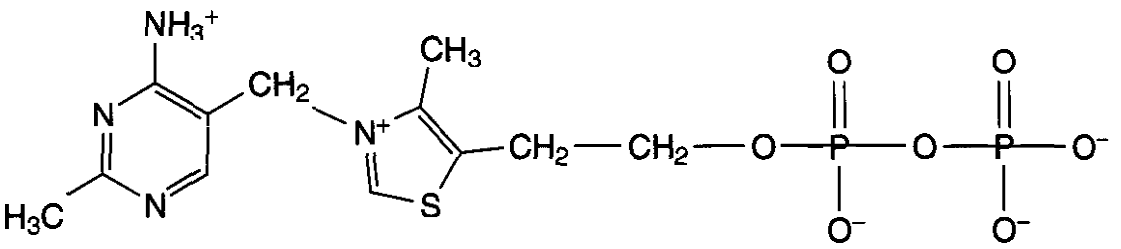


Thiamine Mononitrate



Thiamine HCl
(Thiamine Chloride HCl)





Thiamine Pyrophosphate

Figure 8.25. Commercial forms of thiamine and formation of thiamine pyrophosphate.

The vitamin B complex, of which thiamine is considered the first one discovered and characterized, generally includes the group of water-soluble vitamins found in rice polishings, bean extracts, yeast, and liver. There are no chemical relationships in the B complex. The nomenclature is very confusing. The common name originally implied something about the chemical nature of the vitamin. Even the concept of water-soluble is somewhat misleading, given that some of the vitamins in this group would be considered poorly soluble by most pharmaceutical standards. The one thing the B complex vitamins have in common is that nearly all function either as a coenzyme or a structural component of a coenzyme.

3.5.1 Chemistry. Thiamine consists of a pyrimidine joined to a thiazole ring by a methylene bridge (Fig. 8.25). The thiazole nitrogen is a quaternary with a permanent positive charge. There are two commercial salts. Thiamine hydrochloride is, in reality, thiamine chloride hydrochloride. It is a double salt, consisting of an amine hydrochloride on the pyrimidine amine and a chloride on the thiazole quaternary nitrogen. Thiamine nitrate is correctly named in that the nitrate anion is found on the quaternary nitrogen, and the pyrimidine amine is not protonated. Once the vitamin enters the acidic stomach, it will exist as the chloride hydrochloride salt.

Thiamine hydrochloride is very water soluble (1g/1 mL). It is also very hygroscopic, making it difficult to use in dry formulations. This salt is commonly used in liquid and injectable formulations. Thiamine nitrate is sufficiently water soluble (1g/35 mL) that it can be used in liquid formulations because the RDAs are less than 2 mg per day. Because it is nonhygroscopic, it is commonly used in dry dosage forms.

3.5.2 Uptake and Metabolism. A saturable active transport system in the jejunum provides efficient uptake of the vitamin into the intestinal mucosa cell. Thiamine kinase in the intestinal mucosa cell transfers a pyrophosphate from the ATP to the propyl alcohol at po-

sition 5 of the thiazole ring, forming thiamine pyrophosphate (TPP) (Fig. 8.25). The latter product is the coenzyme form of the vitamin. There is some evidence that this phosphorylation is the rate-limiting step and controls the absorption of the vitamin. The coenzyme is transported to the tissues where needed.

Thiamine pyrophosphate has two important coenzyme roles, both of which focus mostly on carbohydrate metabolism (Figs. 8.26 and 8.27). The active portion of the coenzyme is the thiazole ring. The first step in the oxidative decarboxylation of a-keto acids requires TPP. The two most common examples are pyruvate and a-ketoglutarate, oxidatively decarboxyated to acetyl CoA and succinyl CoA, respectively. The same reaction is found in the metabolism of the branched-chain amino acids valine, isoleucine, leucine, and methionine. In all cases, TPP is a coenzyme in a mitochondrial multienzyme complex, consisting of TPP, lipoic acid, coenzyme A, FAD, and NAD. Note the number of vitamins required for the oxidative decarboxylation of a-keto acids: thiamine (TPP), pantothenic acid (coenzyme A), riboflavin (FAD), and niacin (NAD).

TPP is also the coenzyme in the transketolase reaction (Fig. 8.27) found in the pentose phosphate pathway that interconverts hexoses, pentoses, tetroses, and trioses. This reaction removes carbons 1 and 2 of a ketose and transfers them to an acceptor aldose. Examples include TPP transferring carbons 1 and 2 of xylulose-5-P to ribose-5-P, producing glyceraldehyde-3-P (5 carbons minus 2 carbons) and sedoheptulose-7-P (5 carbons plus 2 carbons). This reaction is reversible. A second reversible reaction has TPP transferring carbons 1 and 2 of xylulose-5-P to erythrose-4-P, producing fructose-6-P (4 carbons plus 2 carbons) and glyceraldehyde-3-P (5 carbons minus 2 carbons).

The dietary reference intakes for thiamine are dependent on carbohydrate consumption. This is because (1) most pyruvate comes from aerobic glycolysis, (2) much of the decarboxylated a-ketoglutarate originates from carbohydrate sources, and (3) the transketolase reaction uses carbohydrates as substrates.

3.5.3 Thiamine Deficiencies. Thiamine deficiencies are reported in the very early medical literature and were called beriberi, a Japanese term. Sailors in the Japanese navy experienced thiamine deficiencies when fed rice in which the polishings had been removed to prevent mold growth. This is somewhat analogous to removing the germ from wheat to prolong the shelf life of flour-containing foods. There are two forms of beriberi, wet and dry. Wet beriberi is characterized by edema and enlarged heart. Dry beriberi is more neurological and can include muscle wasting.

Assuming a reasonably balanced diet, most thiamine deficiencies today are caused by chronic alcoholism. Alcohol reduces the active transport of the vitamin (45). This form of thiamine deficiency is called Wernicke-Korsakoff syndrome. It is common for emergency medical personnel to add thiamine to the intravenous solution being administered to a comatose patient suspected of experiencing substance abuse.

3.5.4 Hypervitaminosis Thiamine. The vitamin is considered very safe. There are no Tolerable Upper Intake Levels. Possibly the rate-limiting phosphorylation step in the intestinal mucosa reduces the risk of toxicity. The percentage of thiamine absorbed decreases as the dose increases.

3.5.5 Dietary Reference Intakes.

AI	
Infants	0.2–0.3 mg/day
EAR	
Children (1–13 years)	0.4–0.7 mg/day
Males (14–18 years)	1.0 mg/day
Females (14–18 years)	0.9 mg/day
Men (19–50+ years)	1.0 mg/day
Women (19–50+ years)	0.9 mg/day
Pregnancy	1.2 mg/day
Lactation	1.2 mg/day
RDA	
Children (1–13 years)	0.5–0.9 mg/day
Males (14–18 years)	1.2 mg/day
Females (14–18 years)	1.0 mg/day
Men (19–50+ years)	1.2 mg/day
Women (19–50+ years)	1.1 mg/day
Pregnancy	1.4 mg/day
Lactation	1.5 mg/day
UL	
None reported	

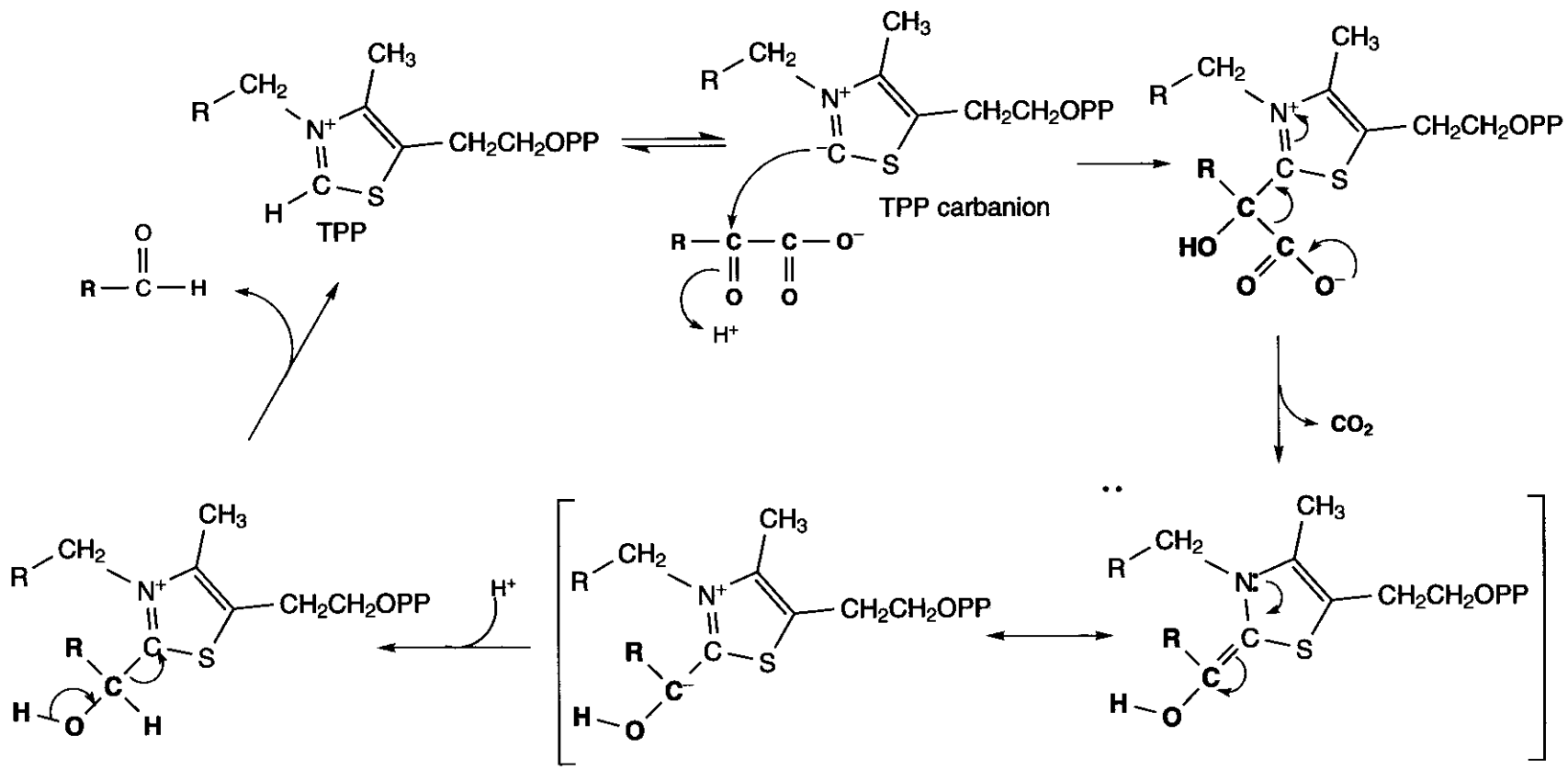


Figure 8.26. Coenzyme role of thiamine pyrophosphate in decarboxylation of α-keto acids.

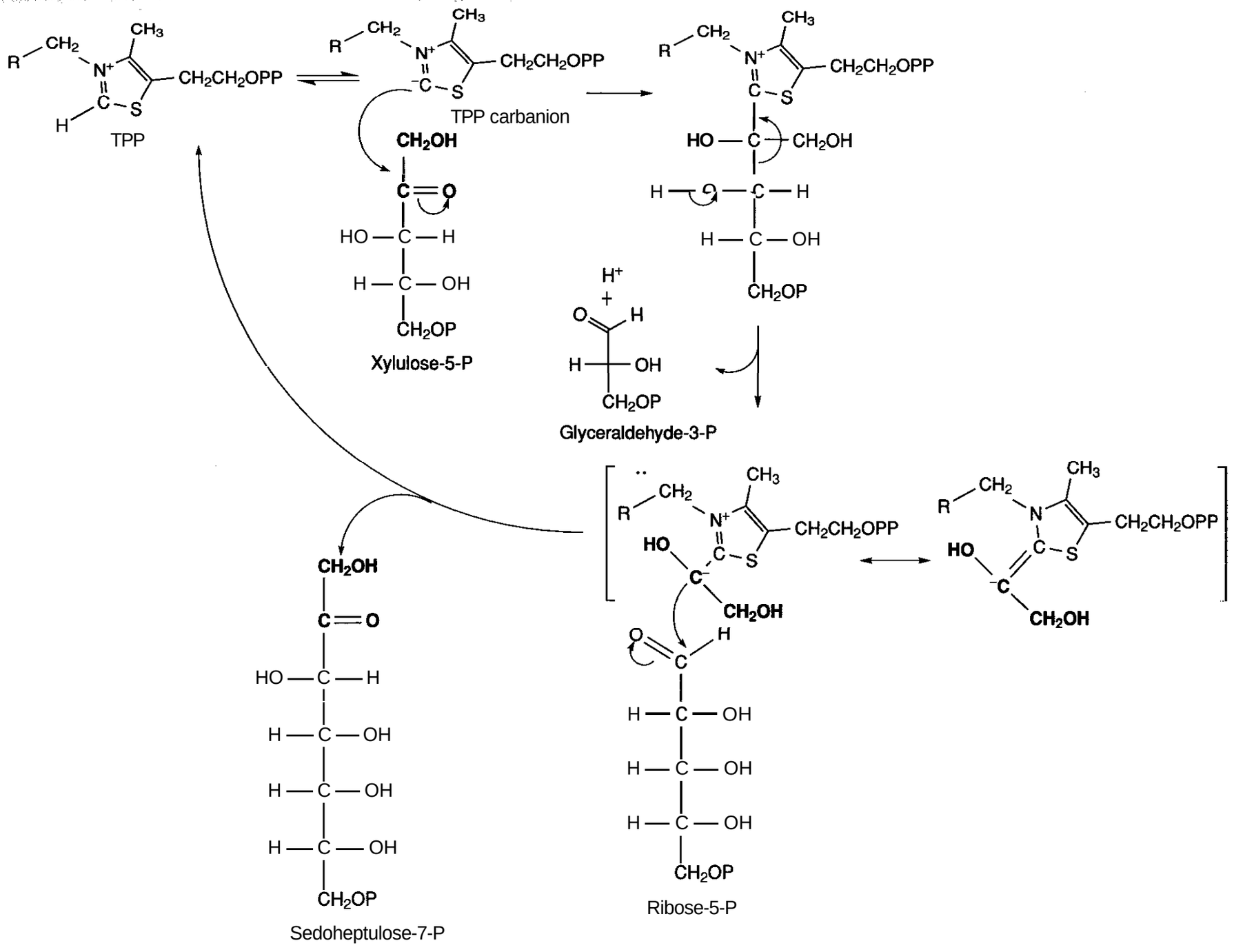


Figure 8.27. Coenzyme role of thiamine pyrophosphate in the transketolase reaction.

3.6 Riboflavin (Vitamin B_2) (46)

Shortly after the discovery of thiamine from yeast concentrates, the presence of a second nutritional factor in such materials was suggested. This second factor was also reported to have a pellagra-preventative activity because it alleviated a deficiency-induced dermatitis in rats. It was called vitamin B_2 in England and vitamin G in the United States.

3.6.1 Chemistry (Fig. 8.28). Riboflavin has a characteristic flavin ring system, which gives it unique spectroscopic and instability properties. There are two commercial forms. Riboflavin itself is poorly water soluble (1 g/10,000 mL) and is limited to oral dry dosage forms. Riboflavin phosphate, as the sodium salt, is very water soluble at 100 mg/mL and is widely used in dry and liquid dosage forms.

3.6.2 Riboflavin Uptake and Chemistry (Fig. 8.28). Most dietary riboflavin is eaten as the FAD or FMN coenzymes. Intestinal pyrophosphatases and phosphatases produce free riboflavin, which is actively transported in the proximal area of the small intestine into systemic circulation. Because of its poor water solubility, it is transported on albumin and immunoglobulin proteins. Conversion to the coenzyme forms occurs inside the cells that need these coenzymes.

3.6.3 Metabolic Role. Riboflavin coenzymes are required for most oxidations of carbon-carbon bonds (Fig. 8.29). Examples include the oxidation of succinyl CoA to fumarate in the Krebs cycle and introduction of α,β-unsaturation in β-oxidation of fatty acids. Riboflavin is also required for the metabolism of other vitamins, including the reduction of 5,10-methylene tetrahydrofolate to 5-methyl tetrahydrofolate (Fig. 8.49), and interconversion of pyridoxine-pyridoxal phosphate-pyridoxamine (Fig. 8.33). Because oxidation/reductions that use FAD or FMN as the coenzyme constitute a two-step process, some flavin coenzyme systems contain more than one FAD or FMN.

3.6.4 Riboflavin Deficiency. With riboflavin's central role in energy metabolism and conversion of folic acid and pyridoxine to their active forms, it is surprising that riboflavin deficiency does not produce a characteristic set of symptoms. One of the reasons may be that it is rare to see a patient who is solely deficient in riboflavin.

3.6.5 Hypervitaminosis Riboflavin. The combination of regulated active transport and conversion to the coenzyme forms prevents hypervitaminosis problems with this vitamin. Toxicities from the water-soluble riboflavin phosphate have not been reported. There are no Tolerable Upper Intake Levels.

3.6.6 Dietary Reference Intakes.

AI	
Infants	0.3–0.4 mg/day
EAR	
Children (1–13 years)	0.4–0.8 mg/day
Males (14–19 years)	1.1 mg/day
Females (14–19 years)	0.9 mg/day
Men (19–70+ years)	1.1 mg/day
Women (19–70+ years)	0.9 mg/day
Pregnancy	1.2 mg/day
Lactation	1.3 mg/day
RDA	
Children (1–13 years)	0.5–0.9 mg/day
Males (14–19 years)	1.3 mg/day
Females (14–19 years)	1.0 mg/day
Men (19–70+ years)	1.3 mg/day
Women (19–70+ years)	1.1 mg/day
Pregnancy	1.4 mg/day
Lactation	1.6 mg/day
UL	
None reported	

3.7 Niacin (Nicotinic Acid)/Niacinamide (Nicotinamide) (47)

The history of niacin revolved around trying to find a way to prevent and cure pellegra, the late-stage deficiency disease caused by a niacin deficiency. Pellagra has been a serious nutritional disorder in the United States, mostly in the southeast. Two thousand deaths from pellagra were reported in 1941. This is ironic because nicotinic acid, later known as niacin, was first reported during the structure elucidation of the alkaloid nicotine.

Like some of the other deficiency diseases, there was disagreement between those who

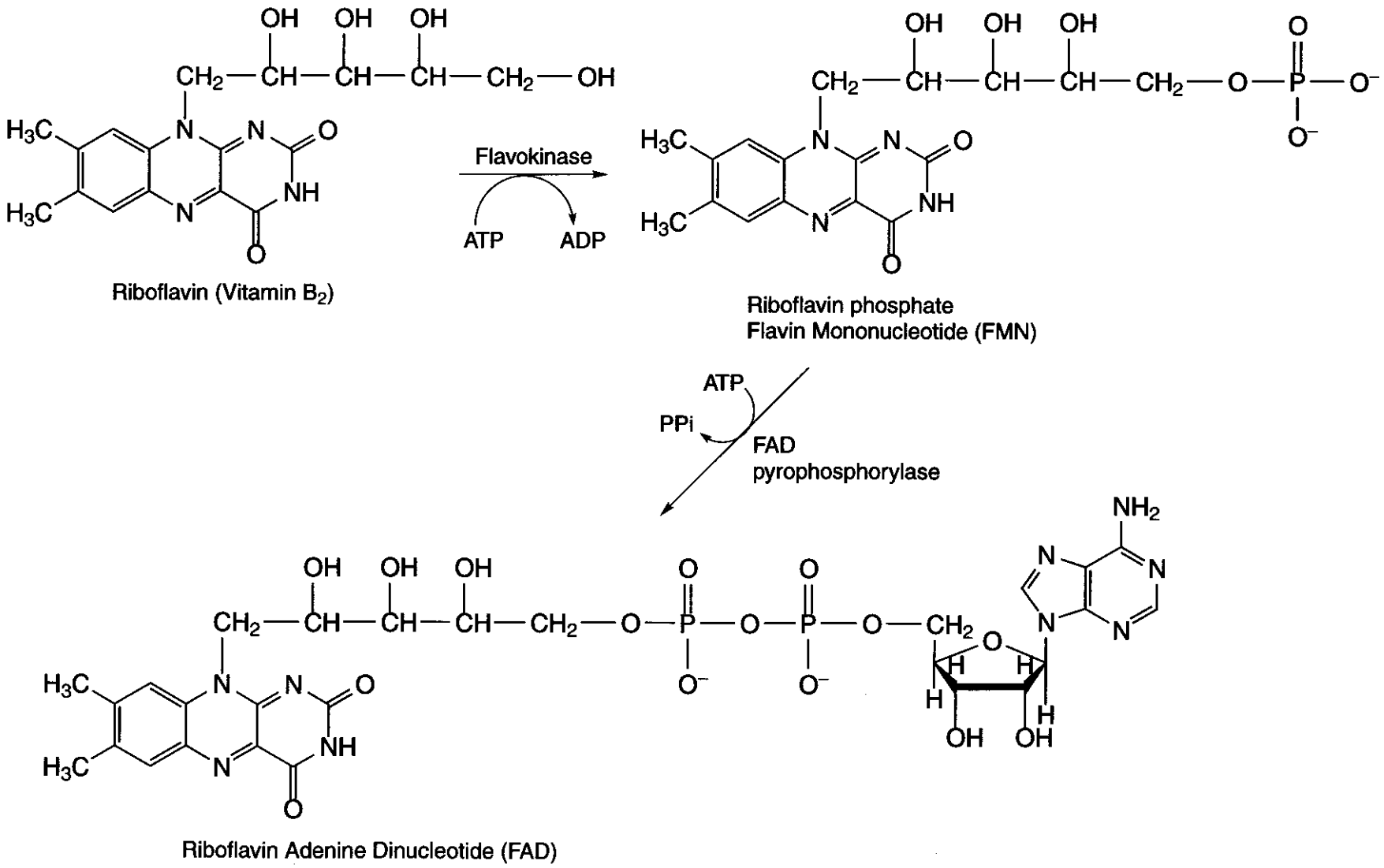


Figure 8.28. Riboflavin dosage and coenzyme forms.

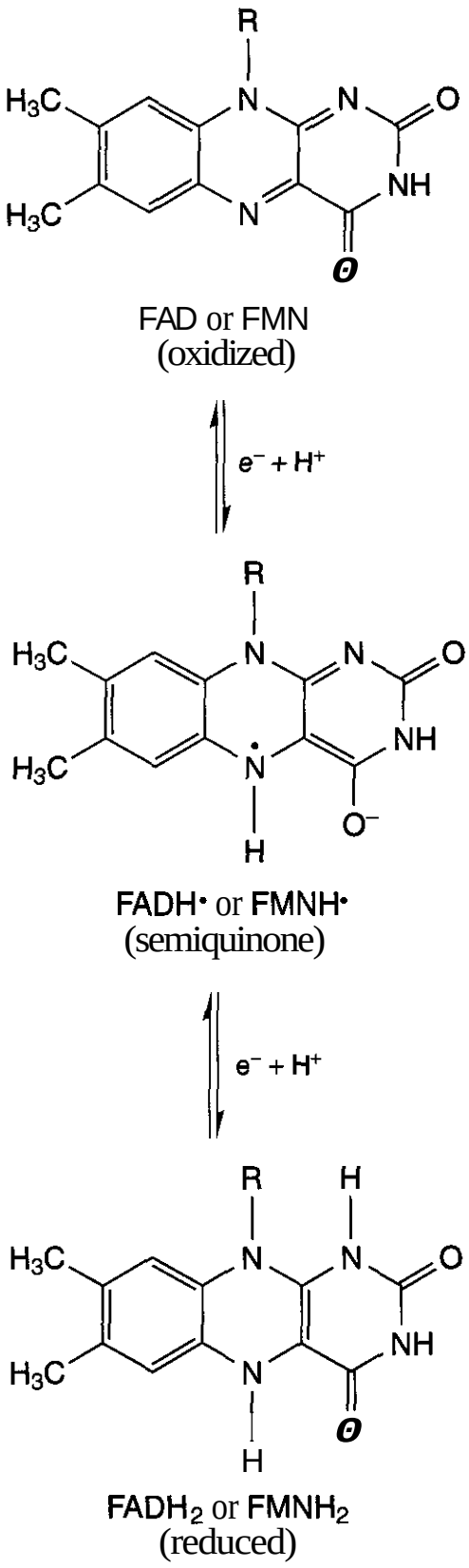


Figure 8.29. FAD/$FADH_2$-FMN/$FMNH_2$ oxidation/reduction.

thought pellegra was caused by poor sanitation vs. those who concluded it was a nutritional disorder. Niacin deficiency, even today, is found in economically poor areas. Even when niacin was first isolated from foods, it was ignored because it did not cure beriberi.

3.7.1 Niacin/Niacinamide Chemistry, Uptake and Metabolism. Niacin is structurally the simplest of the vitamins, but has some of the most complex biochemistry of the vitamins. Structurally, it is pyridine-3-carboxylic acid (Fig. 8.30). Strictly speaking, it is nonessential because the essential amino acid tryptophan is a source (Fig. 8.31) (48). The biosynthetic route does not produce "free" niacin by decarboxylation of quinolinic acid. In a complex reaction, quinolinic acid loses the 2-carboxyl group and adds 5′-phosphoribose to form nicotinate mononucleotide (Figs. 8.30 and 8.31) (49, 50). Niacin and niacinamide, in pharmaceutical dosage forms, undergo a similar ribosylation reaction. Most vitamin products contain niacinamide because niacin can cause a distracting vasodilation that leads to flushing in the face and scalp.

The two commercial forms of the vitamin, niacin and niacinamide, are rapidly absorbed from both the stomach and intestine. As the dose increases, absorption decreases. It is not clear whether there is a feedback mechanism operating or the transport system becomes saturated. Conversion to the coenzyme forms occurs in the cells where NAD and NADP are needed.

NAD is the primary coenzyme required for oxidation/reduction of carbon-oxygen bonds and is required for oxidative catabolism (glycolysis, β-oxidation, Krebs cycle). NADP is the coenzyme in biosynthetic routes (fatty acid and cholesterol synthesis) and will be part of oxidation/reduction reactions involving both carbon-oxygen and carbon-carbon bonds.

The active part of the coenzyme is the pyridine ring (Fig. 8.32). When the substrate is labeled with deuterium, it has been shown that NAD systems can be categorized by the deuterium ending up on the A or B face of the pyridine ring. Examples of NAD dehydrogenases where the hydride anion attaches to the A face are isocitrate dehydrogenase, malate dehydrogenase, lactate dehydrogenase, and alcohol dehydrogenase. Dehydrogenases involving the B face include α-ketoglutarate dehydrogenase, glucose-6-phosphate dehydrogenase, glutamate dehydrogenase, and glyceraldehyde-&phosphate dehydrogenase.

3.7.2 Niacin Deficiency. Niacin deficiency, manifested as pellagra, is characterized by the four Ds: dermatitis, diarrhea, depression, and death. The dermatitis is characterized by a pigmented rash developing on skin exposed to

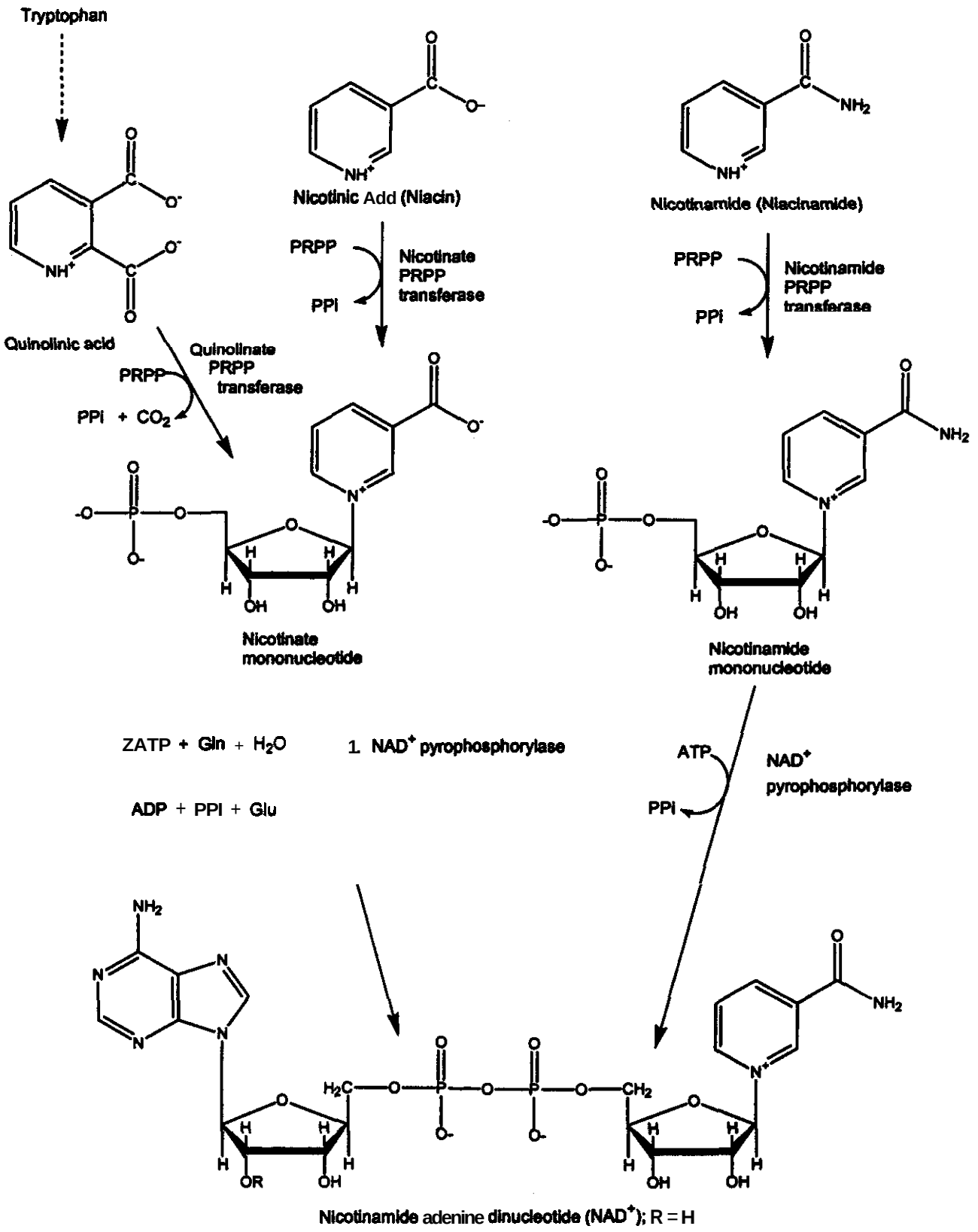


Figure 8.30. Biosynthesis of NAD from niacin and niacinamide.

heat. Changes to the gastrointestinal tract can lead to vomiting, constipation, or diarrhea. Depression is one of the neurological symptoms that also can include apathy, headache, fatigue, and memory loss.

Deficiencies were common in populations whose main calorie source was corn **(51, 52).** Zein is the main protein found in corn and it is very low in both niacin and tryptophan. When corn is ground with lime water, the small amounts of niacin and tryptophan become more bioavailable. Populations who consume corn meal by this process have a smaller incidence of pellagra **(53).** At the same time it must be remembered that **pellegra** is the extreme form of a niacin deficiency. The Dietary Reference Intakes for this vitamin use clinical chemistry **as**says listed in Table 8.2 to determine **DRIs** rather than the first appearance of pellagra symptoms.

3.7.3 Hypervitaminosis Niacin. Niacin is considered nontoxic, and there are no Tolerable Upper Intake Levels based on its use as a vitamin. These refer only to niacin and niaci-

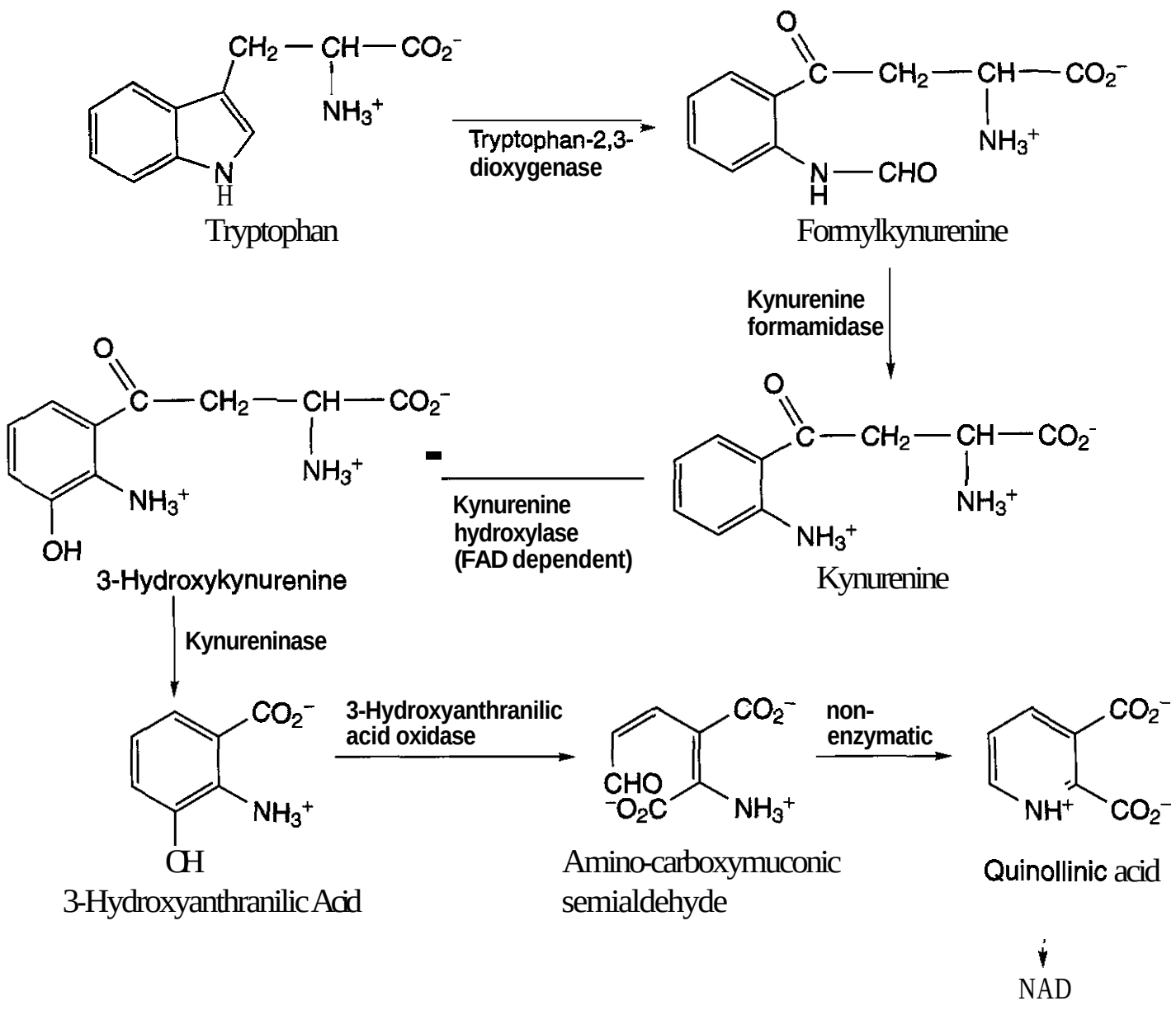


Figure 8.31. Biosynthesis of NAD from tryptophan.

namide, but not tryptophan and niacin equivalents (see next section). Large doses of niacin and niacinamide do have adverse reactions. These are sometimes seen with patients who are prescribed niacin in doses up to 2 g daily for hyperlipidemia, including both hypercholesterolemia and hypertriglyceridemia. For the former, there is the desired decreased LDL and increased HDL. Adverse reactions include vasodilation from niacin, particularly in the head area, caused by increased intercranial blood flow and hepatic complications. Niacin had been used for Raynaud's syndrome to treat the vasoconstriction seen with this disease. Liver toxicities have been experienced by patients prescribed sustained-release niacin products for hyperlipidemia (54, 55). The UL to RDA ratio is 2, but the UL is the dose before adverse reactions are experienced. Vasodilation from niacin occurs close to its RDA.

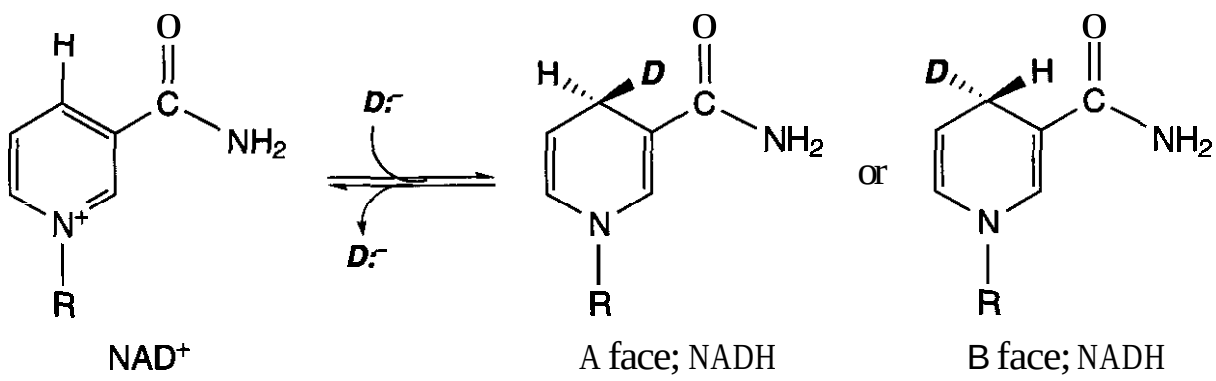


Figure 8.32. Stereochemistry of NAD reduction.

3.7.4 Dietary Reference Intakes. Many of the DRI units are milligram niacin equivalents (mg NE). These units take into account the fact that approximately 60 mg tryptophan produce 1 mg of niacin. For adult males, the RDA is between 960 mg of tryptophan and 16 mg of niacin, given that 960 mg of tryptophan is equivalent to 16 mg of niacin (56, 57).

AI	
Infants (0–5 months)	2 mglday of preformed niacin
Infants (6–11 months)	4 mg NE/day
EAR	
Children (1–13 years)	5–9 mg NE/day
Boys (14–18 years)	12 mg NE/day
Girls (14–18 years)	11 mg NE/day
Men (19–70+ years)	12 mg NE/day
Women (19–70+ years)	11 mg NE/day
Pregnancy	14 mg NE/day
Lactation	13 mg NE/day
RDA	
Children (1–13 years)	6–12 mg NE/day
Girls (14–19 years)	1.3 mg NE/day
Boys (14–19 years)	16 mg NE/day
Men (19–70+ years)	16 mg NE/day
Women (19–70+ years)	14 mg NE/day
Pregnancy	18 mg NE/day
Lactation	17 mg NEIday
UL	
Infants (0–12 months)	Source of intake should be formula and food only
Children (1–13 years)	10–20 mg/day of niacin
Adolescents (14–18 years)	30 mglday of niacin
Pregnancy (14–18 years)	30 mglday of niacin
Pregnancy (19 years and older)	35 mglday of niacin
Lactation (14–18 years)	30 mglday of niacin
Lactation (19 years and older)	35 mglday of niacin

3.8 Vitamin B_6 Family

This group was discovered in the 1930s during feeding experiments on rats. Its importance was realized tragically when the heat process for an infant formula reduced the bioavailabilitiy of the vitamin. Children developed convulsive disorders. Before realizing that the problem was caused by an induced pyridoxine deficiency, it was thought a contaminant had been introduced. The vitamin is a coenzyme for amino acid and glycogen metabolism.

3.8.1 Uptake and Metabolism. The vitamin B_6 family consists of pyridoxine, pyridoxal, pyridoxamine, pyridoxine phosphate, pyridoxal phosphate (PLP), and pyridoxamine phosphate (Fig. 8.33). The commercial form is pyridoxine. Pyridoxal phosphate is the coenzyme form. It and pyridoxamine phosphate are from animal tissues. Pyridoxine is from plant tissues. All phosphorylated forms are hydrolyzed in the intestinal tract by phosphatases before being absorbed passively. Conversion to the phosphorylated forms occurs in the liver. Notice that niacin (NAD) and riboflavin (FMN, FAD) are required for interconversion among the vitamin B_6 family. The phosphorylated forms are transported to the cells where needed. The major excretory product is 4-pyridoxic acid.

3.8.2 Pyridoxal Phosphate Biochemistry (58). Pyridoxal phosphate (PLP) is required for amino acid metabolism and reactions involving amino acids. PLP is covalently bound to the apoenzyme through an enamine (Schiff's base) linkage between an ϵ-amino group of lysine and the aldehyde moiety of PLP (Fig. 8.34). The most common of the PLP-catalyzed reactions are transaminations (Fig. 8.35). One-half of all transamination reactions involve a-ketoglutarate as the acceptor of the amine group forming glutamic acid. Alternatively, glutamic acid donates the amine group and an a-keto acid is the acceptor forming a new amino acid. Examples include:

a-amino acid + a-ketoglutarate ⇆ a-keto acid + glutamic acid

a-amino acid reactant	a-ketoacid product
alanine	pyruvate
aspartate	oxalacetate

Another PLP-catalyzed reaction is decarboxylation of amino acids (Fig. 8.35). These are part of the biosynthesis of neurotransmitters, includ-

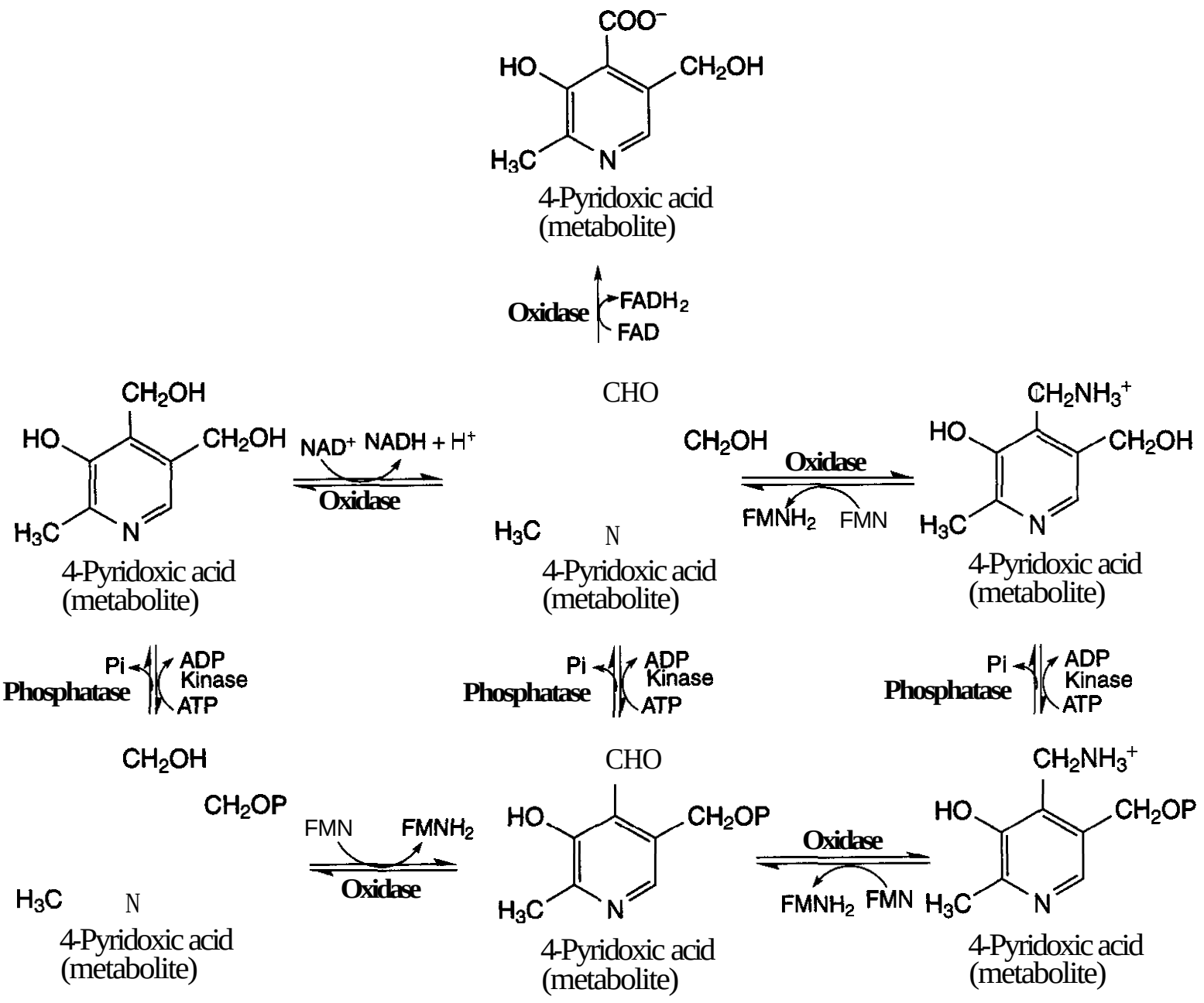


Figure 8.33. Interconversions among the vitamin B_6 family.

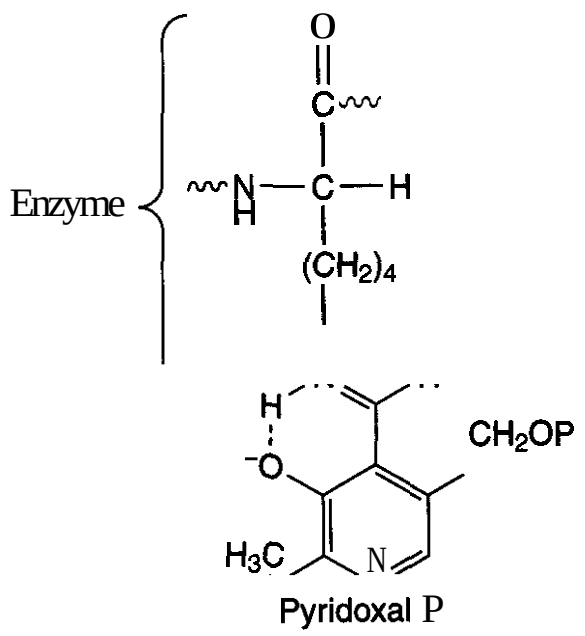


Figure 8.34. Pyridoxal phosphate bound to the enzyme.

ing histamine from histidine; serotonin from tryptophan, dopamine, norepinephrine, and epinephrine from dihydroxyphenylalanine (dopa) (Fig. 8.36); and γ-aminobutyric acid from glutamic acid. Other reactions in which an amino acid is the substrate include formation of δ-aminolevulinic acid in the biosynthesis of heme and two reactions in cysteine biosynthesis from methionine (Fig. 8.52). Although not well understood, PLP is required for phosphorylase-catalyzed glycogenolysis producing glucose-1-

3.8.3 **Vitamin B_6 Deficiency.** There is no distinct deficiency syndrome, but a deficiency can be serious. What is observed is a seborrheic dermatitis, microcytic anemia, and convulsions. The anemia may be caused by decreased heme biosynthesis and

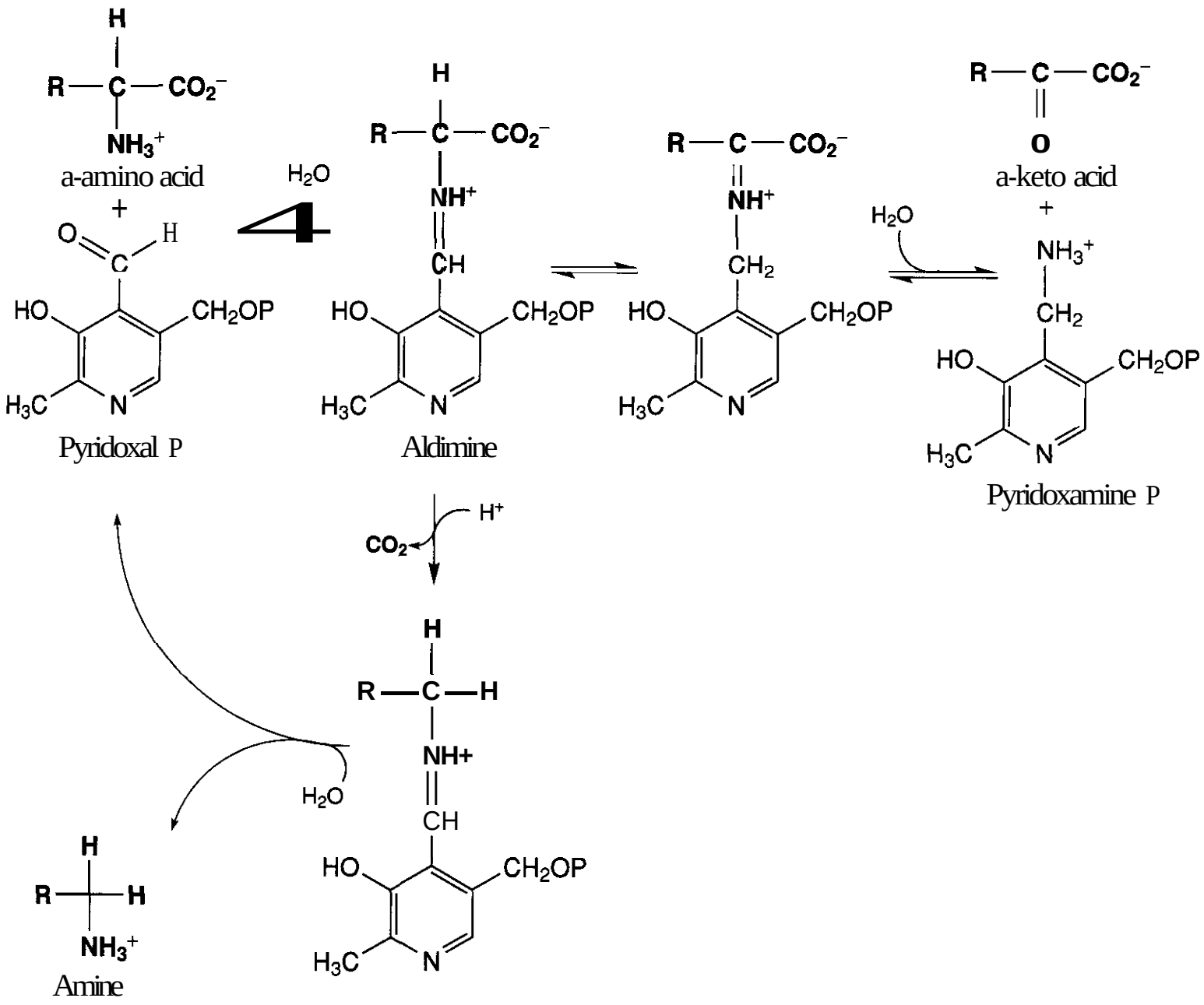


Figure 8.35. Pyridoxine phosphate-catalyzed transamination and decarboxylation.

the convulsions by imbalances in neurotransmitter biosynthesis.

There is reason to conclude that vitamin B_6 deficiency might contribute to arteriosclerosis. There is a correlation between elevated homocysteine levels and incidence of cardiovascular disease (59). There is debate as to whether homocysteine contributes to the damage of cells on the interior of blood vessel or whether homocysteine is a marker of intensive cell repair and formation of replacement cells. Nevertheless, administration of pyridoxine, folic acid, and cyanocobalamin are being recommended along with the two antioxidant vitamins, α-tocopherol and ascorbic acid for arteriosclerosis. Vitamin B_6 is required for two of the steps in the catabolism of homocysteine to succinyl CoA (Fig. 8.52). Note in Fig. 8.52 (bottom) that biotin and a coenzyme form of cobalamin also are required for the final reactions. The homocysteine model is discussed again under the folic acid and cyanocobalamin sections.

3.8.4 Vitamin-Drug Interactions (60). The two most clinically significant interactions are pyridoxal phosphate with L-dopa or isoniacid. Examine Fig. 8.36 and note that dopa decarboxylase requires PLP. This enzyme is found both centrally and peripherally. The latter includes the intestinal mucosa. The precursor to dopamine, L-dopa is indicated for the treatment of Parkinson's disease. L-Dopa is prescribed because little dopamine crosses the blood-brain barrier relative to its precursor L-dopa. A patient with Parkinson's disease prescribed L-dopa and who takes a vitamin supplement with amounts of pyridoxine greater than the vitamin's RDA can experience an in-

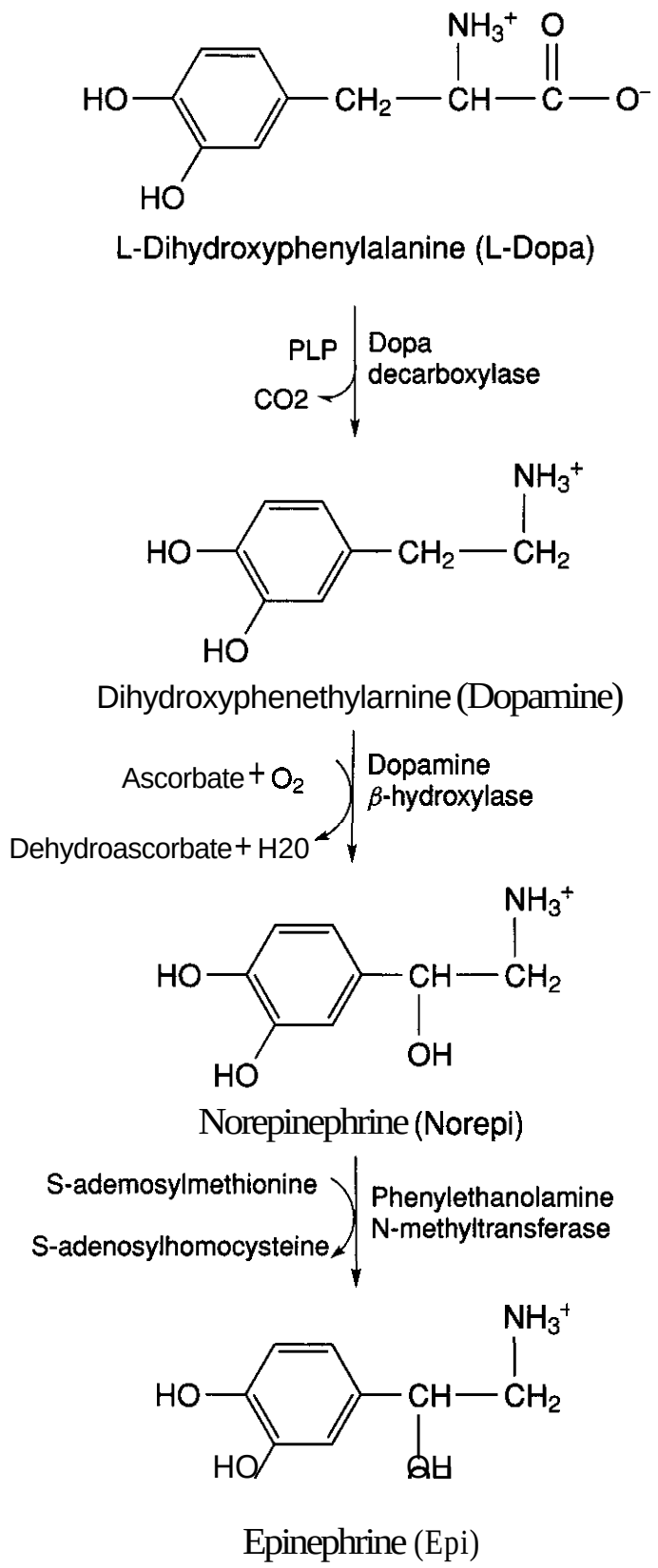


Figure 8.36. Pyridoxal phosphate-catalyzed dopa decarboxylase.

crease in parkinsonian tremors. This is because L-dopa will undergo decarboxylation in the intestinal mucosa and never reach the locations in the brain where it is converted to the needed dopamine.

Isoniazid is widely prescribed for tuberculosis. It can chemically react with pyridoxal and pyridoxal phosphate, thus significantly reducing the availability of this coenzyme (Fig. 8.37) (61). Pyridoxine supplements commonly are recommended to prevent isoniazid-caused peripheral neuropathy, but they do not reduce the effectiveness of isoniazid.

3.8.5 Hypervitaminosis Pyridoxine. A certain mystique has built up around this vitamin, resulting in individuals' overdosing themselves with commercial vitamin supplements. Serious neurological problems have been seen in doses of 2–6 g/day for 2–40 months (62–64). Megadosing below 2 glday seems safe, but all of this information is based mostly on anecdotal reports. There is a Tolerable Upper Intake Level, but the UL to RDA ratio is a comfortable 50–60.

3.8.6 Dietary Reference Intakes.

AI (any form of vitamin B_6)	
Infants	0.1–0.3 mglday
EAR (any form of vitamin B_6)	
Children (1–13 years)	0.4–0.8 mglday
Males (14–19 years)	1.1 mg/day
Females (14–19 years)	1.0 mglday
Men (19–50 years)	1.1 mg/day
Men (51+ years)	1.4 mg/day
Women (19–50 years)	1.1 mglday
Women (51+ years)	1.3 mglday
Pregnancy	1.6 mglday
Lactation	1.7 mg/day
RDA (any form of vitamin B_6)	
Children (1–13 years)	0.5–1.0 mglday
Males (14–19 years)	1.3 mglday
Females (14–19 years)	1.2 mglday
Men (19–50 years)	1.3 mglday
Men (51+ years)	1.7 mglday
Women (19–50 years)	1.3 mglday
Women (51+ years)	1.5 mglday
Pregnancy	1.9 mg/day
Lactation	2.0 mglday
UL (as pyridoxine)	
Children (1–13 years)	30–60 mglday
Adolescents (14–18 years)	80 mglday
Adults (19+ years)	100 mglday
Pregnancy (14–18 years)	80 mglday
Pregnancy (19+ years)	100 mglday
Lactation (14–18 years)	80 mg/day
Lactation (19+ years)	100 mglday

3.9 Pantothenic Acid (65)

Pantothenic acid is essential and is a normal component of our diet. There has been little

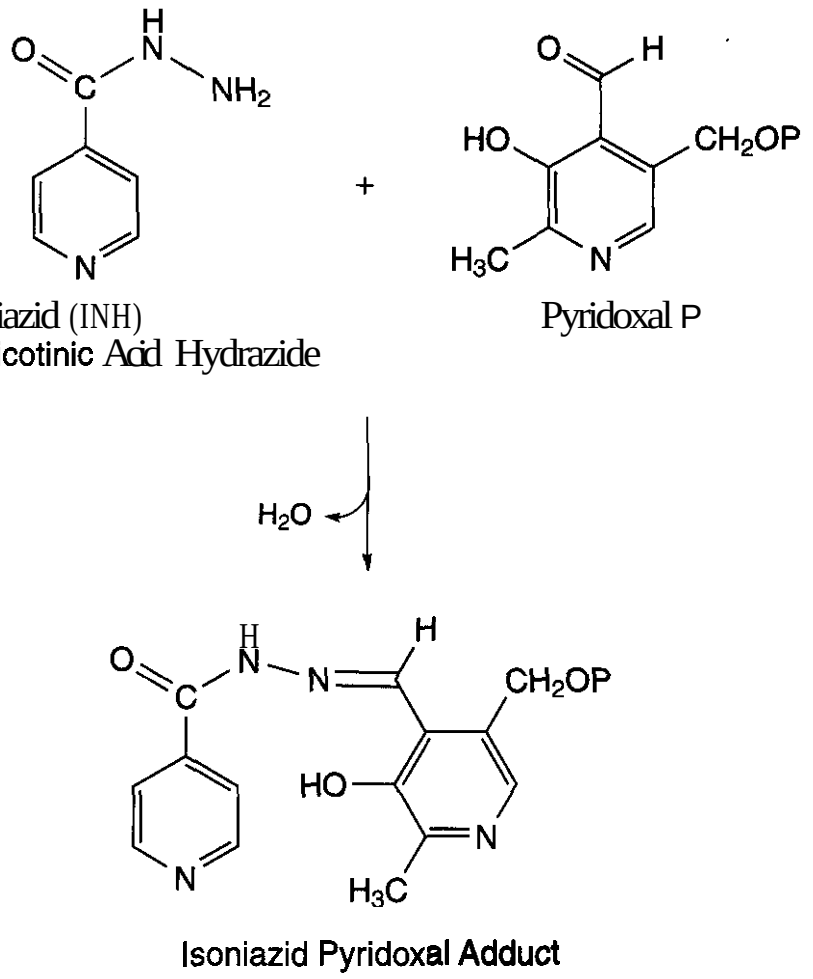


Figure 8.37. Pyridoxal phosphate-isoniazid interaction.

research done on this vitamin and, therefore, it has adequate intakes (AI) and no RDAs.

3.9.1 Chemistry, Uptake, and Metabolic Role. This vitamin, which can be considered a derivative of β-alanine, is asymmetric (Fig. 8.38). The natural form has the $D(+)$ configuration. The $L(-)$ stereoisomer is inactive. The reduced alcohol form, pantothenol, is considered as equally active as the parent acid. Many of the multiple vitamin products use a synthetic, racemic mixture. This means that double the amount of synthetic vitamin must be used to obtain equivalent active vitamin.

Dietary pantothenic acid is consumed as coenzyme A and the intermediates from coenzyme A's biosynthesis (Fig. 8.39). These are hydrolyzed to free pantothenic acid. Absorption is by saturable, active transport.

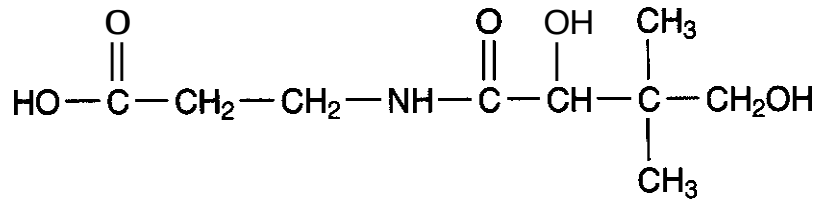
Calcium pantothenate is commonly used in dry dosage forms. It is moderately hygroscopic, with a solubility of 1 g/2.8 mL, and is unstable for autoclaving. Neither the parent pantothenic acid nor the sodium salt is commonly used in dosage forms.

Pantothenol (panthenol) is reasonably stable and freely soluble and is used both in injectable and oral dosage forms. Although widely used in cosmetics including skin creams and shampoos, there is no evidence that this vitamin is effective as a vitamin topically. It apparently has good emollient properties, but these have nothing to do with its systemic role.

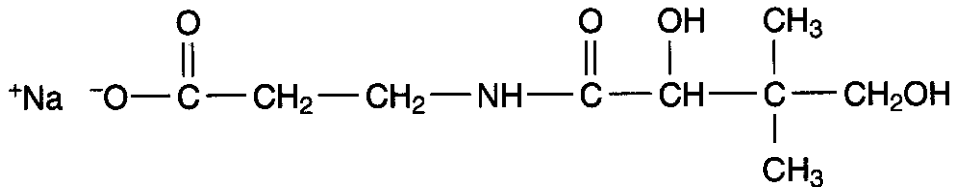
Pantothenic acid is a structural component, but not the active site, of coenzyme A. The acyl thiol esters form on the mercaptan moiety that originates from a cysteine (Fig. 8.39). The biosynthesis of coenzyme A occurs in the tissues requiring it. Because coenzyme A is required for nearly all acyl transfers, biosynthesis takes place in nearly all cells.

3.9.2 Hypervitaminosis Pantothenic Acid. There have been no reports of toxicity and no Tolerable Upper Intake Levels. Because its active transport is saturable, excessive uptake is doubtful. Also, this vitamin does not have the mystique that would prompt marketing "high potency" formulations.

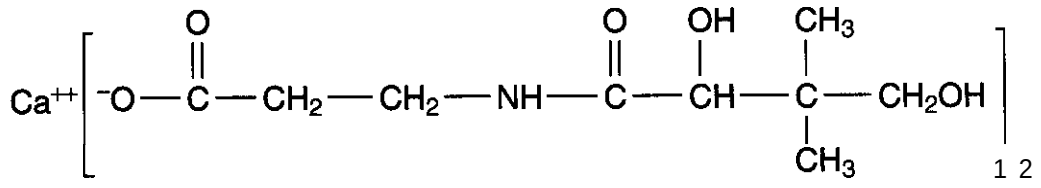
3.9.3 Dietary Reference Intakes. There are too few studies to provide sufficient information to estimate Estimated Average Requirements (EAR) or Recommended Dietary Allowances (RDA).



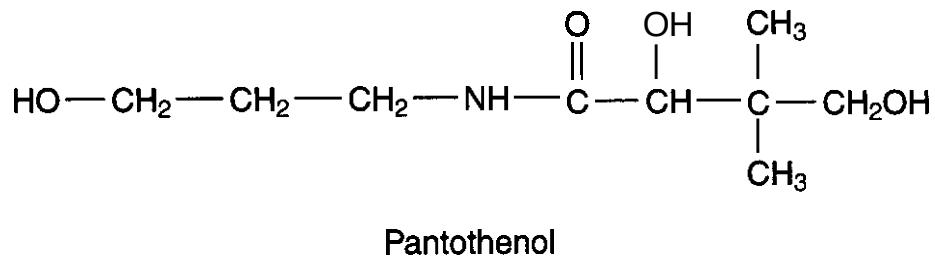
Pantothenic acid



Sodium Pantothenate



Calcium Pantothenate



Pantothenol

Figure 8.38. Forms of pantothenic acid.

AI	
Infants	1.7–1.8 **mg/day**
Children (1–13 years)	2 4 **mg/day**
Everyone else	5 **mg/day**
Pregnancy	6 **mg/day**
Lactation	7 mglday
EAR	
None reported	
RDA	
None reported	
UL	
None reported	

3.10 Biotin (66)

Biotin (Fig. 8.40) is essential, a normal constituent of the diet, and required for four biotin-dependent carboxylation reactions. Eating raw egg white can induce a deficiency.

3.10.1 Uptake. In foods, most biotin is covalently bound (Fig. 8.41) to the apoenzyme, where it is the coenzyme for carboxylation reactions. Intestinal enzymes hydrolyze the amide linkage to produce free biotin. Biotin is actively transported through the intestinal flora into the portal vein and to the liver where it may be stored. It appears that adults may store several months of biotin. From the liver it is transported to tissues where it is needed.

3.10.2 Chemistry. Biotin consists of two 5-membered rings cis-fused to each other that can be drawn either as the keto (urea) or enolic form (Fig. 8.40). The enolic d-isomer is the active stereoisomer, but many times commercial multivitamin products contain the synthetic racemic *d,l* mixture. There is no activity with the 1-stereoisomer.

3.10.3 Metabolic Role. Biotin picks up carbon dioxide that has been activated by combining with an ATP-donated phosphate, producing the mixed anhydride of phosphoric and carbonic acids (Fig. 8.42). The biotin enolate receives the carbon dioxide, producing the keto carbon dioxide-releasing coenzyme.

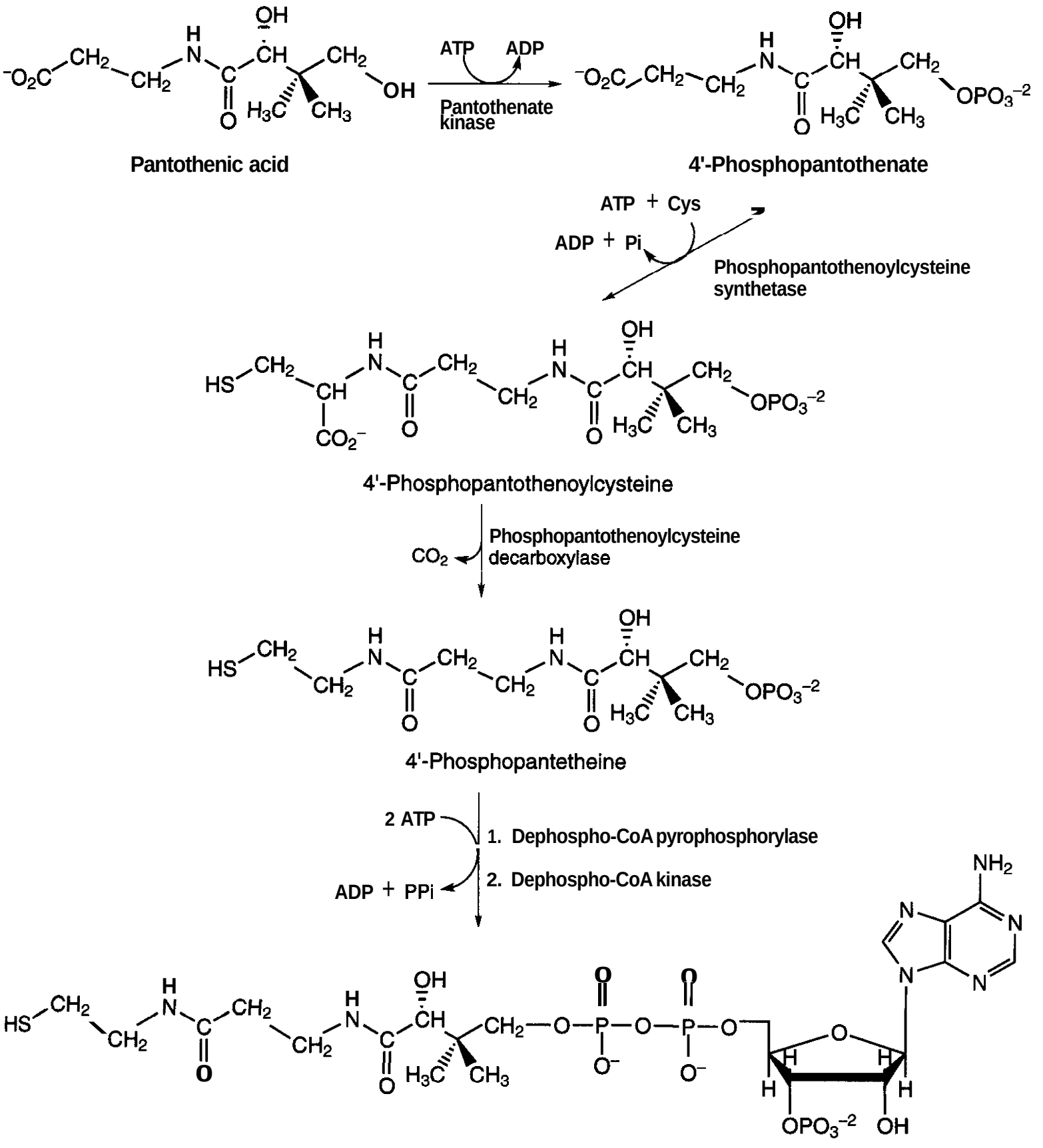


Figure 8.39. Pantothenic acid chemistry.

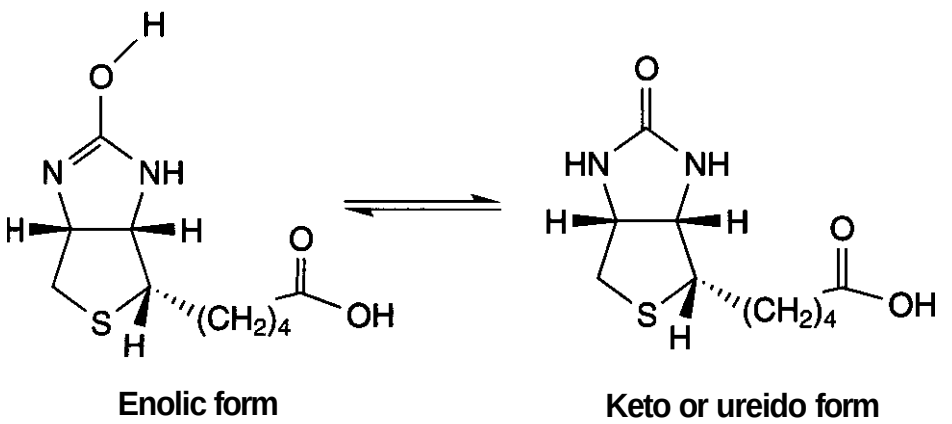


Figure 8.40. Biotin.

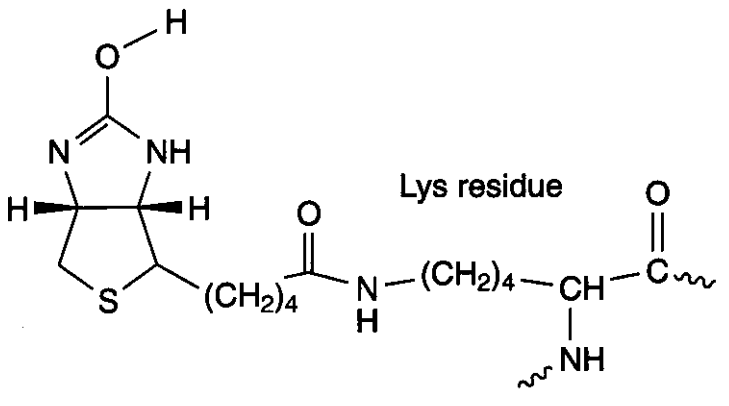


Figure 8.41. Coenzyme form of biotin.

There are four biotin-dependent **carboxylation** reactions, three of which are in the mitochondria. They are:

1. **Pyruvate** carboxylase (Fig. 8.43)
   This reaction, which converts pyruvate to **oxalaceetate,** is in the mitochondria and **hastwo** functions. First, it is the initial reaction in gluconeogenesis to overcome the **14** kcal energy barrier to form **phosphoenol**-pyruvate. Second, this same reaction, sometimes referred to as an anapleurotic reaction, ensures that there is adequate **oxala**-cetate when there are large amounts of acetyl **CoA** entering the Krebs cycle.
2. Acetyl **CoA** carboxylase (Fig. 8.44)
   This reaction, found mostly in the **cytosol,** is the committed step in the synthesis of fatty acids.
3. Propionyl **CoA** carboxylase (Fig. 8.45)
   Propionyl **CoA** is the product from the catabolism of valine, isoleucine, **methio**nine, and odd-numbered fatty acids. The carboxylation reaction, found in the mitochondria, produces methyl malonyl **CoA.** The latter undergoes a cobalamin (vitamin B_{IZ})-catalyzed rearrangement, forming succinyl **CoA,** which is metabolized further in the Krebs cycle.
4. **β-Methylcrotonyl CoA** carboxylase (Fig. 8.46)
   This mitochondria1 reaction permits the final steps in the catabolism of the branched-chain amino acid **leucine.** The final products, acetoacetate and acetyl **CoA,** either are oxidative metabolized to carbon dioxide and water or enter other reactions in lipid metabolism.

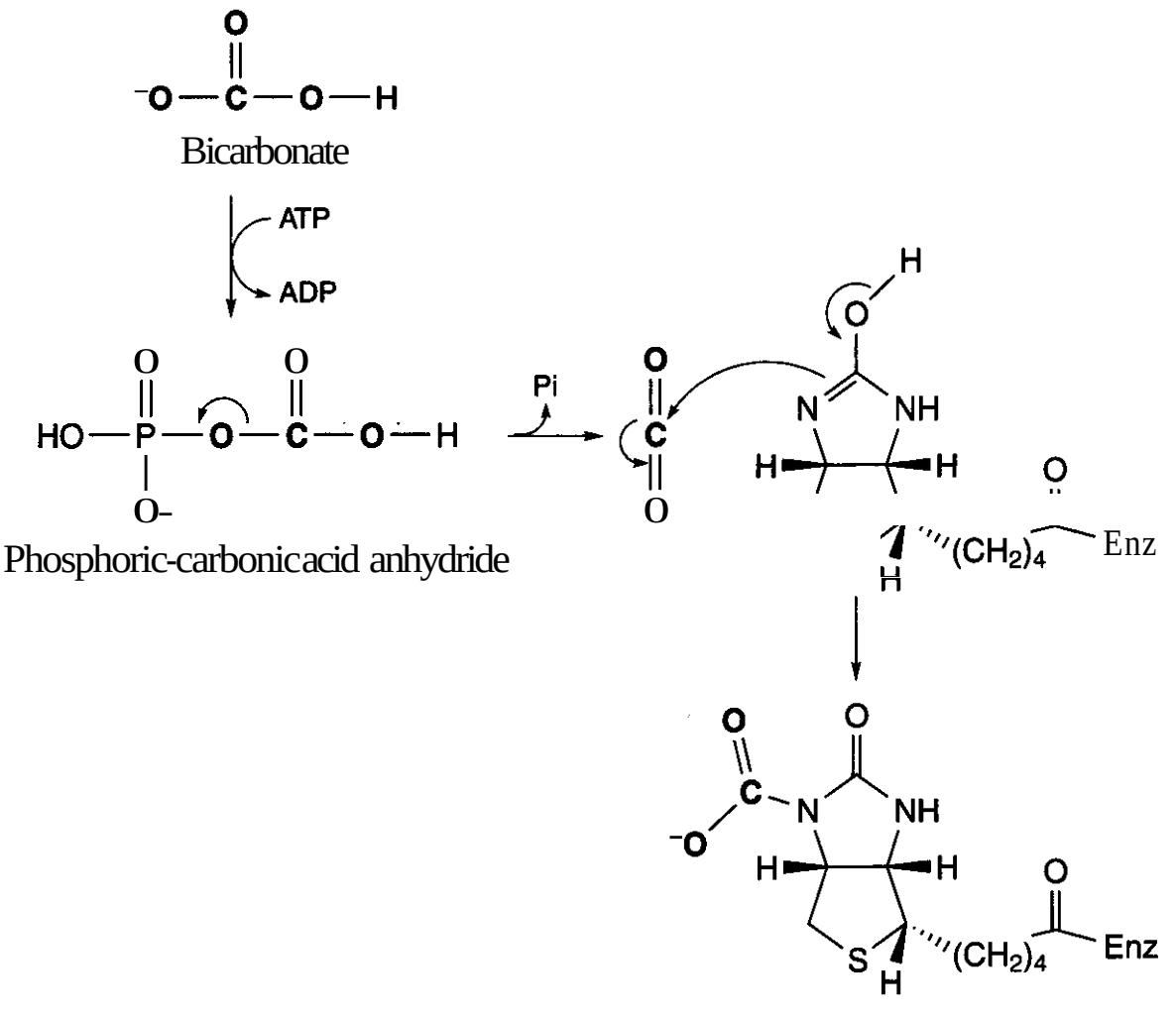


Figure 8.42. Biotin binding carbon dioxide.

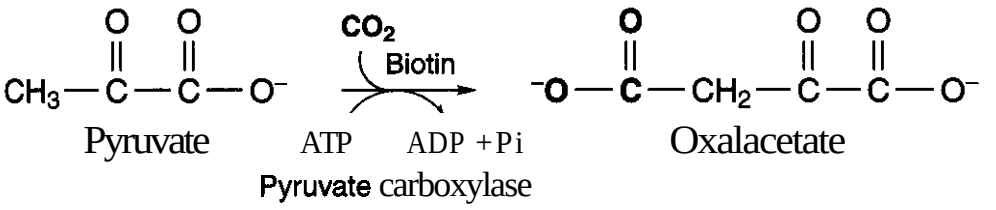


Figure 8.43. Carboxylation of pyruvate producing oxalacetate.

3.10.4 Biotin Deficiency. Relative to many of the vitamins, it is easy to induce a biotin deficiency by feeding volunteers raw egg white. **Avidin,** a basic protein found in egg white, forms salt linkages with acidic biotin and prevent its transport across the intestinal barrier. Cooked egg white is not a problem. Because biotin is found in the yolk, eating whole raw egg will not induce a deficiency. Deficiencies also were caused in patients on total parenteral nutrition (TPN) because biotin was not included in the early formulations. Symptoms include dermatitis, loss of hair color, and central neurological effects.

3.10.5 Hypervitaminosis Biotin. None has been reported in humans and there are no Tolerable Upper Intake Levels.

3.10.6 Dietary Reference Intakes. These have been difficult to determine. There has been some speculation that humans might obtain part of their biotin requirements from the intestinal flora in the colon. The question that has not been adequately answered is whether there is significant absorption of bacteria-produced biotin from the colon.

AI	
Infants	5–6 μg/day
Children (1–13 years)	8–20 μg/day
Adolescents (14–18 years)	25 μg/day
Adults	30 μg/day
Pregnancy	30 μg/day
Lactation	35 μg/day
EAR	
None reported	
RDA	
None reported	
UL	
None reported	

3.11 Folic Acid (67)

Because all vitamins are essential, it is difficult to state that one vitamin is more important than another. Nevertheless, folic acid, with its coenzyme role in purine biosynthesis, can be considered crucial for some of the cells' most fundamental biochemistry, cell division. This vitamin is intimately tied to vitamin B_{12} (cobalamin), which has made estimating its DRIs difficult. Also, conditions that can cause a folic acid deficiency also can result in a vitamin B_{12} deficiency.

3.11.1 Chemistry. The commercial form of the vitamin is folic acid (Fig. 8.47). It consists of a pteridine ring attached to a *p*-aminobenzoic acid that is attached to the a-mine of glutamic acid. Two biosynthetic changes must occur before it is active. First, it must be reduced to tetrahydrofolate by dihydrofolate reductase in a two-step reduction (Fig. 8.48). Notice that niacin is required for this reduction. Second, a polyglutamate chain must be attached to the γ-carboxyl of the parent glutamic acid (Fig. 8.47). The remaining linkages of the polyglutamate chain are traditional a-amino-a-carboxyl peptides.

The natural vitamin is made up of a family of polyglutamates all connected to the initial glutamic acid at the previously described γ-carboxyl group. The length of this polyglutamate chain varies with the source of the vitamin, but lengths of 3, 5, and 7 amino acids are seen. The most common polyglutamate found in food is 5-methyltetrahydrofolate polyglutamate (Fig. 8.47).

3.11.2 Uptake. The dietary polyglutamates are cleaved to the monoglutamate vita-

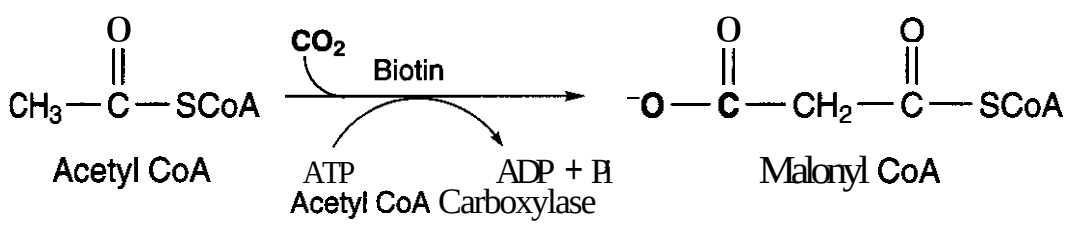


Figure 8.44. Carboxylation of acetyl CoA producing malonyl CoA.

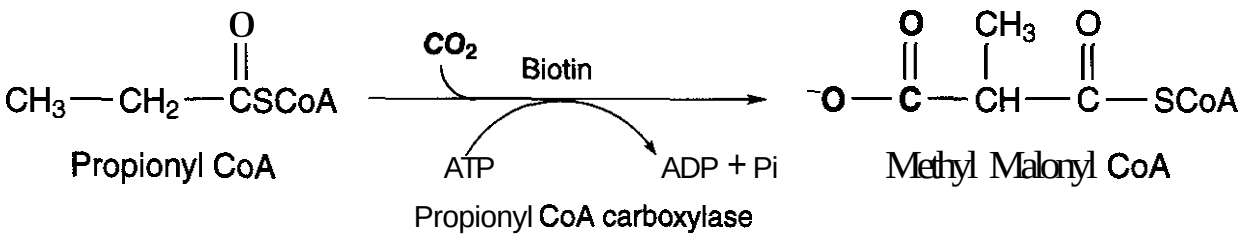


Figure 8.45. Carboxylation of propionyl CoA producing methylmalonyl CoA.

min by a y-L-glutamylcarboxy peptidase, commonly called conjugase. Folic acid is absorbed as the monoglutamate. Conjugase is found in the brush border of the intestine. Therefore, chronic inflammatory conditions in the intestine lead to low conjugase activity, which can result in significant decreased folic acid absorption.

The absorbed vitamin must be converted to the coenzyme form. This requires adding back a five- to seven-member glutamate chain and commercial folic acid must undergo the two-step reduction to tetrahydrofolic acid. There are two common abbreviations for the reduced form, FH_4 and THF. These reactions apparently occur in a wide variety of tissues. The liver contains about a 3- to 6-month supply of the vitamin, presumably in the polyglutamate form.

3.11.3 Metabolic Roles. There are five forms of tetrahydrofolate polyglutamate, some of which are coenzymes (Fig. 8.49). The most highly oxidized is 10-formyl tetrahydrofolate polyglutamate, produced by addition of formic acid to tetrahydroformate polyglutamate. It is the coenzyme for two reactions in purine biosynthesis: (1) the synthesis of formylglycine ribotide (FGAR) from glycine amide ribotide (GAR) and (2) the formation of aminoimidazole carboxamide ribotide (AICAR) to formamidoimidazole carbxamide ribotide (FAICAR) (Fig. 8.50).

5,10-Methenyl tetrahydroformate polyglutamate is the cyclic enamime formed from 10-formyl tetrahydroformate polyglutamate. It also is formed from the catabolism of histidine and can be considered an intermediate between the 10-formyl compound and, upon reduction, 5,10-methylene tetrahydroformate polyglutamate. The latter is the coenzyme required for the interconversion of serine and glycine and the methylation of deoxyuridylic acid forming deoxythymidylic acid (Figs. 8.49 and 8.51). Most of the one-carbon units carried on position 5 or 10 or 5,10 bridge come from serine.

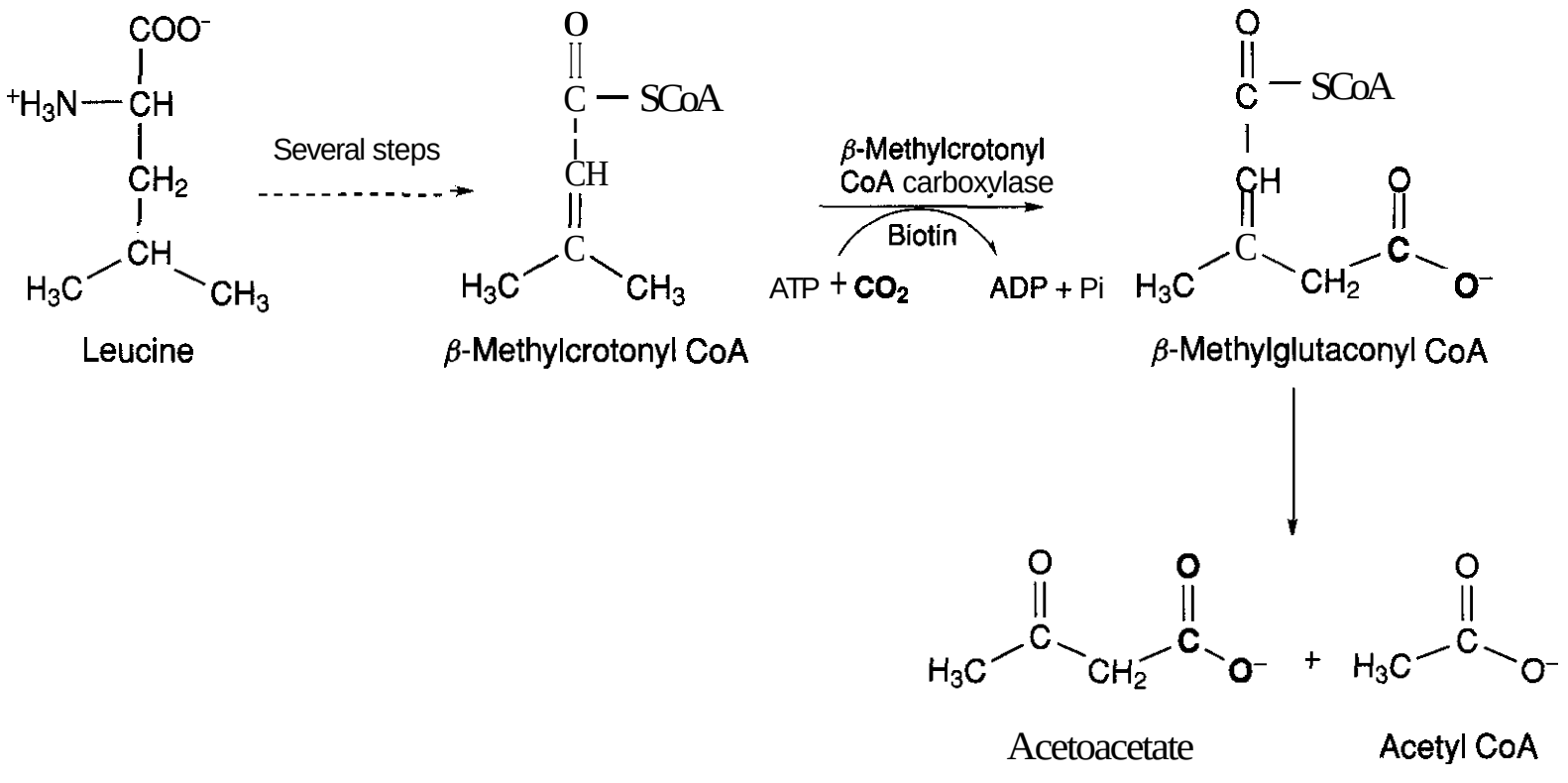
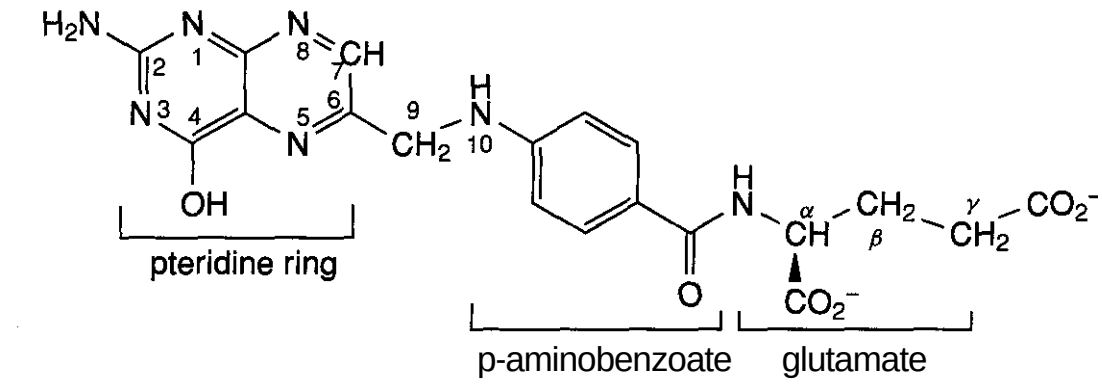
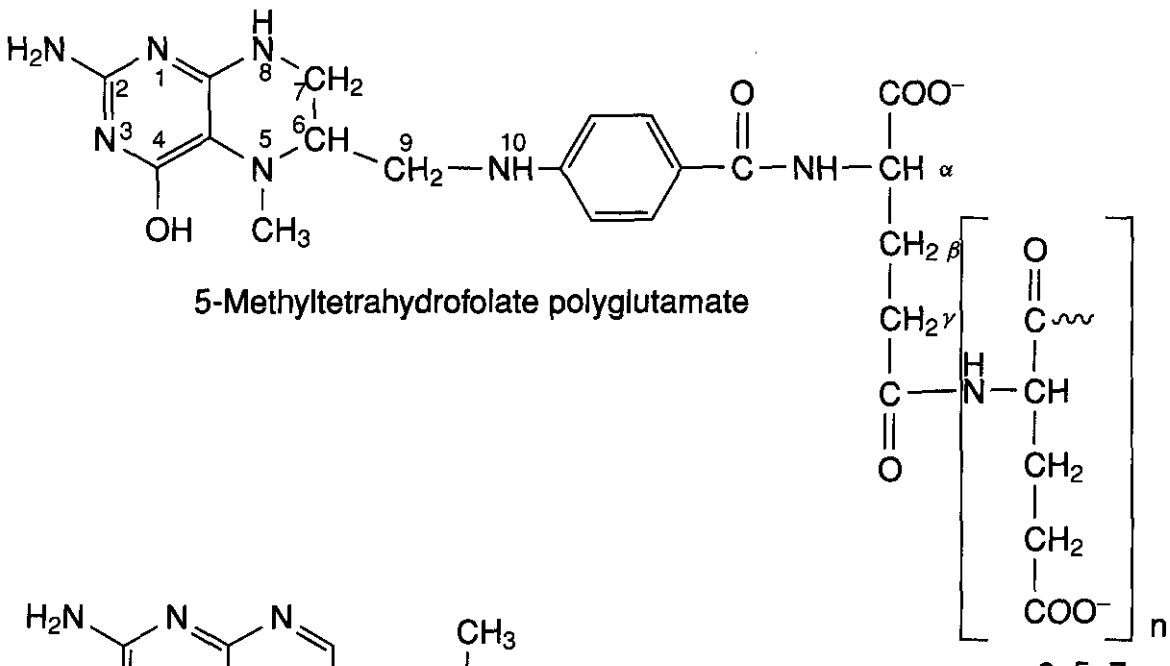


Figure 8.46. Role of biotin in leucine catabolism.

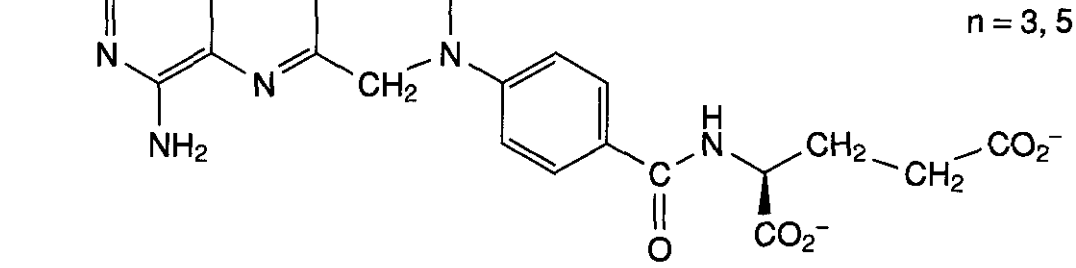


Folic Acid (FA)



5-Methyltetrahydrofolate polyglutamate

n = 3, 5, 7



Methotrexate

Figure 8.47. Common forms of folic acid and methotrexate.

The most reduced coenzyme is 5-methyl tetrahydrofolate polyglutamate. It is the source of the methyl group added to homocysteine regenerating methionine and tetrahydrofolate ready to accept a one-carbon unit from formate or serine. This last reaction is where folic acid and vitamin B_{12} come together (Figs. 8.49, 8.52, and 8.53). The implications of this reaction and how folic acid can mask pernicious anemia are discussed in the section on vitamin B_{12} (cyanocobalamin). Note that the formation of 5-methyl-THF normally is not reversible. Tetrahydrofolate can be regenerated only if there is adequate methyl cobalamin coenzyme.

The fifth tetrahydrofolate compound is 5-formyl THF (folinic acid, citrovorum factor). This compound is not a coenzyme, but it can be converted to any of the active coenzyme forms. It is administered after treatment with the dihydrofolate reductase inhibitor, methotrexate (Fig. 8.47), as a form of rescue therapy. Because it already is in the reduced tetrahydrofolate form, it does not need dihydrofolate reductase to become an active coenzyme.

3.11.4 Folic Acid Deficiency. It is obvious that folic acid is a very important vitamin for biosynthetic reactions, particularly those required for the biosynthesis of purines, methyl-

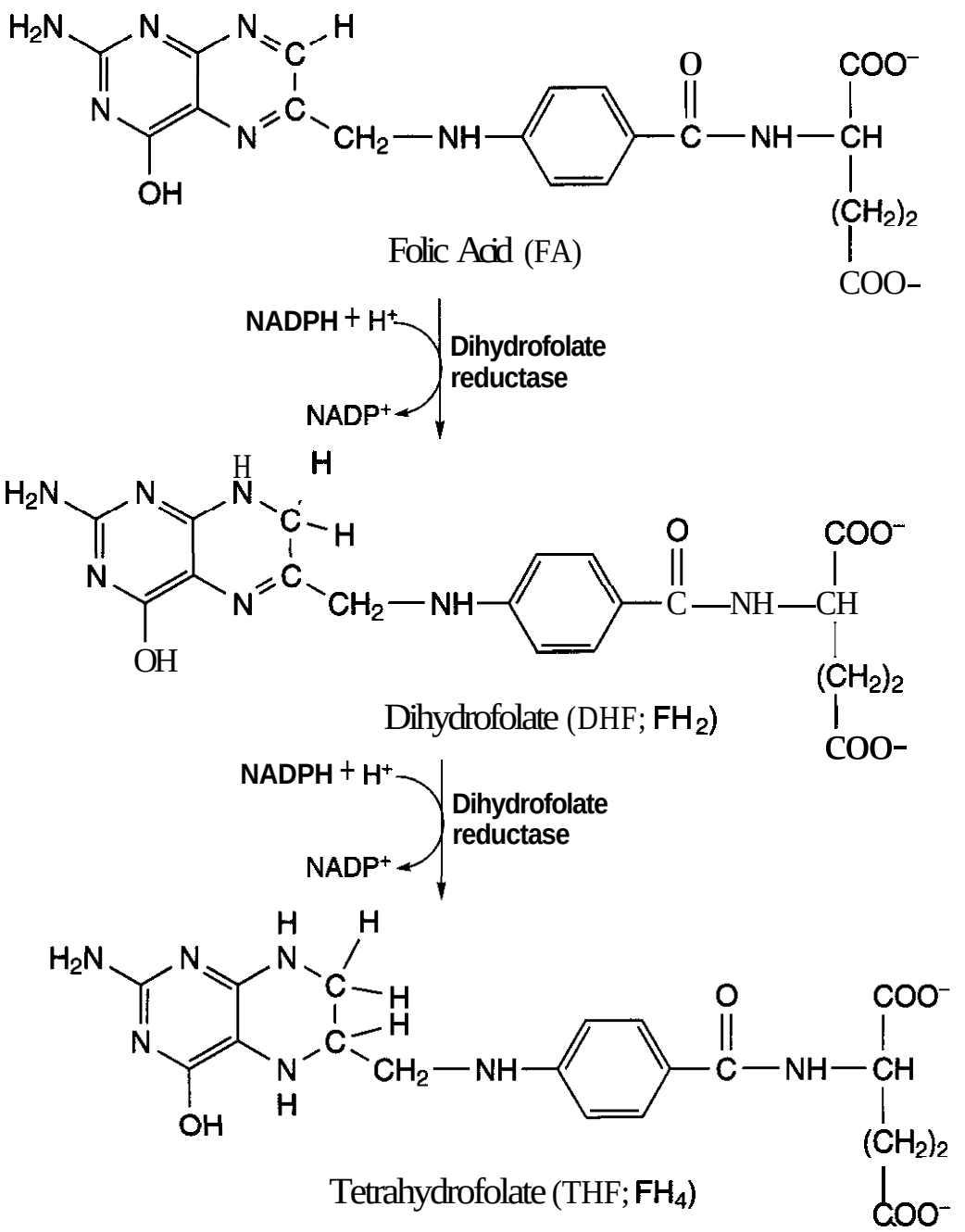


Figure 8.48. Reduction of folic acid to tetrahydrofolate.

ation of deoxyuridylic acid, and regeneration of methionine from homocysteine. The main deficiency is a characteristic megaloblastic anemia attributed to a shortage of nucleotides required for the production of erythrocyte precursor cells.

Another clinical sign of folic acid deficiency is neural tube defects, including spina bifida and anencephaly. Neural tube defects constitute one of the main reasons that federal regulations mandate supplementing cereal grain-based foods with folic acid along with thiamine, riboflavin, and niacin (68, 69). Although prenatal multiple vitamins contain adequate amounts of folic acid, pregnant women may not start taking these products until the second or third month of the their pregnancies, and this may be too late.

A third indication of inadequate folic acid is elevated blood homocysteine levels, with attendant increased risk of cardiovascular disease. This hypothesis is based on an observation that individuals with increased blood vessel plaque buildup also show increased levels of homocysteine. The elevated homocysteine can be corrected, at least partially, with folate supplements. Figure 8.52 illustrates why the three vitamins, pyridoxine, folic acid, and vitamin B_{12}, are indicated for elevated levels of homocysteine. Pyridoxal phosphate (PLP) and cobalamin (B_{12}) are required for the catabolism of homocysteine to succinyl CoA. Methyl cobalamine (methyl B_{12}) is required for the conversion of homocysteine back to methionine.

There are many causes of folic acid deficiencies. Inadequate nutrition during periods of increased requirements is one of the main causes of megaloblastic anemia of pregnancy and neural tube defects. Alcoholism is considered the leading cause of folic acid deficiency

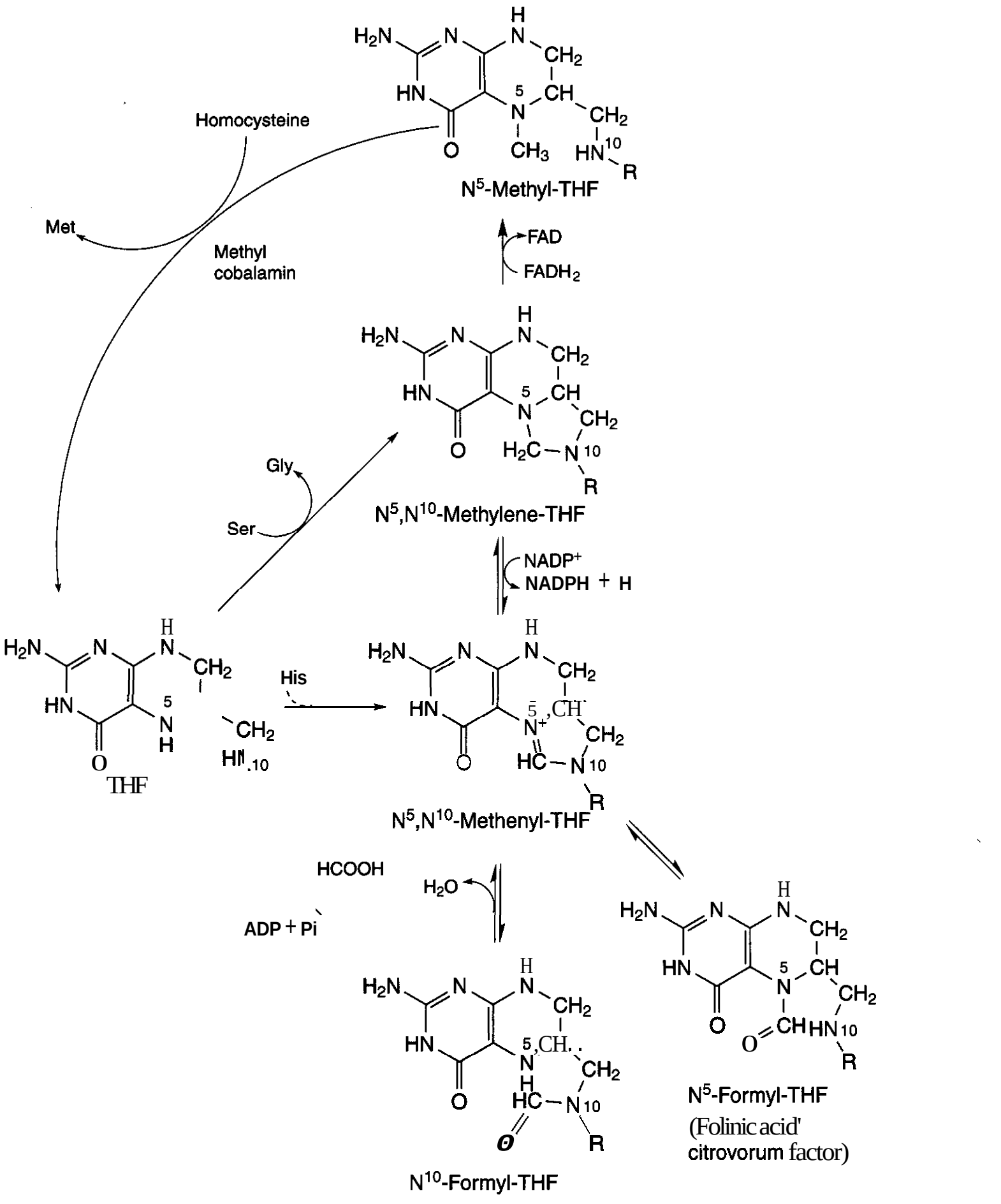


Figure 8.49. Formation of folic acid coenzymes.

in the United States (70, 71). There are two ways that excessive alcohol consumption can interfere with folic acid activity: impairment of folic acid reduction to the active THF forms and interference with folic acid storage and release from the liver. A third cause of folic acid deficiency is chronic inflammation of the intestinal mucosa. Inflammation can reduce production of the required conjugase enzyme, which removes the polyglutamate chain,

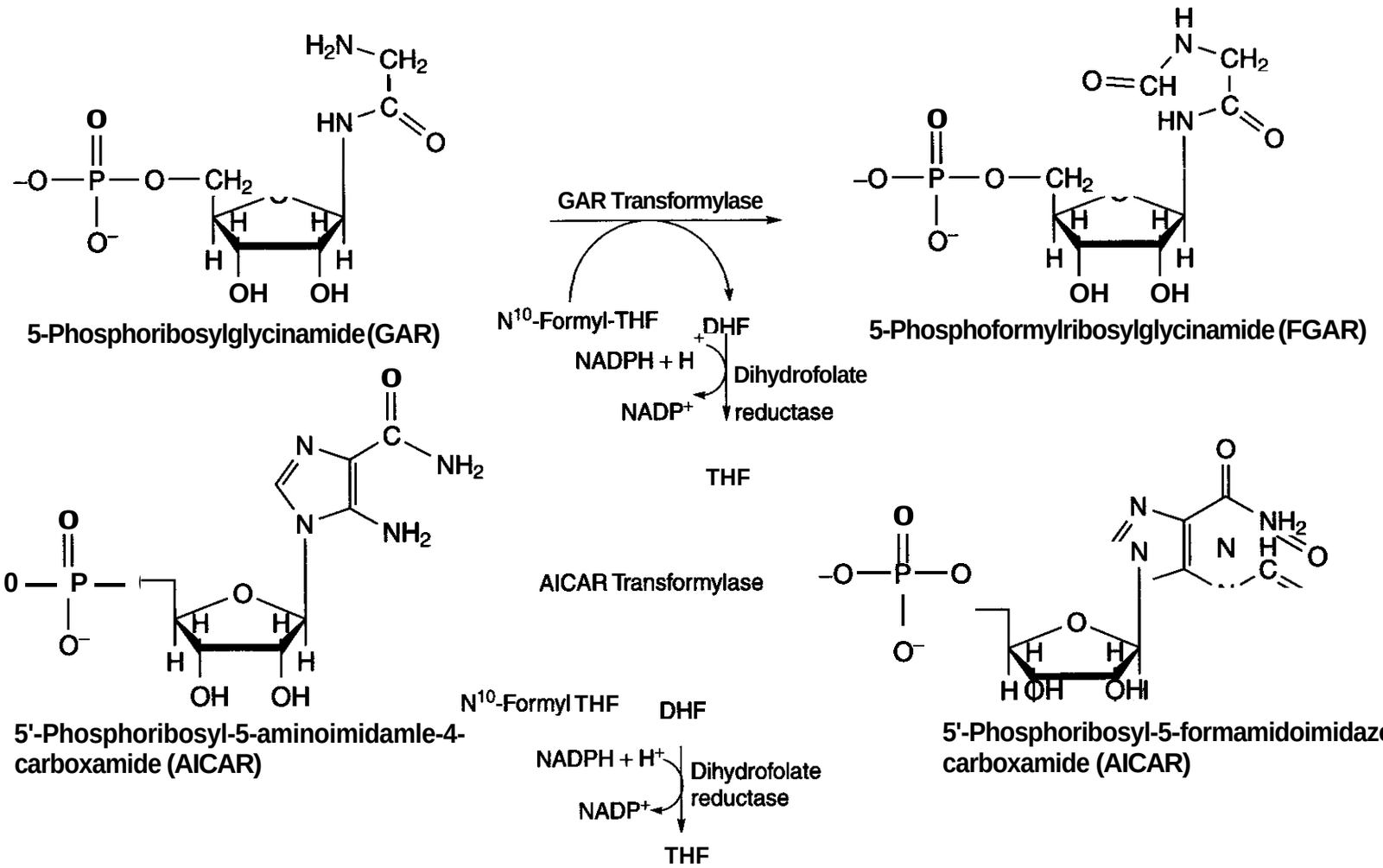


Figure 8.50. Coenzyme roles for 10-formyl **THF.**

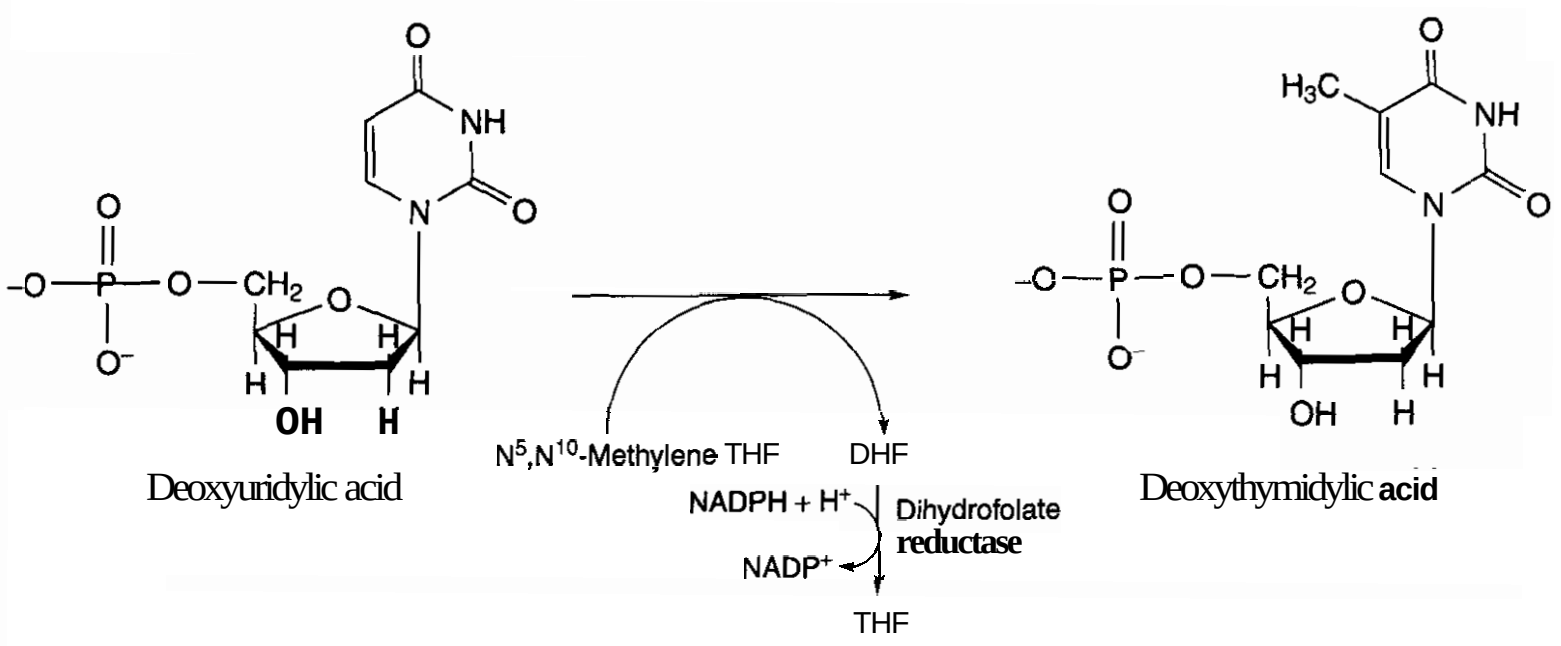


Figure 8.51. Methylation of deoxyuridylic acid forming deoxythymidylic acid.

and/or an inflamed mucosa inhibits folate transport. Finally, anticonvulsants such as phenytoin somehow interfere with folic acid uptake or utilization.

The dihydrofolate reductase inhibitor, methotrexate (Fig. 8.47), was developed as an anticancer drug, whose inhibition of formation of folic acid coenzymes would block purine synthesis. In other words, it was designed to induce a folic acid deficiency. Notice in Figs. 8.50 and 8.51 that formation of dTMP, FGAR, and AICAR also causes the oxidation of tetrahydrofolate to dihydrofolate. The latter must be reduced by dihydrofolate reductase to tetrahydrofolate before active coenzyme can form again. Thus, not only does methotrexate inhibit the initial formation of the tetrahydrofolate moiety, it blocks regeneration of the coenzyme form.

3.11.5 Hypervitaminosis Folic Acid. This apparently is not a problem. Transport across the intestinal mucosa may be regulated by a feedback mechanism or the rate of hydrolysis of the polyglutamate chain or a combination of both. What is very important is that taking the vitamin in doses above 400 μg (800 μg in pregnant and lactating women) can mask the macrocytic anemia seen with pernicious anemia caused by a cyanocobalamin deficiency (Fig. 8.53). The Tolerable Upper Limit is based on trying to avoid this masking. Therefore, the UL to RDA ratio is low (2–2.5) in adults.

3.11.6 Dietary Reference Intakes.

AI	
Infants	65–80 μg/day
EAR	
Children (1–8 years)	120–160 μg/day
Children (9–13 years)	250 μg/day
Adolescents (14–18 years)	330 μg/day
Adults (19–50+ years)	320 μg/day
Pregnancy	520 μg/day
Lactation	450 μg/day
RDA	
Children (1–8 years)	150–200 μg/day
Children (9–13 years)	300 μg/day
Adolescents (14–18 years)	400 pglday
Adults (15–50+ years)	400 μg/day
Pregnancy	600 μg/day
Lactation	500 μg/day
UL (from fortified food or supplements)	
Children (1–3 years)	300 μg/day
Children (4–8 years)	400 μg/day
Children (9–13 years)	600 μg/day
Adolescents (14–18 years)	800 pglday
Adults (19+ years)	1000 μg/day
Pregnancy (14–18 years)	800 μg/day
Pregnancy (19+ years)	1000 pglday
Lactation (14–18 years)	800 μg/day
Lactation (19+ years)	1000 μg/day

3.12 Vitamin B_{12} (Cobalamin) (72)

This chemically very complex vitamin is required for two reactions, the methylation of ho-

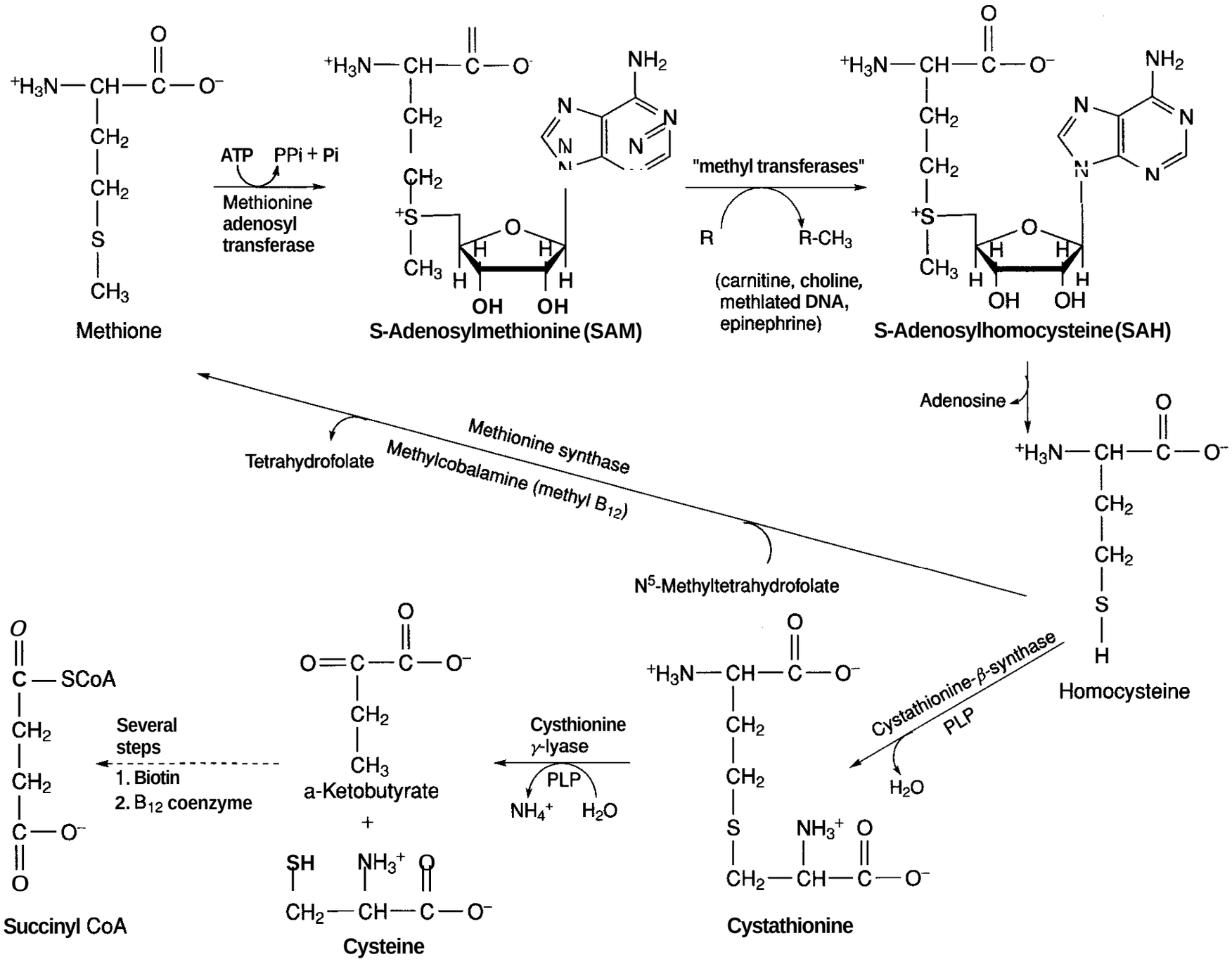


Figure 8.52. Methionine metabolism.

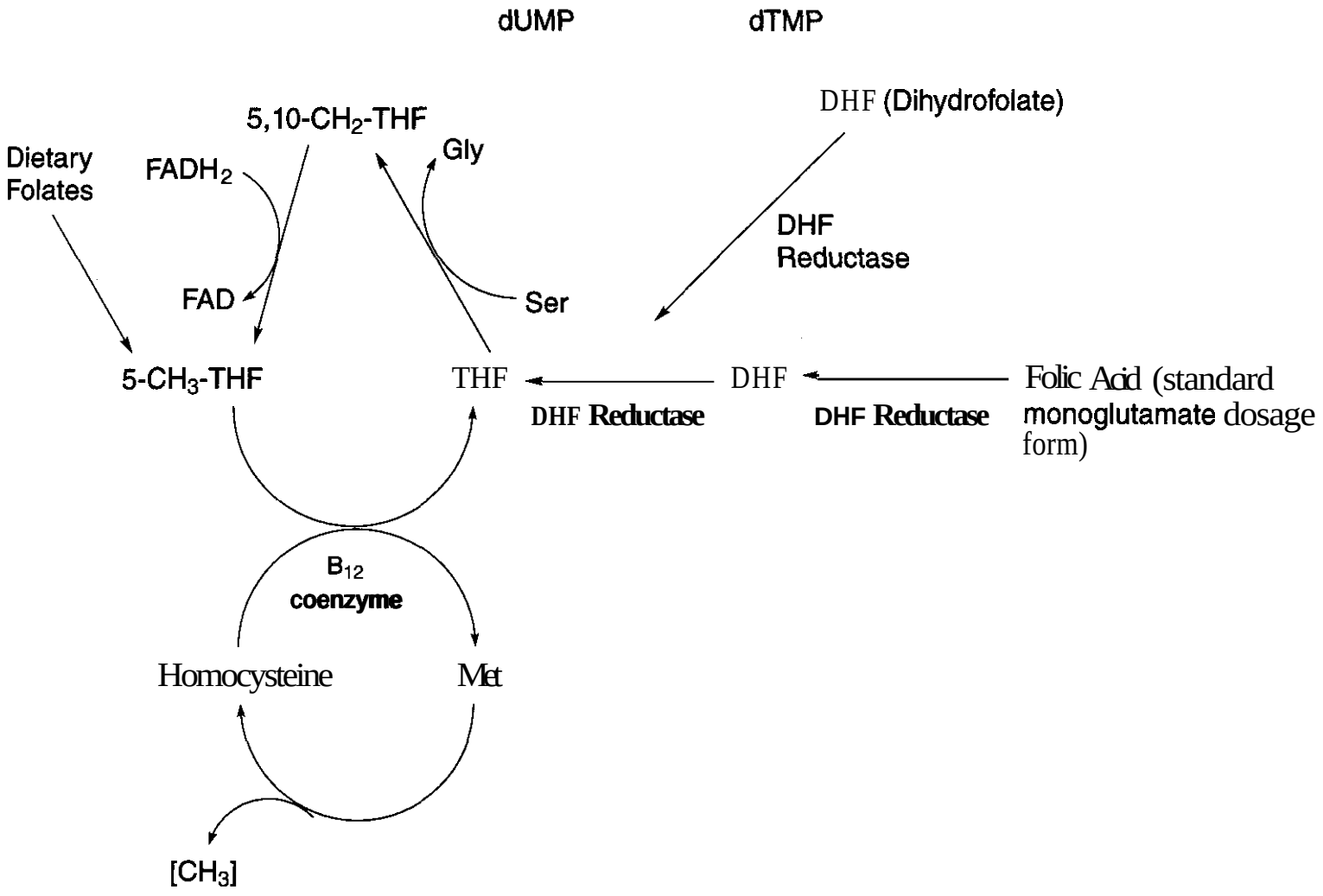


Figure 8.53. Methyl trap hypothesis.

mocysteine to methionine and rearrangement of methyl malonyl CoA to succinyl CoA. A deficiency leads to pernicious anemia, at one time a disease whose prognosis was death. Because folic acid can mask the blood picture of a cobalamin deficiency, it has become important for physicians to order tests specific for the vitamin.

3.12.1 Chemistry. The cobalamin family consists of a corrin ring (Fig. 8.54). It is similar to that of the porphyrin ring system, except that there is no methylene or methine bridge between pyrrole rings A and D, and it contains cobalt rather than iron. The commercial form sold in the United States is cyanocobalamin. The hydroxy dosage form also has been used. The coenzyme forms include methyl and adenosyl cobalamin. The commercial vitamin is produced from bacterial fermentation.

3.12.2 Uptake. Uptake of the vitamin from food and vitamin products is complex. Indeed, most deficiencies are not from inadequate diet, but result from defects in the uptake process. Dietary cobalamin requires a fully functioning stomach and ileum of the small intestine (73). Parietal cells in the stomach produce hydrochloric acid, to free the vitamin from the food, and "intrinsic factor," a glycoprotein that binds cobalamin. This complex attaches to specific receptors in the ileum. Eventually, the vitamin passes into systemic circulation and is transported by a series of plasma-binding proteins, the transcobalamins. About 50% of the absorbed vitamin reaches the liver, with the remainder transported to other tissues.

Humans are efficient recyclers of vitamin B_{12} because of enterohepatic circulation. The vitamin is secreted in the bile. Upon combining with intrinsic factor, the absorption process is repeated. This recirculation probably explains why dietary deficiencies are uncommon and why the inability to produce intrinsic factor results in vitamin B_{12} deficiency, even though there may be adequate dietary intake.

3.12.3 Biochemical Role. Cobalamin is a coenzyme in only two reactions, but they are basic to the health of the individual. Methyl

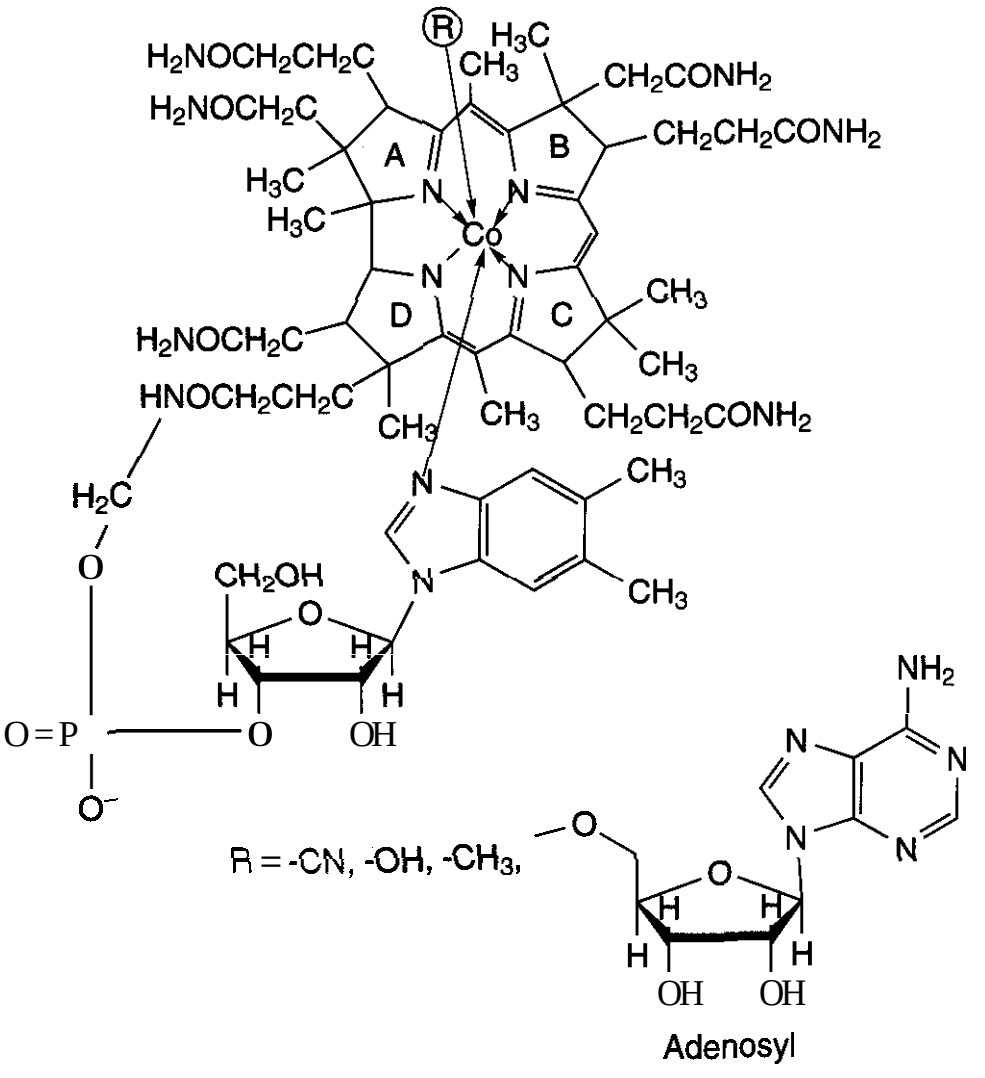


Figure 8.54. Vitamin B_{12} and coenzyme forms.

cobalamin is required for the regeneration of methionine from homocysteine. 5-Methyltetrahydrofolate polyglutarnate is also required (Figs. 8.49 and 8.53). The second reaction is the rearrangement of methylmalonyl CoA to succinyl CoA with adenosylcobalamin as the coenzyme (Fig. 8.55). Odd-numbered fatty acids and the amino acids valine, isoleucine, and

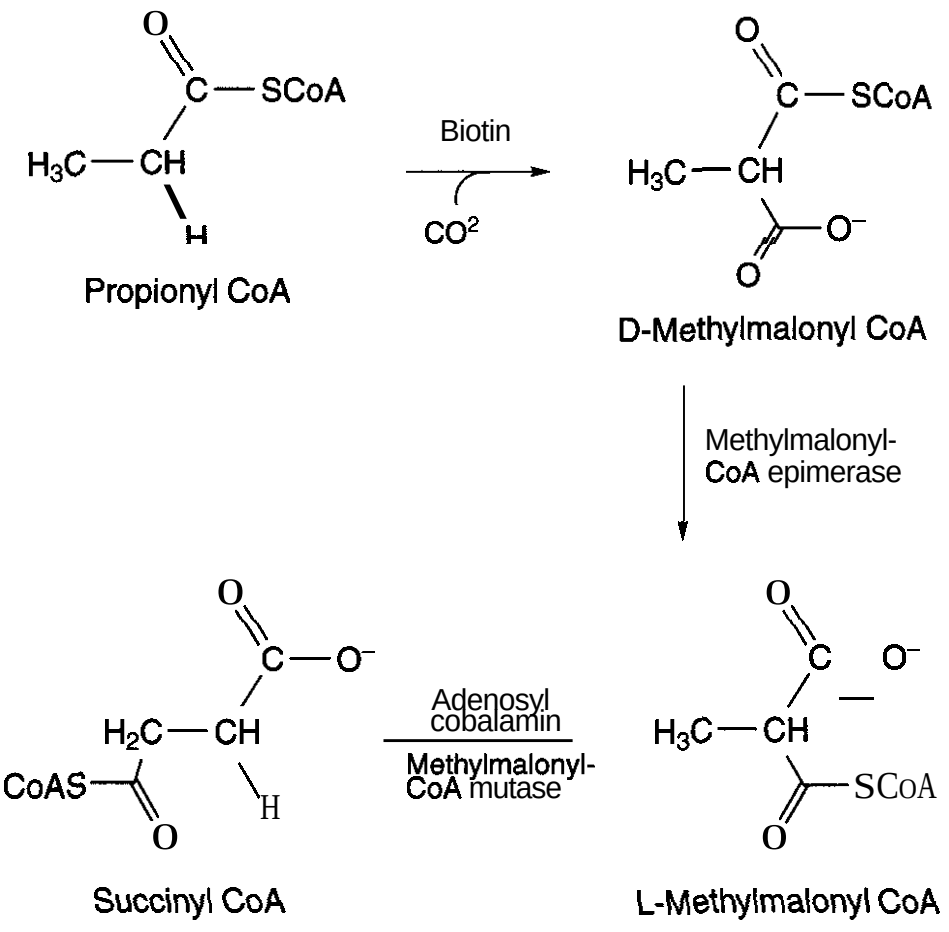


Figure 8.55. Cobalamin-catalyzed mutase reaction.

methionine produce propionyl CoA as part of their catabolism. Biotin-catalyzed carboxylation (Fig. 8.45) yields D-methylmalonyl CoA, which must be epimerized to the *L*-stereoisomer. The latter undergoes a rearrangement to succinyl CoA, which enters the Kreb cycle for final degradation. There has been considerable debate as to the mechanism of this rearrangement, but the general consensus is that there are free-radical intermediates (74–76).

3.12.4 Cobalmin Deficiency. Pernicious anemia is the disease associated with vitamin B_{12} deficiency. It is usually caused by the inability to produce intrinsic factor. Indeed, many times the vitamin must be administered by injection. The blood picture, a megaloblastic anemia, is indistinguishable from that caused by folic acid deficiency. Indeed folic acid supplements can mask the blood picture. This is illustrated in Fig. 8.53. Removal of adenosyl cobalamin eliminates the regeneration of tetrahydrofolate during the methylation of homocysteine to methionine. Folic acid supplements provide a fresh source of tetrahydrofolate coenzymes. DNA synthesis can continue and new erythrocytes form. Excess folic acid also may compete for the available vitamin, further exacerbating vitamin B_{12} deficiency.

Pernicious anemia can be lethal if not treated because of nerve damage. There are two explanations for the cause of this damage, both involving an excess of methylmalonyl CoA. Methylmalonyl CoA is a competitive inhibitor of malonyl CoA during fatty acid synthesis. This may impede repair of the myelin sheath surrounding nerves. Alternatively, methylmalonyl CoA replaces malonyl CoA as a substrate in fatty acid synthesis, producing fatty acids with methyl substituents. These are incorporated into the lipids components of the myelin sheath, producing a nonfunctioning myelin sheath.

3.12.5 **Hypervitaminosis B_{12}.** The vitamin is considered nontoxic. There has been some concern that the presence of the CN^- anion in the commercial vitamin might cause problems with megadoses. However, 1000 μg of cyanocobalamin contains only 0.02 mg of CN. There are no Tolerable Upper Intake Levels.

3.12.6 Dietary Reference Intakes.

AI	
Infants	0.4-0.5 μg/day
EAR	
Children (1–13 years)	0.7–1.5 μg/day
Adolescents (14–18 years)	2.0 pglday
Men and Women (19–50+ years)	2 μg/day
Pregnancy	2.2 pglday
Lactation	2.4 pglday
RDA	
Children (1–13 years)	0.9–1.8 μg/day
Adolescents (14–18 years)	2.4 μg/day
Men and Women (19–50+ years)	2.4 pglday
Pregnancy	2.6 pglday
Lactation	2.8 pglday
UL	
None reported	

3.13 Ascorbic Acid (Vitamin C) (77)

Deficiencies of this vitamin have been associated with the early sailors who lacked fresh fruit and vegetables. However, it was a problem on land and was seen in the Irish potato famine, the California gold miners, and territorial prisons. There long has been a mystique surrounding this vitamin, with interest sharply increasing when Linus Pauling published his book *Vitamin C and the Common Cold,* forcing the medical, nutritional, and biochemical professions to reexamine carefully the role of this essential nutrient in human health. A significant problem with studying this vitamin is the fact that ascorbic acid is not a vitamin in most animals. It was not until the discovery that guinea pigs also require ascorbate that animal experiments could be conducted.

3.13.1 Chemistry. Ascorbic acid is derived from the aldonic acid form of L-gulose (Figs. 8.56 and 8.57). There are two enolic proton-donor groups, with the one at position 3 being the most acidic with a pK_a value of 4.1. Ascorbic acid is easily oxidized to the dehydro form without loss of vitamin activity, but the lactone ring now hydrolyzes easily, producing inactive open chain product.

Most animals, except for primates and guinea pigs, produce their own ascorbic acid

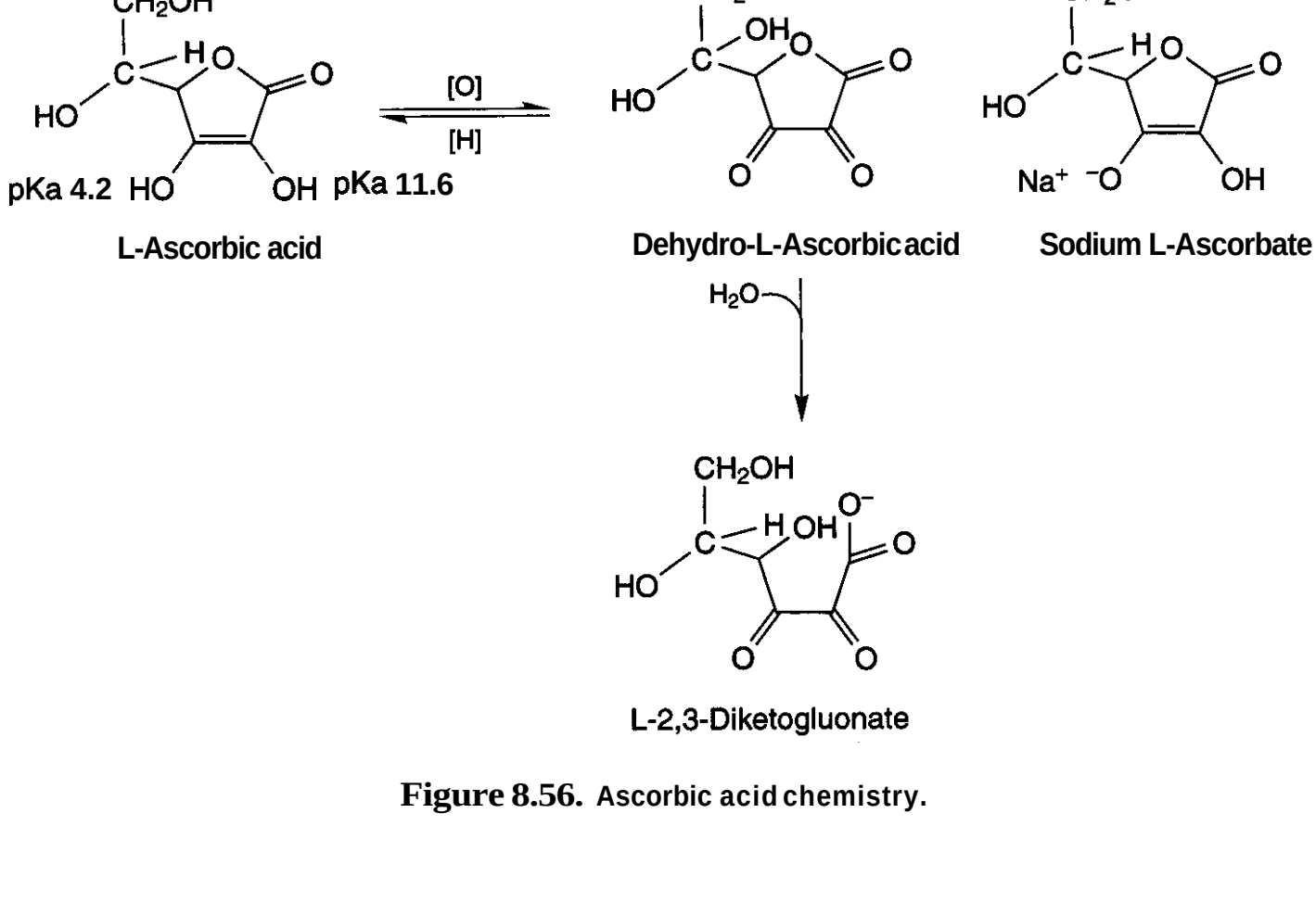


Figure 8.56. Ascorbic acid chemistry.

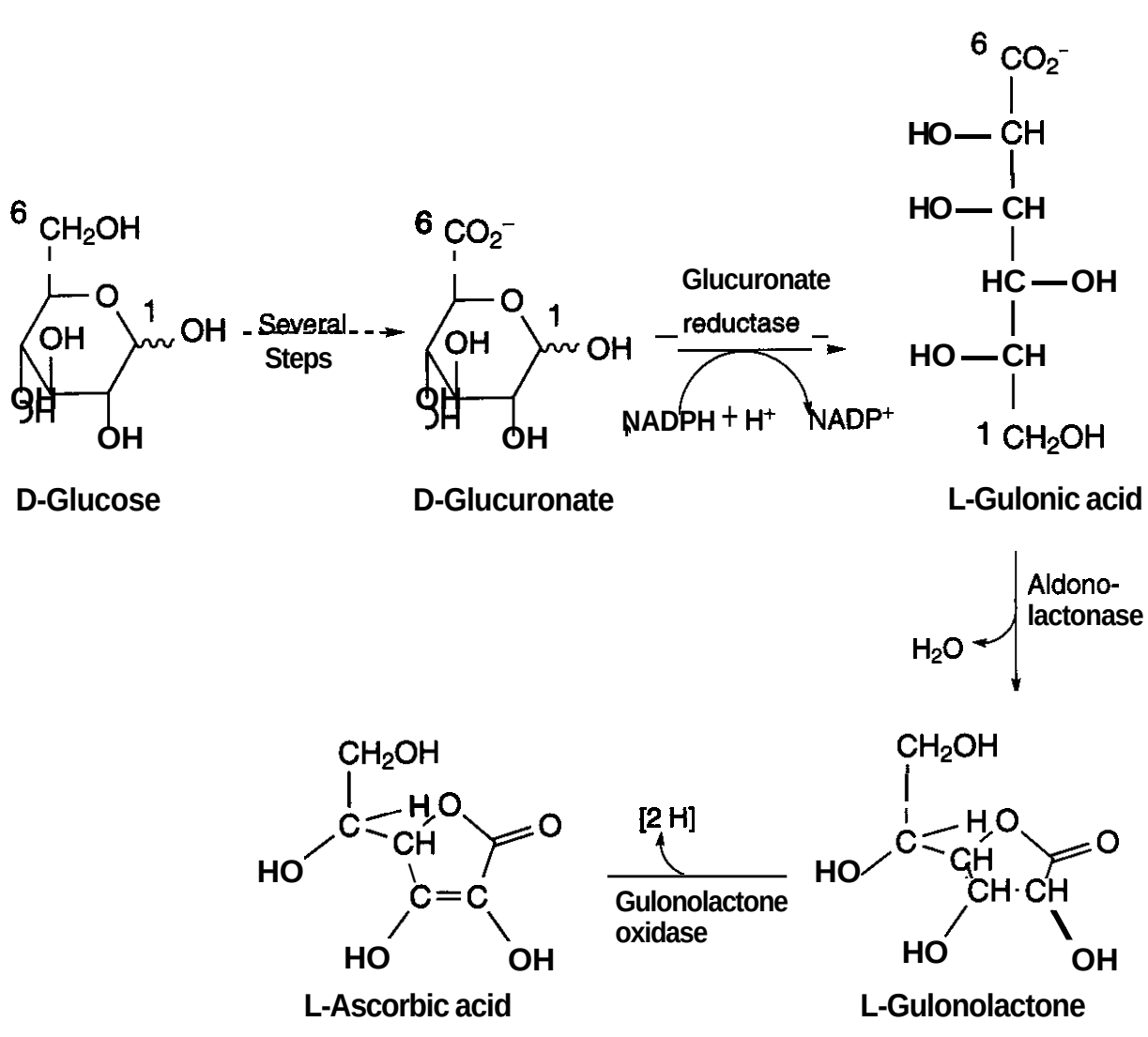


Figure 8.57. Outline of ascorbic acid biosynthesis.

Table 8.5 Metabolic Roles of Ascorbic Acid (Vitamin C)

Enzyme	Reaction	Contribution
Dopamine-β-hydroxylase	Hydroxylate dopamine phenethyl chain	Synthesis of norephrine
Peptidyl-glycine monooxygenase	Amidate carboxyl end of peptide hormones	Biosynthesis of peptide hormones
4-Hydroxyphenyl-pyruvatedioxygenase	Hydroxylate phenylalanine	Synthesis of tyrosine
Proline hydroxylase	Posttranslational Hydroxylation of proline	Crosslinking of collagen
Lysine hydroxylase	Posttranslational hydroxylation of lysine	Crosslinking of collagen
Trimethyl lysine hydroxylase	Hydroxylation of trimethyl lysine	Carnitine synthesis
4-Butyrobetaine hydroxylase	Oxidation of 4-butyrobetaine aldehyde	Carnitine synthesis
Cytochrome P450 isozymes	Oxidation of steroids	Corticosteroid biosynthesis

from glucose (Fig. 8.57). The pathway follows the standard route to glucuronic acid. The aldose carbon is reduced to an alcohol and, following normal carbohydrate-naming convention, the former carbon **6** of D-glucuronic acid becomes carbon 1 of L-gulonic acid. Cyclic L-gulonolactone forms and is oxidized to L-ascorbic acid. Humans and primates lack gulonolactone oxidase.

3.13.2 Uptake. Ascorbic acid is absorbed from the intestine by a sodium-dependent active transport system that is saturable. As the concentration of vitamin C increases in the intestinal tract, the absorption changes to passive diffusion. Once in systemic circulation, there are specific transporters based on cell types.

3.13.3 Metabolic Roles. Ascorbic acid is an electron donor required for a variety of oxidative processes. It is readily regenerated by glutathione, NAD, and NADP and thus has a long biological half-life. Currently, there are eight known human enzymes that require ascorbic acid, and they are listed in Table 8.5. The precise metabolic roles have not been completely elucidated, but it appears that in the metalloenzymes, ascorbate reduces the active metal site. In addition to these specific enzymes, ascorbic acid seems to function as a free-radical scavenger in the aqueous phase of plasma and cells.

3.13.4 Ascorbic Acid Deficiency. Scurvy is the classical disease associated with ascorbate deficiency. It is a disease of the connective tissue and probably is caused by inadequate crosslinking attributed to a lack of hydroxylated proline and lysine. Many consider scurvy to be an advanced stage of ascorbate deficiency. Chronic deficiencies may also (1) increase risk for malignancies, as evidenced by oxidized DNA markers and increased concentrations of reactive oxygen species; (2) decreased immune function, as evidenced by less vitamin in neutrophils and lymphocytes; **(3)** cardiovascular disease caused by the inflammatory response on the blood vessel walls; and (4) cataract formation caused by decreased concentrations of ascorbate in the ocular tissues.

3.13.5 Hypervitaminosis C. The vitamin is considered very safe. At one time, many of the over-the-counter products contained significant amounts of sodium ascorbate, which would be contraindicated in people on low sodium diets. Today's products are virtually sodium free unless labeled otherwise. Nevertheless, there are intermittent reports of adverse reactions associated with high doses. Therefore, there are Tolerable Upper Intake Levels, but these are very high relative to the RDAs. The UL to RDA ratio averages about 20.

3.13.6 Dietary Reference Intakes.

AI	
Infants	**40–50** mglday
EAR	
Children **(1–8** years)	**13–22** mglday
Boys **(9–18** years)	**39-63 mg/day**
Girls **(9–18** years)	**39–56** mglday
Men **(19–70+** years)	**75 mg/day**
Women (19–70+)	**60** mglday
Pregnancy	**66–70** mglday
Lactation	**96–100** mglday
RDA	
Children **(1–8** years)	**15–25** mglday
Boys **(9–18** years)	**45–75 mg/day**
Girls **(9–18** years)	**45-65** mglday
Men **(19–70+** years)	**90** mglday
Women (19–70+)	**75** mglday
Pregnancy	**80–85** mglday
Lactation	**115–120 mg/day**
UL	
Infants	Not established; use formula and food only
Children **(1–8** years)	**400–650** mglday
Boys and Girls (9–**13** years)	**1200 mg/day**
Adolescents **(14–18** years)	**1800** mglday
Adults (19+ years)	**2000** mglday
Pregnancy	**1800–2000** mglday
Lactation	**1800–2000 mg/day**

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CHAPTER NINE

Lifestyle and Over-the-Counter Drugs

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1 INTRODUCTION

The landscape of the pharmaceutical industry has undergone significant changes in recent years. The changes were brought about by increased demand from consumers to take control of their health **(1–3),** direct-to-consumer **(DTC)** advertising of prescription drugs (4, 5), technological advances in genomic research (6), and the search for blockbuster drugs by pharmaceutical companies **(7).** Today, consumers are more informed and want to take control of their health. The best example is the increased popularity of dietary supplements and the consumer lobbying to pass the Dietary Supplements Education and Health Act in the United States (8, **9).** The relaxation of the direct-to-consumer advertising regulation in **1997** provides an excellent avenue for the pharmaceutical industry to promote new therapeutic categories of prescription drugs such as lifestyle drugs that cater to lifestyle en-

hancement to the consumers. The desire of the aging baby-boomer to maintain an active lifestyle (10) and the difficulty of searching for blockbuster drugs in traditional therapeutic areas provide an excellent environment for the birth of lifestyle drugs.

The landscape of self-care has also undergone significant changes in recent years as consumers become more informed and as more highly effective prescription drugs (Rx) are switched to over-the-counter (OTC). The demand of the baby-boomer consumers to look and feel young has given rise to the popularity of dietary supplements (13, 14) and the so-called "cosmeceuticals" (10, 11). Some of the more successful Rx-to-OTC switches (e.g., hair-growth, smoking cessation) in recent years are related to lifestyle enhancement. Such lifestyle-enhancing products help create new therapeutic areas in the OTC drug arena.

This chapter will attempt to show how lifestyle drugs and over-the counter-drugs relate to each other and the challenges that they face. At first glance, they seem to be oceans apart; the former are mainly available by prescriptions and the latter are available without prescription. However, lifestyle drugs bear some resemblance to the over-the-counter drugs in that both are primarily for non-life threatening conditions and are heavily promoted by direct-to-consumer advertisement. Moreover, consumers and patients are taking an active role in choosing these two groups of products. Furthermore, they both cater to improve the quality of life of the consumers. Finally, lifestyle prescription drugs with low side effects will be ideal candidates for Rx-to-OTC switch. The following sections will provide an overview of OTC drugs, selected examples of lifestyle drugs, and the future of both groups.

2 SELF-MEDICATION

Before we discuss OTC drugs and lifestyle drugs, we would like to provide a brief overview of self-medication to help gain an appreciation of the changing attitudes of consumers. A recent survey conducted by Roper Starch Worldwide Inc. on behalf of the Consumer Healthcare Products Association (CHPA) showed that American consumers are increasingly comfortable with self-medication for minor medical problems. Some of the findings (2) are as follows:

- 73% would rather self-treat than see a doctor
- 96% say they are confident about their healthcare decision
- 80% used at least one OTC product to treat minor ailments
- approximately 90% of consumers read the label for possible side effects the first time they use an OTC product
- 57% of consumers say they are using or considering the use of dietary supplements
- 80% of those who take dietary supplements are happy with the outcomes

The above attitudes toward self-medication strongly suggest that American consumers cherish self-reliance and convenience. Similar attitudes are held by European consumers (3, 15) and are beginning to shape Japanese consumers (16). Today, consumers are generally better informed because of DTC advertising on television and the vast amount of medical information available on the internet. Consumers use OTC products to treat a variety of minor ailments such as headache, upset stomach, cough, and cold.

3 OVER-THE-COUNTER DRUGS

In the United States, there are two classes of drug products: prescription and nonprescription. Nonprescription drugs are often referred to as over-the-counter drugs because they are available for general sale to consumers without a prescription (17). Consumers can purchase OTC drug products off the shelf from a variety of mass distribution outlets such as convenience, grocery, and drug stores, with the exception of Schedule V Controlled Substance and insulin products, which are kept behind the pharmacist counter and dispensed by pharmacists. In European countries, most OTC drugs are only available at the pharmacy, although in some countries, e.g., U.K., the simplest products are widely available through gen-

eral sale (18, 19). However, there is a growing trend to allow more non-prescription products to be sold outside the pharmacy. When we speak about OTC products, we also include medical devices such as pregnancy test kits and dietary supplements. This chapter will focus on OTC drugs only. The emphasis will be on the U.S. OTC drug classification process, with a brief overview of the OTC drug classification processes in Europe and Japan.

3.1 OTC Drug Classification in the United States

To gain a better understanding of the OTC classification process, we need to take a look at the history of the regulatory process in the United States.

3.1.1 History of the Regulatory Approval Process. The process of drug approval underwent an evolutionary change throughout the 20th century (20, 21). Major changes are invariably triggered by some public health hazards. The first major piece of legislation at the beginning of this century was the 1906 Pure Food and Drug Act, which mandated that the labeling on the package be truthful. There was no requirement for safety and efficacy as we know today. The New Drug Application (NDA) process did not begin until the passage of the original 1938 Federal Food, Drug and Cosmetic (FD&C) Act, which only demanded that the manufacturer submitted an NDA with safety data before marketing a new drug. The manufacturer could market the product if the FDA had no objection to the NDA within 60 days. It was up to the manufacturer to decide whether or not the new drug would be prescription or OTC. The distinction between prescription drugs and OTC drugs is based on the Durham-Humphrey amendment in 1951, which limited the following three classes of drugs to prescription status (22):

1. Certain habit-forming drugs
2. Drugs that cannot be safely used without professional supervision
3. Drugs limited to prescription under an NDA

A subsequent 1962 amendment (Kefauver-Harris) to the 1938 FD&C Act in 1962 clarified that drug products must be both safe and efficacious and be approved by the FDA before marketing (23). As a result of the 1962 amendment, the FDA was required to retrospectively review all NDAs approved between 1938 and 1962 for both efficacy and safety. The FDA, in collaboration with the National Academy of Sciences and National Science Foundation, reviewed all NDAs submitted between 1938 and 1962 through the Drug Efficacy Study Implementation (DESI) review program (22–25). This review focused primarily on prescription products. After the completion of the review of the prescription products, FDA turned their attention to OTC products.

3.1.2 OTC Drug Review. In the 1960s, the OTC drug market back then was similar to today's market in that there was a proliferation of products based on the same actives (24–28). For this reason, it became impossible to review every OTC product on the market. For practical reasons, the FDA decided to review active ingredients by therapeutic categories instead of individual finished products. In 1972, the FDA initiated the OTC Drug Review process. The goal of the OTC Drug Review was to determine which active ingredients and their associated conditions of use might be considered to be generally regarded as safe (GRAS) and efficacious (GRAE) and not misbranded. The FDA set up a total of 17 public advisory panels to review close to 90 categories of OTC drugs (24, 25). All OTC products, including Grandfather OTC products introduced before 1938, were also reviewed. The review was conducted in three rule-making phases.

***3.1.2.1* Phase 1: Advisory Panel Review.** The panels were asked to categorize the active ingredients in each class of products and their claims into one of three categories based on review of data submitted by manufacturers, scientific data in the literature, and their own experiences. The three categories (24, 25) are as follows:

- Category I: generally recognized to be safe and effective for the claimed indications
- Category II: not generally recognized as safe and effective or unacceptable for the claimed indications

Table 9.1 *OTC* Monographs

Already Published	Still Pending
Acne	Antidiarrheal
Anorectal	Antiseptic first aid
Antacid	Antiseptic (professional)
Anthelmintic	Antiperspirants
Antibiotic first aid	Cough/cold combinations
Anticaries	External analgesic
Antiemetic	Internal analgesic
Antiflatulent	Laxative
Antifungal	Menstrual
Cholecystokinetic	Oral discomfort
Corn/callus removers	Oral health care
Cough/cold antihistamine	Otic (ear drying)
Coughlcold antitussive	Overindulgence
Coughlcold bronchodilator	Poison treatment
Coughlcold expectorant	Skin bleaching — (reproposal)
Coughlcold nasal decongestant	Skin protectants
Dandruff/seborrhea/psoriasis	Vaginal drug products (douches)
Deodorants (internal)	
Male genital desensitizers	
Nighttime sleep aids	
Otic (earwax)	
Pediculicide	
Stimulant	
Sunscreen	
Wart remover	
Weight control	

Source: Ref. 28 (Reproduced with permission from Consumer Healthcare Products Association).

- Category III: insufficient data available to permit final classification into Category I or Category II

The above three-category classification system is not in use today. Drugs applied for OTC use are classified either as "monograph" or "non-monograph" status. The findings of the advisory panels were published in the Federal Registry as "advance notice of proposed rulemaking" (ANPR), which sets forth the advisory panel's recommendation for OTC monograph by category of use (Table 9.1), including recommendations on general recognition of safety and effectiveness for each active ingredient. This phase of review took about 10 years to complete.

3.1.2.2 Phase 2: FDA Review. After publication of an advance notice of proposed rulemaking, any interested party may submit comments and additional new data to support their point of view. The FDA reviews all the comments and newly submitted data and published their rulings in a "tentative final monograph" (TFM) or proposed rule, which sets forth FDA's revisions to the ANPR relating to active ingredients and conditions of use. The TFM groups actives together for specific indications to form what we now call a "monograph" (Table 9.1). The rulings on those actives and indications where no claims can be made are called "negative monographs" (Table 9.2). In a similar manner to the ANPR, additional comments and data could be submitted to clarify or refute the rulings.

3.1.2.3 Phase 3: The Final Monograph. After reviewing comments to the proposed rule (or TFM), the FDA publishes the "Final Monograph" in the form of a "Final Rule" in the Federal Register, which is then incorporated into the Code of Federal Regulations (21CFR part 330). The final monograph consists of a list of active ingredients approved for the specific indications, labeling, general provisions,

Table 9.2 Negative Monograph (Non-Monograh)

Aphrodisiac	Hypo/hyperphosphatemia
Benign prostatic hypertrophy	Ingrown toenail
Boil treatment	Insect repellant (oral)
C/C anticholinergic	Nail biting/thumbsucking
Daytime sedatives	Oral wound healing
Digestive aids	Prevention of inebriation
Exocrine pancreatic insufficiency	Smoking deterrents
Fever blisterlcold sore (internal)	Stomach acidifiers
Hair grower	Vaginal contraceptives

Source: Ref. 28 (Reproduced with permission from Consumer Healthcare Products Association).

and testing procedures. During the OTC Drug Review, misbranded products that did not meet the monograph requirements would need to be reformulated, or their labels needed to be revised to stay on the market. A drug manufacturer can market an OTC product based on those active ingredients for the intended indications described in the monograph without the need of an NDA submission. However, a drug manufacturer cannot make claims not described in the monograph unless they submit additional data to petition for a revision of the final monograph or justify their deviations and obtain approval through an NDA. Even though the monographs are based on active ingredients and not on the dosage forms, the implicit assumption is that the dosage forms (e.g., tablet, capsule, liquid) will not affect the efficacy and safety of the active. Whereas acetaminophen immediate-release tablets, capsules, and liquids can be marketed without an NDA submission, marketing a sustained-release acetaminophen tablet will still require a submission of an NDA deviation.

Although most of the final monographs have been published, there are still 17 final monographs awaiting completion (Table 9.1). The OTC review process is dynamic in the sense that final monographs *can* be amended. New ingredients or indications can also be petitioned for monograph status based on material time and material extent (29). For example, foreign OTC products and existing OTC actives approved through an NDA *can* be petitioned for monograph status after a certain period of marketing.

3.1.3 OTC Classification After OTC Drug Review. The OTC Drug Review is based on the principle set down in the Durham-Humphrey amendment that if a drug can be OTC, it should be OTC (23, 30). Prescription status should be an exception. However, the history of drug approval suggests otherwise. Most new chemical entities are initially approved as prescription drugs, which may then be switched after a period of at least 5 years of marketing experience and meeting certain switch criteria (29). Direct OTC approval of new chemical entity is a rare phenomenon.

Close to 30 drugs were reclassified from prescription to OTC status during the OTC Drug Review (24). Some of the common OTC cough cold actives such as diphenhydramine, brompheniramine, chlorpheniramine, and pseudoephedrine, together with hydrocortisone, were switched during that time. The switch can go in both directions. Metaproterenol sulfate inhaler was switched by FDA during the OTC Drug review and was later reversed back to prescription status because of the objection of the medical community. Today Rx-to-OTC switch is achieved by submitting an NDA for the same indication or new indications or new dose. Three-year market exclusivity will be granted to the sponsor if new clinical studies that are "essential" to the switch of an existing prescription product are conducted (31, 32). All the H_2 blockers that were switched received this type of exclusivity because additional studies were conducted to support the heartburn prevention claims. Five years exclusivity will be granted for a new OTC chemical entity that has not been marketed previously. A supplemental NDA is filed if a direct switch is desired without additional clinical studies. In this case, there is no marketing exclusivity other than what may already exist through patent protection. The

first generation vaginal antifungal products and minoxidil products represented direct switches and received no exclusivity. With or without exclusivity, successful Rx-to-OTC switches represent major growth opportunities for OTC companies.

3.1.4 Criteria for Reclassification of Drug/Rx-to-OTC Switch. The terms reclassification and Rx-to-OTC switch can be used interchangeably (25, 27, 30–37). There are many drivers for Rx-to-OTC switch. Pharmaceutical companies use Rx-to-OTC switch to increase the lifespan and profitability of a prescription drug that is coming off patent. Switch can also expand the market by tapping into a new patient population that previously can not be reached as a prescription product. An example is Imodium (38). Healthcare providers and governments who fund national healthcare insurance would like to advocate for the switch for cost-containment. Each Rx-to-OTC switch is unique and is evaluated on a weight-of-the evidence of science and data. Rx-to-OTC switch does not limit to self-limited, acute conditions. The scientific/regulatory paradigms of reclassification of drugs used by the OTC Drug Review are still applicable to today's Rx-to-OTC switches (30, 36, 37). Reclassification is based on the principles of benefit-risk assessment of safety and efficacy of the switched drug (33). The switch criteria center around the ability of a prescription product to meet the safety, effectiveness, and labeling requirement of being an OTC self-care product for a specific indication. Safety, effectiveness, and labeling are the three most important criteria in deciding potential Rx-to-OTC switches. The following sections describe the requirements of safety, effectiveness, and labeling for Rx-to-OTC switches in more detail.

3.1.4.1 **Safety.** Safety is especially important for OTC products because they are used without professional supervision. Safety does not mean that there is no potential toxicity nor abuse potential (33). It does imply a low incidence of adverse reactions or significant side effects under adequate labeling and a low abuse potential for harm (39). No drug is devoid of side effects, but the margin of safety should be much wider for OTC drugs compared with that of prescription drugs. The FDA demands that the drug manufacturer establishes a minimum effective dose and defines a daily allowable dose to minimize undue side effects. The switched Rx drugs should be free of any carcinogenic potential in humans. Reproductive toxicity, if any, should be addressed by proper OTC labeling. An example is the pregnancy/nursing warning for aspirin-containing OTC products because of the potential of Reyes syndrome (40). Any toxicity observed in animals should also be shown to be species-specific and have no relevance to humans.

3.1.4.2 **Effectiveness.** FDA regulation defines effectiveness as a reasonable expectation that the drug will have the claimed therapeutic effect in the targeted population when used according to instructions in the label (41). Effectiveness is demonstrated by controlled clinical studies. The clinical development for an Rx-to-OTC switch program generally starts with a dose ranging study to determine the minimum dose to produce a therapeutic response in most subjects in the target population. Oftentimes, a lower dose is switched to increase margins of safety. Actual use studies are then performed in the target population to demonstrate the effectiveness of the minimal effective dose and identify any rare side effects in OTC-like setting. For these reasons, the number of patients involved can be quite substantial.

3.1.4.3 **Labeling.** Clear and understandable labeling to average consumers, including those individuals with low literacy, is crucial to the safe and effective use of an OTC product without professional supervision. The label should contain information such as active ingredient, indications, warnings, directions for use, and proper storage condition. An average consumer of the target population of users should be able to understand the product label to self-recognize the signs and symptoms, select the right OTC product, understand the warnings, and self-treat appropriately as directed (30). Warnings may include contraindications, precautions, situations to avoid, what to do in the case of overdose, and drug–drug interactions (42). However, not all warnings are included in the label, only those that are clinically significant and important to the safe

and effective use of the product by the consumers. Labels should therefore be properly design and tested.

Label comprehension studies are designed to test the comprehensibility and readability of the proposed label under general conditions of purchase to ensure safe and effective use of the OTC products. They become more important for more complex Rx-to-OTC switches. In general, the Rx-to-OTC switch sponsor will work with FDA to define the comprehension study objectives before conducting the study. Several rounds of label comprehension studies are performed to refine the comprehensibility and readability of the label before initiating an actual use study in the target population in a simulated OTC setting to confirm the effectiveness of the product using the finalized label. Oftentimes, a self-selection study is also conducted to assess the ability of the consumer to select or de-select the product. An Investigational New Drug Application (IND) is not always required for label comprehension studies. An IND is required only if the sponsor would like to discuss the studies with FDA.

The content of the label of a monograph product should be consistent with the allowable indications and wordings specified by the relevant monograph. For Rx-to-OTC switch products, the label should be consistent with the label approved in the NDA or supplemental NDA. To make labels easier for consumers to read and comprehend, the FDA revised the labeling requirements for OTC products in 1999 to standardize the content, format, font size, headings, terminology and sequence of information (43). The new OTC Drug Facts label (44) contains six specific headings as depicted in Fig. 9.1 and resembles the Nutrition Fact label of food and nutritional products.

3.2 OTC Classification in Europe

In Europe, the OTC regulatory landscape is still fragmented despite the Mutual Recognition Procedure (MRP) and Directive 921261 EEC (18, 19, 45–47). Directive 92/26/EEC intends to provide a uniform framework for the classification of the medicinal products into prescription and non-prescription and facilitates the switch process (46, 47). However, its application is left with the regulatory approval agency of the individual country at the time when a marketing license is granted. This decentralized approach results in lack of consistency from country to country. As a result, some products are Rx in some countries but are OTC in other countries (Table 9.3).

A closer look at Article 3 of Directive 921261 EEC revealed that a product is prescription if it meets the following requirements.

1. It may present a danger if used without medical supervision.
2. It could easily be misused or abused, thereby causing harm to the consumers.
3. It causes side effects that require further medical investigations.
4. It needs to be administered parenterally.

Article 4 of the Directive further stated that medicinal products not classified as prescription status should be non-prescription. The principles for classification of medicinal products in Directive 92/26 are very similar to those in the United States. Despite the establishment of the Mutual Recognition Procedure, there is as yet no unified interpretation of the Directives. The interpretation of the Directives is left largely to the regulatory agency of each country. For example, the United Kingdom and Germany consider a switch with reference to an active, whereas in the majority of European countries, the switch is determined on a product by product basis (18, 19). Despite the lack of uniformity in the switch process, most social insurance systems in European countries are aggressively promoting Rx-to-OTC switch as a means to contain healthcare costs. The MCA in the U.K. has already proposed a wide range of potential switches in the next few years (48).

European countries do not have a Monograph system to allow for rapid marketing of OTC products. All OTC products require registration and marketing authorization before marketing (46, 47). Marketing authorization can be sought on a national level for marketing in just one country or using the Mutual Recognization Procedure for marketing in more than one country. The marketing authorization is renewable every 5 years. When using the MRP, the company first files a marketing authorization application in a one of the

Drug Facts

Active ingredient
- Name & amount per unit

Purpose
- What is the product for, e.g pain reliever, cough suppressant ?

Uses
What symptom or diseases the product intend to treat?

Warnings (generally contain the following information)
- When not to use the product?
- Consult a doctor before taking the product.
- Possible side effects or drug-drug interactions
- When to stop taking the product and contact the doctor?
- If pregnant or breastfeed, ask a healthcare professional before use.

Keep out of reach of children

Directions (generally contain the following information)
- Do not take more than directed
- How much to take?
- Frequency & duration of treatment
- What age groups the product is for?

Other information *(generally contains the following infonnation)*
- What is the proper storage condition?
- Amount of calcium, potassium or sodium in the product

Inactive Ingredients *(generally contain the following information)*
- Inactive ingredients in the product

Figure **9.1.** An example of the new **OTC Drug Facts** label.

EU countries, which is designed as the reference member state (RMS). Once the RMS approves the product, it will then send an assessment report to other EU member countries the company desires to market the product. The other EU countries are called the concerned member states (CMSs). They will approve the product if they concur with the assessment of the RMS. The CMS can disagree with the assessment of the RMS based on safety concerns. The company and the dissenting CMS will go into arbitration. Oftentimes, the many objections are political in nature, making the MRP a rather protract and chaotic process. Because the company cannot launch the product in any CMSs unless the arbitration is resolved, they will generally withdraw the application from the member country undergoing arbitration to prevent undue delay. The European Commission is considering overhauling the MRP (47).

The non-prescription product landscape is further complicated by the different modes of distribution from country to country (49). There are two major distribution channels for non-prescription products in Europe; namely, pharmacy only and general sale outlets, unlike the United States, where OTC products are for general sale only. For pharmacy-only products, some will need to be dispensed by the pharmacist. Rx-to-OTC switch products are usually placed in this category. The purpose of pharmacy-only products is to provide professional consultation to consumers. However, a

Table 9.3 Examples of Different Classifications of OTC Products Across Several European Countries, Australia, and the United States

	U.S.	Australia	France	Germany	Italy	Sweden	Switzerland	UK
Diclofenac	Rx	OTC	OTC	Rx	OTC	Rx	OTC	OTC
Ketoprofen	OTC	OTC	OTC	OTC	OTC	Rx	Rx	OTC
Clotrimazole (vaginal)	OTC	Rx	OTC	OTC	Rx	OTC	Rx	OTC
Loratadine	Rx	OTC	Rx	OTC	Rx	OTC	Rx	OTC
Acyclovir	Rx	OTC	OTC	OTC	Rx	Rx	OTC	OTC
Cimetidine	OTC	Rx	OTC	Rx	OTC	Rx	Rx	OTC
Famotidine	OTC	Rx	OTC	Rx	Rx	OTC	OTC	OTC
Ranitidine	OTC	OTC	OTC	Rx	Rx	OTC	OTC	OTC
Nicotine (gum)	OTC	OTC	OTC	OTC	OTC	OTC	OTC	OTC
Nicotine (patch)	OTC	Rx	OTC	OTC	OTC	OTC	Rx	OTC

Source: **Ref. 19.**

study by the U.S. General Accounting Office (GAO) did not find any differences of benefits in either mode of distribution (49).

3.3 OTC Classification in Japan

Drugs in Japan are classified into prescription, non-prescription (OTC), and quasi-drug. Prescription drugs are those drugs that require physician supervision (50–53). Non-prescription drugs are primarily for mild actions and are only available in the pharmacy or drug store. Deregulation reform reclassified 15 categories of non-prescription drugs to create a new class of drugs called quasi-drugs, which can be sold in convenience stores and supermarkets starting in Spring 1999 (51). Quasi-drugs are primarily used for external uses or preventive purposes. They include the popular tonic drinks, which are unique to the Japanese OTC market.

All three classes of drug are regulated under the Pharmaceutical Affairs Law administered by the Ministry of Health and Welfare. Prescription drugs and quasi-drugs are approved by Ministry of Health, Labor, and Welfare (MHLW) only, whereas non-prescription drugs are approved by both the MHLW and local prefectural governments. OTC drugs are approved by Evaluation Division III of Pharmaceuticals and Medical Devices Evaluation Center, which is under the MHLW. The country is administratively divided into 47 prefectural governments (52). Registration application submitted to MHLW is through the prefectural government.

Similar to European countries, all OTC products require regulatory approval (Shonin) and a license (Kyoka) to market the product (52). The approval time is generally longer than in the United States. OTC drug application is categorized into six classes (50, 52). The application system allows for both direct OTC approval and Rx-to-OTC switch. Minoxidil was switched directly to OTC status without being a prescription product first. To expedite and simplify OTC drug registration, the MHLW transferred its approval authority to prefectural governments (52). Specific monographs were established for each of these categories.

Rx-to-OTC switch in Japan has for a long time followed the principle—safety first and efficacy second (53). For this reason, a lower dose is often switched. Label comprehension is not a big issue for Japanese OTC products because OTC products are sold primarily in the pharmacy and a lot of graphics and cartoons are used to communicate warnings and directions of use. Rx-to-OTC switches are usually of low profile in Japan. The reason is that physicians seldom support and advocate the switch. Because physicians both prescribe and dispense prescription drugs, the switch will decrease their business. There are a couple of other reasons for poor performance of some of the Rx-to-OTC switches. Consumers view OTC products as less effective because of lower dose and less safe because of warnings listed on the label and in advertising. Moreover, co-pay for prescription drugs used to be

Table 9.4 A Few Definitions of Lifestyle Drugs

Source	Definition
Gilbert et al. (63)	"Drugs that are used to treat either non-health problems that are lifestyle wishes and not medical necessities of health problems that might be better treated by a change of lifestyle"
Herald Tribune (56)	"Drugs that don't necessarily cure illness but can be used to improve daily life by boosting psychological attitudes, energy levels, sexual performance and body image."
Harth and Linse (66)	"Pharmaceuticals which are taken in order to attain a certain psychosocial beauty ideal rather than serve to stabilize the body's vital functions."
Business Week (54)	"Drugs that are taken not for severely impaired diseases but for improving quality of life."

less than that of OTC products. Furthermore, no comparative advertising is allowed for OTC products with approved standard monographs. However, Rx-to-OTC switch will become more important in the future because the government is trying to control healthcare costs by increasing the co-pay.

4 LIFESTYLE DRUGS

Lifestyle drugs received much publicity (54–56) since the launch of the little blue pill, Viagra. The publicity is fueled partly by the hype of the media and partly by the debates about who is going to pay and what are their therapeutic benefits to the consumers (57–61). The scientific field is equally interested in this class of drugs, as reflected by a dedicated Scrip report (62), an article in the *British Medical Journal* (63), and a special series of articles in the *International Journal of Clinical Pharmacology* (64–68).

Several factors, such as advances in technologies, growing consumerism, and demand of baby-boomer consumers to look and feel young, contribute to the popularity of lifestyle drugs. Consumers want not only the absence of disease but also improvement of quality of life. Longer lifespans also give rise to a variety of cosmetic and performance conditions that demand remedies.

Several terms, such as vanity drugs, life-enhancing drugs, and quality-of-life drugs, are used by the media to describe lifestyle drugs (69, 70). These are the terms pharmaceutical companies try to dissociate themselves from because they tend to project a rather negative tone and create an image that lifestyle drugs are to cater to desires rather than to treat real medical problems. So exactly what are lifestyle drugs? There are numerous definitions of lifestyle drugs, depending on whom you talk to. Table 9.4 lists a few definitions of lifestyle drugs. The definitions range from attainment of psychosocial beauty to treatment of health problems that might be better treated by a change of lifestyle.

The term "lifestyle drugs" refers to pharmaceuticals, in particular prescription drugs, that primarily aim to enhance the wellness and improve the "desired" quality of life of an individual rather than alleviating or curing life-threatening diseases. Although all medications are used to improve the quality of life, use of lifestyle drugs by consumers are intended to attain a certain ideal quality of life rather than to treat some life-threatening disease. The definition for lifestyle drugs may be broadened to include drugs for the use of treating serious medical conditions caused by unhealthy lifestyles such as over-eating or for off-label use, such as the use of Prozac, for more self-confidence.

The focus of lifestyle drugs usually centers around prescription drugs because of the issue of who should pay for these medications and their off-label use. The term lifestyle drugs should not be limited to prescription drugs only because some of them have already been switched to OTC. Table 9.5 lists a few examples of lifestyle drugs and their indications. These examples cover a broad range of drug products. The range of indications is equally broad; they range from preventive and cosmetic to serious medical conditions such as obesity and smoking.

Table 9.5 Examples of Lifestyle Drugs

Indications	Drug
Image and look	
Wrinkles	Tretinoin, vitamin A, botulium toxin type A, sunscreen
Hair loss	Minoxidil hasteride
Facial hair removal	Elornithine
Nail fungus	Butenafine, terbinafine, itraconazole
Increase muscle mass or height	Growth hormone
Quality of life improvement	
Incontinence	Oxybutynin, tolterodine
Impotence	Sildefil, apomorphine
Influenza	Zanamivir
Lifestyle	
Postpone menstruation	Norethisterone
Smoking cessation	Nicotine replacement therapy, bupropion
Contraception	Oral contraceptives
Weight control	Orilstat, sibutramine, bupropion
Dyspepsia	Proton pump inhibitor
Sun protection	Sunscreens
Psychological	
Anti-depression	Fluoxetine
Obsessive-compulsive disorder	Sertraline
Social anxiety disorder	Paroxetine

Source: Refs. **63, 69, 71.**

Because the majority of lifestyle drugs are not used to treat life-threatening diseases or to alleviate pain, and the cost of coverage is huge, the majority of debate has been focused on who is going to pay (57, 61). Most countries have a national healthcare insurance system that needs to control rising healthcare costs. Coverage for lifestyle drugs puts considerable strain on the limited resources of the insurance system. The agency will therefore need to rationalize the service and treatments. They need to set priorities and determine which treatments are medically useful and which treatments are for lifestyle enhancement and convenience only. The issue of what is medically needed is complicated by the heavy promotion of lifestyle drugs by the pharmaceutical industries and transition of treatment from specialists to general practitioners. Without a doubt, the following scenario will occur. Pharmaceutical industries will continue to push the envelop of medical innovations and find ways to meet unmet consumer lifestyle needs to increase their profitability. The healthcare provider and national health insurance system will continue to find ways to control the cost of healthcare. The solution may be partially solved by switching the safer lifestyle drugs to OTC because consumers are willing to pay to obtain the benefit, and the fact that they are already paying for many of these lifestyle drugs.

The other controversy is what benefits do lifestyle drugs offer the consumers because they are primarily treating non–life-threatening diseases. Diseases such as incontinence and impotency are not life-threatening, but they create undue psychological stress on the suffers. Furthermore, even for lifestyle drugs that have legitimate therapeutic uses, they can still be abused by healthy individuals for purely performance enhancement. For lifestyle drugs that **are** used to treat diseases arisen from unhealthy lifestyles, drug coverage is only a partial and least-preferred solution. The best solution is for patients to take control of their health by living a healthy lifestyle. All these controversies will continue to fuel the debate for coverage and benefits of lifestyle drugs.

The following sections provide four examples of lifestyle drugs: hair growth compounds for appearance enhancement, sexual disorder compounds for performance remedy, smoking

Table 9.6 Approved Treatments for Hair Growth Disorders

Generic Name	Brand Name	Originator	Indication	Dosage Form	Daily Dose	Current Regulatory Status
Minoxidil (1)	Rogaine	Upjohn	Androgenetic alopecia (men and women)"	2%,5% solutions	Topical application twice daily	OTC
Finasteride (2)	Propecia	Merck	Androgenetic alopecia (men only)	1 mg tablet	1 tabletlday	Prescription
Eflornithine hydrochloride (3)	Vaniqa	BMS	Hirsutism (women's facial hair)	13.9% cream	Topical application twice daily	Prescription

"The 5% is for men only.

cessation compounds for lifestyle change, and sunscreens for lifestyle purposes. They span the spectrum of OTC and prescription products.

5 HAIR GROWTH DISORDERS

Common baldness, also known as male-pattern baldness, affects approximately 50% of men by age 50 (72), although it can start as early as the teen years. Till the late 1980s, common baldness was viewed as a merely cosmetic condition that has no health implications; with the introduction of hair growth agents, the condition was medicalized and redefined as androgenetic alopecia. Androgenetic alopecia affects women as well, but in a different pattern that is usually referred to as female-pattern baldness, diffuse hair loss, or diffuse androgen-dependent alopecia. An estimated 30% of Caucasian women, and 15–20% of all women, develop the condition before age 50 (73, 74). Whereas hair loss, like other lifestyle conditions, is not a direct threat to a person's well-being, it can be a distressing and psychologically disturbing condition because of lower satisfaction with one's body image (75, 76). In one of the clinical studies on minoxidil, the majority of participants thought that personal presentation of self was of equal to or greater importance than their job performance (77). Obviously, the condition has a more severe psychological impact on female subjects, which was confirmed in two separate studies (78, 79).

Excessive hair growth in cosmetically undesirable areas is another hair growth disorder of little clinical importance but great lifestyle impact. The condition, which is medically known as idiopathic hirsutism, is defined as increased hair growth in the androgen sensitive areas of women with regular ovulatory menstrual cycle and normal serum androgen levels (80, 81). Hirsutism is a common condition that can have a severe psychological effect on young women and negatively affect their quality of life. The argument that preserving or restoring physical appearance can have profound psychological benefits provides the basis for medicalizing cosmetic disorders such as hair loss, hirsutism, or aging skin wrinkling and discoloration.

5.1 Clinical Use

5.1.1 Current Drugs. Table 9.6 lists the current therapies for hair growth disorders. The effects of the two hair growth agents (minoxidil and finasteride) have not been directly compared in clinical trials, and no clinical studies have been performed on the combination of the two drugs either, but a study in an animal model for male pattern hair loss suggests that the combination of 0.5 mg/day oral finasteride and 2% topical minoxidil has an additive effect on hair growth (82, 83). Eflornithine is the only drug currently approved for the reduction of unwanted facial hair in women. In addition to approved therapies,

hormonal treatments are still commonly used both for female pattern androgenetic alopecia and hirsutism. Estrogens (ethinylestradiol or oral contraceptives) and antiandrogens (cyproterone acetate, spironolactone, and flutamide) are used either alone or in combinations for either condition (73, 81). Finasteride was found to be effective for the treatment of idiopathic hirsutism at a dose of 5 mglday, although it is not approved for the indication. This section will mainly focus on the two approved hair growth agents.

5.1.2 Adverse Effects. The side effects of topical minoxidil are mainly local, caused by skin irritation and contact dermatitis. Systemic side effects are uncommon because of limited percutaneous absorption, but diffuse hypertrichosis of the face and limbs has been reported with the 5% solution and was attributed to systemic absorption of the drug (84). Although topical minoxidil does not change blood pressure in healthy subjects, it increases heart rate by 3–5 beats/min and slightly increases the left ventricular end-diastolic volume, cardiac output, and left ventricular mass (85). These effects are not considered clinically significant, and the potential for cardiovascular side effects is very low.

The clinical dose of finasteride is well tolerated. The main side effects reported in phase III clinical studies were sexual function disorders including decreased libido, ejaculation disorders, and erectile dysfunction. All these sexual disorders were mild-to-moderate and were reversed on discontinuation of the drug, and in some patients, they resolved even with continued therapy (86). A 5-mgdose in hirsute women was well tolerated with no significant side effects, although the risk of developing abnormalities in the external genitalia of male fetus, if the drug is taken or a crushed tablet is handled by a pregnant woman, causes a great limitation to its use in women at child-bearing age.

Topical eflornithine has a very good safety profile. The only side effects observed in the clinical trials at a higher frequency than placebo were related to skin irritation and included stinging or burning skin and rash at the site of application.

5.1.3 Pharmacokinetics. Minoxidil is poorly absorbed through skin, and systemic accumulation after topical application is unlikely (87). After topical application, minoxidil appears in the systemic circulation at clinically insignificant levels. The total amount recovered in urine is less than 4% of the applied dose. Applying the drug to the entire scalp is equivalent to a systemic dose of 2.4–5.4 mg/day (88). A total of 41–45% of the applied dose remains on the scalp or is lost on the pillowcase. Dermal metabolism of minoxidil is negligible (89). Systemic minoxidil elimination, after topical administration, is zero order, which indicates that it is controlled by zero order percutaneous absorption. The main route of elimination after oral absorption is hepatic metabolism. The primary metabolite is a glucuronic acid conjugate at the N-oxide position of the pyrimidine ring. Twenty percent of the orally administered drug is excreted unchanged in urine, and 95% of the total dose is recovered in urine within 48 h.

Finasteride is rapidly absorbed after oral administration, with peak plasma concentration reached in 1–2 h. The oral bioavailability is about 80% and is not dose-dependent. Food has no effect on the bioavailability of finasteride. Moderate accumulation occurs with multiple dosing, and steady state is reached within 3 days. Finasteride has a large volume of distribution (76 L) as a result of wide tissue distribution. Approximately 90% of the circulating drug is bound to plasma proteins. Finasteride undergoes extensive hepatic metabolism. Finasteride metabolism occurs in the liver through an oxidative pathway by cytochrome P450 3A4 enzymes. The two major metabolites are ω-hydroxyfinasteride and a monocarboxylic acid derivative. Their *in vitro* activity is less than 20% of that of finasteride, but their *in vivo* activity has not been studied. Virtually no unchanged drug is excreted in urine; 56.8% of the metabolized drug is excreted in bile and 39.1% in urine. The mean elimination half-life after multiple oral dosing is 4.8 h, and the mean clearance after intravenous infusion is 9.9 L/h. The mean serum half-life is about 6 h in middle-aged men and slightly longer in the elderly, but dosage adjustment is not necessary (86, 90). The biological half-life of finasteride is longer than its

serum half-life. After discontinuation of the drug, dihydrotestosterone (DHT) takes at least 2 weeks to return to baseline levels.

Eflornithine absorption after percutaneous administration is minimal (<1% of the applied dose). Commonly used hair removal methods such as shaving, plucking, or tweezing, when performed within 2 h before application, did not increase the percutaneous absorption. Steady state is reached after 4 days of twice daily application. Eflornithine is excreted unchanged in urine, and no metabolism has been observed. The apparent steady-state half-life is approximately 8 h.

5.2 Physiology and Pharmacology

5.2.1 Physiology of Hair Growth. Hair growth goes through a three-phase cycle (74): anagen (growth phase), catagen (involution), and telogen (rest). At the end of the telogen phase, hair is shed and the next cycle begins. In a normal scalp, approximately 90–95% of hair is in the anagen phase. The duration of anagen determines the hair length. Hair diameter is determined by the volume of the hair bulb. The scalp's terminal hair follicles are predetermined to grow long thick hair, whereas the vellus hair follicles on most of the body are predetermined to grow short and fine hair. In androgenetic alopecia, the cycle of scalp hair growth is altered, with gradual reduction in the length of anagen phase leading to a reduction in the ratio of anagen to telogen hair (86). This leads to progressive miniaturization of the scalp hair in a recognizable pattern. The main difference between the male and female androgenetic alopecia, other than the visible pattern, is that bald men have reasonable hair densities (hairs per square centimeter) even though the hairs in bald areas are predominantly vellus (short and thin), with some short hairs that have normal diameters. In women with androgenetic alopecia, although the scalp has fewer hairs per square centimeter, the hair that remains is similar in diameter and length to hair fibers in non-balding women (73). It is not clear if these differences have any implications on the pharmacological therapy of the condition.

5.2.2 Role of 5-α-Reductase. Skin is a target tissue for androgens; androgen receptors are present in the hair follicles and sebaceous glands of the scalp skin. Although testosterone acts directly on androgen receptors in several tissues such as skeletal muscles, male beard hair follicles, and male external genitalia, it has to be metabolized to the more potent androgen dihydrotestosterone to act on androgen receptors in the scalp hair follicles and prostate among other tissues. 5α-reductase is an important enzyme that mediates testosterone intracellular conversion to dihydrotestosterone (DHT). There are two types of 5α-reductase. Type I is primarily present in the skin, especially the sebaceous glands, epidermal and follicular keratinocytes, and sweat glands, as well as in the liver and kidneys. Type II is mainly found in the gonadal tissue (prostate, seminal vesicles, and fetal genital skin), dermal papilla, and in the liver and scalp hair follicles (91, 92). Recent evidence proved that type II exists in the root sheaths of the scalp hair follicles and predominates in the dermal papillae, contrary to the earlier studies that suggested predominance of type I at this location (86, 92). 5α-reductase converts testosterone to dihydrotestosterone, the androgen responsible for androgenetic alopecia. Type I 5 a-reductase is responsible for one-third of circulating DHT, whereas the type II isoenzyme is responsible for two-thirds of circulating DHT (93). The active androgen DHT binds androgen receptors in the susceptible hair follicles. The hormone-receptor complex binds to specific gene sites to stimulate gradual transformation of large terminal follicles to miniaturized follicles (74, 91).

5.2.3 Mechanisms of Action. The two approved hair growth agents act by totally different mechanisms. Minoxidil, the older agent, acts by stimulating the microcirculation in the balding scalp, whereas finasteride, the newer agent, acts by altering androgen metabolism in the hair follicles, through inhibition of type II 5α-reductase.

5.2.3.1 ***Minoxidil.*** Minoxidil increases the blood flow to the follicular dermal papilla by a direct vasodilation effect on the arteriolar blood vessels (94, 95). Vasodilation is caused by the active metabolite minoxidil sulfate.

Minoxidil is converted to its active metabolite minoxidil sulfate by liver sulfotransferase sulfation (96). Minoxidil sulfate acts directly on the cutaneous blood vessels and induces smooth muscle relaxation. It has been suggested that minoxidil sulfate acts as a potassium channel agonist to enhance potassium permeability. This results in hyperpolarization and causes reduction in agonist-stimulated Ca^{2+} influx and hence decreased cytoplasmic free Ca^{2+} concentration, and therefore causes smooth muscle relaxation (97, 98). Minoxidil sulfation occurs both in proliferating keratocytes and in hair follicles, which suggests that an additional mechanism that results in direct stimulation of the hair follicle independent of vasodilatation might be involved (99, 100). In vitro data suggest that the direct effect of minoxidil involves increased biosynthesis of glycosaminoglycans (100). It has also been shown that minoxidil inhibits lysyl hydroxylase, an enzyme that catalyzes the hydroxylation of certain lysine residues in the polypeptide precursors of procollagen (91). Hydroxylysine is important for the formation of intermolecular crosslinks in the collagen fiber. It is not known whether minoxidil's effect on hair growth is related to its effect on collagen metabolism.

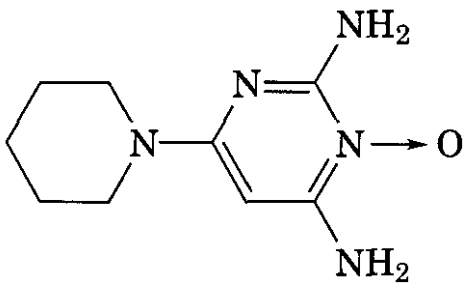
Minoxidil increases the duration of anagen hair and causes the growth of larger and normally formed follicles compared with the pretreatment short miniaturized terminal hair follicles, which yields thicker longer hair and decreased shedding. Minoxidil, however, does not increase the total hair count (74, 95). Because of the non-specific mechanism of minoxidil, the drug can promote hair growth regardless of the cause of hair loss. In addition to its effectiveness in androgenetic alopecia, it promotes hair growth in unrelated conditions such as alopecia areata, congenital hypotrichosis, and loose androgen syndrome (74). Vertex hair loss responds better to minoxidil therapy than frontal hair loss. The effect of minoxidil seems to plateau after approximately 1 year of therapy and reverses when the treatment is stopped.

5.2.3.2 Finasteride. Finasteride is a specific and competitive inhibitor of type II 5α-reductase. It is 100-fold more selective to the type II isoenzyme compared with type I (101). It has been shown through in vitro studies that finasteride competes with testosterone for the same binding site on 5α-reductase, but it has no effect on the binding of testosterone or DHT on androgen receptors (102). The inhibition of type II 5α-reductase blocks the peripheral conversion of testosterone into DHT and therefore results in decreased DHT concentration both in the circulation and in target tissue. Sixty-five percent suppression of serum DHT is reached within 24 h of oral dosing with a 1-mg tablet (93), and a median of 68.4% reduction occurred in men who were treated for 1 year (86). Therapeutic doses of 0.2–5 mg/day for 4–6 weeks reduced the scalp DHT level by up to 65%. The relative contributions of the reduction in scalp DHT and tissue DHT to finasteride effect on male pattern baldness has not been identified. The circulating levels of testosterone and estradiol increase slightly but remain in the normal range. Finasteride does not have any direct androgenic or antiandrogenic effect and does not cause any significant changes in the levels of luteinizing hormone (LH) and follicle stimulating hormone (FSH). The therapeutic dose of finasteride (1 mg/day) causes significant increases in hair count and hair growth both in frontal hair loss and vertex hair loss male subjects (86). As with minoxidil, finasteride does not revive hair follicles that are inactive (i.e., in older men who are bald) and is therefore recommended to be used only by younger men with partial hair loss. At least 3–4 months of therapy with either minoxidil or finasteride is needed before any hair growth effect can be seen. The therapeutic effect declines when treatment is discontinued, with complete reversal within a few months in the case of minoxidil and 12 months in the case of finasteride (86, 93).

5.2.3.3 Eflornithine. Eflornithine acts by inhibiting the enzyme ornithine decarboxylase (ODC) in the hair follicles of the human skin (93). The enzyme is necessary for the synthesis of polyamines. Animal data indicates that inhibiting ODC inhibits cell division and synthetic function and therefore inhibits hair growth. It is postulated that eflornithine causes irreversible inhibition of the enzyme.

5.3 Chemistry and Structure-Activity Relationships

5.3.1 Minoxidil. Minoxidil (**1**) is 2,4-diamino-6-piperidino-pyrimidine-3-oxide. It was developed as an antihypertensive from triaminotriazines. Triaminotriazines were found to be potent vasodilators in dogs and cats because

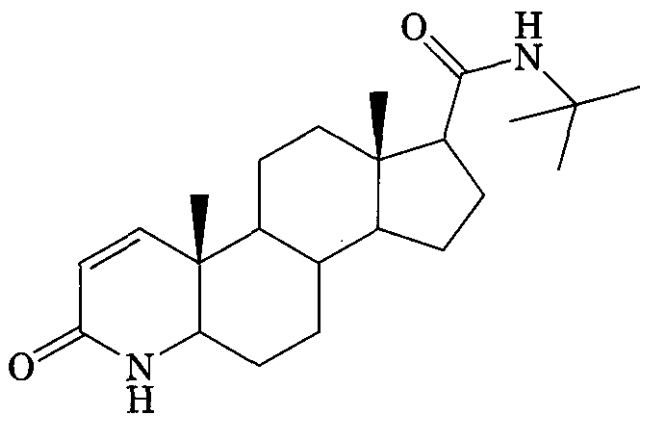


(1) Minoxidil

of the formation of N-oxides. In humans, however, they were inactive because of the inability to form N-oxides. This led to the development of minoxidil by isosteric replacement by triaminopyrimidine of a triaminotriazine moiety.

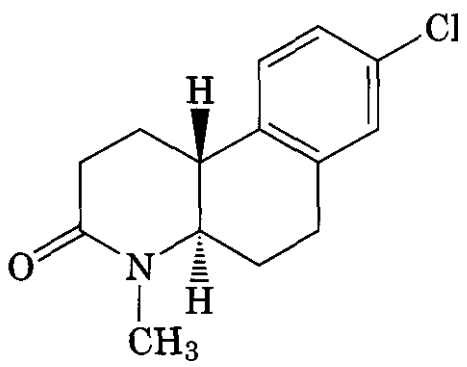
The structural requirements for minoxidil's hypotensive effect (103), and its lysyl hydroxylase inhibitory effect (91) have been reported, but the relevance of these requirements to the hair growth effect of minoxidil is unknown. One of the distinct features of minoxidil is the nitroxide group in position 1 of the pyrimidine ring. This group is essential for the vasodilator effect. However, for the effect on lysyl hydroxylase, removal of the nitroxide oxygen results only in partial loss of activity. A secondary or tertiary amine substituent on position 4 of the pyrimidine ring is also required for hypotensive activity. In minoxidil, this requirement is met by a piperidinyl group. Replacing the piperidinyl group with another source for a tertiary or secondary nitrogen such as pyrrolidinyl, morpholinyl, or an N-methylpiperazinyl group does not cause detrimental effects on the antihypertensive activity but results in total loss of lysyl hydroxylase inhibitory activity. Hydroxylation at position 3 or 4 of the piperidine ring (by metabolic transformation) results in marked reduction in hypotensive activity. The same 3- or 4-hydroxy piperidinyl derivatives are as potent as the parent compound in suppressing lysyl hydroxylase.

5.3.2 5α-Reductase Inhibitors. Finasteride **(2)** is a 4-azasteroid derivative of the 3-oxo-5α-



(2) Finasteride

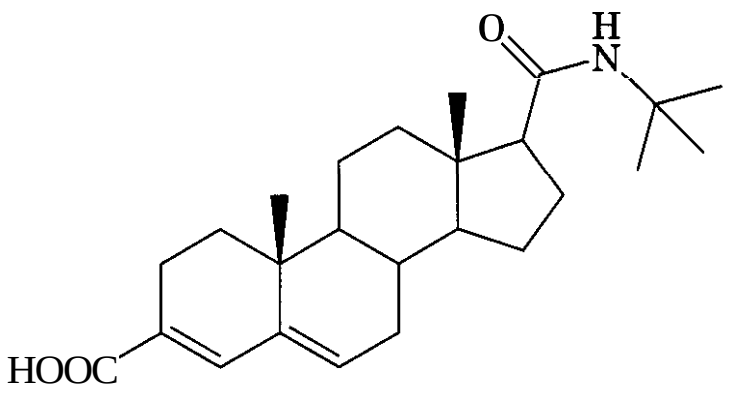
steroids. An important structure requirement for 5α-reductase inhibition of azasteroids is stable configuration in ring A of the steroid molecule that mimics the transition state in the conversion of testosterone to DHT (104, 105). This allows for a tight binding between the inhibitor and the active site of the enzyme. Most of the 5α-reductase inhibitors in development are either 4- or 6-azasteroids. However, steroidal carboxylic acids and non-steroidal tricyclic compounds with structural resemblance to azasteroids are also potent inhibitors of 5α-reductase (106). Molecular modeling of the binding between the enzyme and substrates suggests that there is a requirement for groups to mimic the carbonyl group of ring-A in the steroid structure (107). An interesting nonsteroidal series of compounds that satisfy this requirement is benzoquinolinones. Among this class is Lilly's LY 191704 (3), a potent non-competitive 5α-reductase inhibitor with high selectivity to type I isoenzyme.



(3) LY191704

The 4- or 6-aza moiety is another important structural requirement. A notable exception

to this requirement is epristeride (4), a type II 5α-reductase inhibitor with no nitrogen in the steroidal ring structure. The position of this



(4) Episteride

nitrogen in the steroidal skeleton does not affect the potency of 5α-reductase inhibition or isoenzyme selectivity. Most of the 4- and 6-azasteroids that have been reported have higher selectivity to isoenzyme II, with only few exceptions (e.g., Merck's L 733692). The position of the aza moiety seems to affect the mechanism of inhibition (108). It has been reported that the inhibition of 5α-reductase by 6-azasteroids is reversible, whereas the 4-azasteroid derivatives such as finasteride cause irreversible inhibition. Benzoquinolinones have a nitrogen that mimics that of 4-azasteroids. In 4-azasteroids, a methyl group on the nitrogen produces greater activity than a hydrogen. A Δ^1 double-bond at the C1-C2 position (in ring A) results in substantial loss of inhibitory action on hair follicle 5α-reductase. However a A^5 double-bond in ring D has little effect on enzyme inhibition.

A bulky lipophilic moiety on C-17 is another structural requirement for maximum 5α-reductase inhibition. A long C-17P sidechain increases the activity (101). Replacing the tert-butyl group on the C17 amide nitrogen of finasteride with a phenyl group results in a slight decrease in potency against type II isoenzyme and an increase in the potency against type I (109). Further introduction of an alkyl group on the same nitrogen results in a drastic loss of activity against both isoenzymes because of changes in the conformational preference (from trans to cis). 6-Azasteroids with C17 anilide substitution are also potent inhibitor of the enzyme. 2,5-Disubstitution of the phenyl ring in the anilide series increases the potency against type I 5α-reductase while retaining higher potency against type II (110). An arylcycloalkyl group on the C17 amide of 6-azasteroids similarly results in high potency against both isoenzymes with higher selectivity to type II.

5.4 History

The interest in pharmacological therapy for male pattern hair loss started as early as 1965, when a topical preparation of testosterone was tested for presumed benefits. Topical testosterone failed to show any efficacy (86). The interest then declined until reports about stimulation of hair growth in hypertensive patients being treated with the then experimental drug minoxidil renewed the hopes.

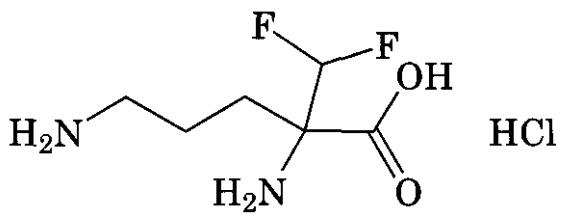
The initial research leading to the discovery of minoxidil started in 1960 when researchers from the Upjohn Company performed empirical pharmacological screening on *N,N*-diallylmelanine (DAM)—a compound they ordered from the American Cyanamid chemical catalog (111). The first pharmacological action that was observed was reduction of gastric acidity in pyloris-ligated rats, but after oral administration to dogs, DAM produced a delayed and very prolonged blood pressure reduction. However, a subsequent clinical trial showed no clinical effect in hypertensive patients. This led to the identification of an active metabolite in dogs that was absent in humans and was responsible for the hypotensive effect of DAM. Subsequently, this metabolite (DAMN-0) was synthesized, and it was determined that it acted by direct vasodilatation. A single-dose clinical trial that was conducted in late 1961 showed a marked reduction in blood pressure and a long duration of action that exceeded 24 h. Consequently, a multiple dose study was initiated. A combination of salt retention side effect and a mistake in interpreting instructions led to the discontinuation of the study. The situation was further complicated by the discovery of a cardiac lesion that developed in a long-term toxicology study in dogs. All this led to the loss of drug candidacy of DAMN-0. Despite this, Upjohn researchers continued to be interested in the direct vasodilator action of DAMN-0 and continued their search for better drug candidates. During the following years, they synthesized hundreds of

analogs, all bearing the N-oxide moiety. Ten candidates were selected and tested in dogs. It was found that the same side effects were associated with the entire class of compounds (salt and water retention, tachycardia, and cardiac lesion). Upjohn researchers selected minoxidil as the best candidate and decided to proceed with clinical trials cautiously and only in patients with life-threatening drug-refractory hypertension. The Investigational New Drug Application of minoxidil was filed in 1968. The initial clinical trial showed dramatic reduction in blood pressure of severely hypertensive and drug-refractory patients. The expected side effects (salt retention and tachycardia) occurred, but were reversed by co-administration of diuretics and β-blockers, respectively. The following trial demonstrated superiority over hydralazine and led to widespread investigators' interest in minoxidil. As a result, the FDA approved an emergency-use protocol in late 1970. Initially, each case had to be approved by an FDA clinical reviewer, but by 1971, an Upjohn clinical monitor was authorized to approve the treatment. Gottlieb et al. (112), the clinical investigators of these initial clinical trials, was the first to report the hair growth stimulating effect of minoxidil. Two dermatologists who were consulted about the phenomenon decided to test a topical solution on the upper arm and were able to demonstrate localized action at the site of application (111). In 1971, Upjohn started absorption and toxicology testing of the minoxidil topical solution. In 1972, the first case of hair growth on a bald scalp of a severely hypertensive patient who was being treated with minoxidil was reported (111). Because of the continuing concerns over oral minoxidil's side effects, Upjohn delayed the filing of topical minoxidil until it gathered convincing evidence of safety that was sufficient for approval of oral minoxidil. The IND for the topical solution was filed in 1977, and the FDA approval of oral minoxidil for hypertension came in 1979. This recognition of safety made it possible for Upjohn to continue pursuing the hair growth indication. Initial hair growth clinical trials began in 1978. Although these studies provided some evidence of clinical efficacy, with hair growth stimulation in a large percentage of subjects, statistically significant differences were not demonstrated until the project moved to large phase III multi-center clinical trials (111). Upjohn's efforts were culminated in 1988 by the approval of minoxidil 2% topical solution for male pattern androgenetic alopecia. In 1991, the FDA approved it for female pattern hair loss. The OTC switch of minoxidil topical solution was approved in 1996 after an NDA application. In 1997, the FDA granted its approval of 5% minoxidil topical solution for initial marketing as an OTC medication. Minoxidil (5%) was never marketed as a prescription drug.

Unlike minoxidil, the discovery of finasteride was a typical case of modern drug discovery—a drug designed to target specific receptors that mediate the disease it is aimed at. The first step that led to the development of finasteride was discovery of the role of 5α-reductase, which provided a better understanding of the separate roles of testosterone and DHT. The role of 5α-reductase was understood through studies on the genetic disorder 5α-reductase deficiency, which produces a form of male pseudohermaphroditism (106, 113, 114). Those studies suggested that the clinical signs of the disorder imply that 5α-reductase has a role in conditions such as benign prostatic hyperplasia, androgenetic alopecia, acne, and hirsutism (106). Consequently, researchers at Merck synthesized a series of azasteroids to target the enzyme and hence provide potential treatments for these conditions. After the isolation of the two 5α-reductase isoenzymes, it was found that type II 5α-reductase, which is inhibited by finasteride, is the isoenzyme responsible for most of the effects ascribed to 5α-reductase. Furthermore, it was found that the patients with male pseudohermaphroditism caused by 5α-reductase deficiency are only deficient in type II and have normal levels of type I isoenzyme (114). Finasteride was first administered to humans in early 1986. Biochemical efficacy was demonstrated in the first clinical study with marked reduction in circulating DHT levels (102). Clinical studies were conducted on finasteride for benign prostate hyperplasia (BPH), hirsutism, male-pattern baldness, and acne. The drug was first marketed for benign prostatic hyperplasia as a 5-mg tablet under the brand name Proscar after its FDA ap-

proval in 1992. The hair growth indication was being pursued at the same time. Propecia, a 1-mg finasteride tablet was approved for the treatment of male-pattern hair loss in 1997.

Eflornithine (5) was originally developed by Merrell Dow (now Aventis) as an antineoplastic and antiprotozoal agent. It was approved in 1990 in the intravenous form for the treatment of sleeping sickness (trypanosomiasis) under the brand name Ornidyl. Inhibition of hair growth was observed during the clinical trials. The indication was licensed to Bristol-Myers Squibb (BMS), which partnered with Gillette on the development program for reduction of unwanted hair in women. Gillette did the early development work and BMS conducted the clinical trials and handled regulatory filing. Eflornithine gained FDA approval in July 2000 and was subsequently launched in September of the same year. An OTC switch of eflornithine is a very likely long-term goal.



(5) Eflornithine Hydrochloride

5.5 Current and Future Trends

Both androgenetic alopecia and hirsutism are very active areas of research. With the increased knowledge of the biochemical and physiological basis of the conditions, novel compounds are being designed to target specific enzymes or receptors that are involved. Most of the compounds under development fall into three major categories: 5 a-reductase inhibitors, topical antiandrogens, and potassium channel agonists. The first two mechanisms are targeted for both scalp hair growth stimulation and body/facial hair growth inhibition. The third category is specific for hair growth promoting agents. Potassium channel agonists are believed to induce local vasodilatation and therefore enhance the microcirculation in the scalp similar to minoxidil sulfate. They cause smooth muscle relaxation by hyperpolarizing the cell membrane and inhibiting acetylcholine-activated voltage-sensitive Ca^{2+} influxes and thus attenuating the acetylcholine-induced tonic contraction.

Among the most promising 5 a-reductase inhibitors is Glaxo's dutasteride (GG-745, GI-198745). Glaxo SmithKline has submitted an NDA with the FDA for BPH in 2001. Dutasteride, a 4-azasteroid, is an inhibitor of both type I and type II isoenzymes and is in phase II clinical trials for androgenetic alopecia. Table 9.7 lists some of the compounds in various stages of development for hair growth disorders. Many of these compounds, particularly antiandrogens and 5 a-reductase inhibitors in earlier phases of development, are being tested for multiple indications.

6 SEXUAL DISORDERS

The recent extraordinary interest in sexual disorders was primarily spurred by the introduction of sildenafil (Viagra), the first effective oral medication for erectile dysfunction. The unparalleled global media hype that followed the launch of Viagra greatly increased the public awareness of erectile dysfunction and of sexual disorders in general. The enormous public response to sildenafil also stirred a debate over the reimbursement of such therapies and of lifestyle drugs in general. Several health authorities argued that impotence is not a disease and does not pose any health risk to the individual or the society. To counter this argument, drug companies that are involved in the field of sexual disorders intensified their efforts in gathering evidence on the impact of sexual dysfunction and its successful treatment on the quality of life of the individuals and their partners. This gave a new life to other sexual disorders that, until recently, were overlooked by the public and researchers as well. The most common sexual disorders are erectile dysfunction, premature ejaculation, and female sexual dysfunction.

Erectile dysfunction, also known as impotence, is defined as the inability to achieve or maintain an erection adequate for sexual satisfaction. It is estimated that 30 million men in the United States have partial or total erectile dysfunction (117). The number is expected to increase as the American population continues to age. Studies have shown that over 50%

Table 9.7 Compounds in Various Stages of Development for Hair Growth Disorders (115,116)

Generic Name/ Laboratory Code	Originator	Chemical Class	Mechanism of Action	Indication
P-1075	Leo (Denmark)	Pyridyl cyanoguanidine	Potassium channel agonist	Alopecia
Dutasteride	Glaxo-Smithkline	4-Azasteroid	5 a-Reductase inhibitor	Alopecia, BPH
Tricomin (PC 1358)	ProCyte	Tripeptide copper compounds	Unknown	Alopecia
Turosteride	Pharmacia	Non-steroidal teteracyclic structure closely resembles 4-azasteroid	5 α-Reductase inhibitor	BPH, alopecia, acne, hirsutism
CS 891	Sankyo (Japan)	Unknown	5 a-Reductase inhibitor	BPH, alopecia
L 733692	Merck	4-Azasteroid	Type I 5 α-Reductase inhibitor	Male pattern baldness
MK 434	Merck	4-Azasteroid	5 a-Reductase inhibitor	BPH, alopecia, acne
EM 250 (SCH 54726)	Endorecherche (Canada) and Schering Plough	17-Hydroxysteroid	Antiandrogen	Acne, alopecia
RU-58841	Aventis	Benzonitrile derivative	Non-steroidal topical antiandrogen	Acne, alopecia
LY 191704	Lilly	Benzoquinolinone	Type 1 5 a-reductase inhibitor	BPH, alopecia, acne
KC-399	Chugai (Japan)	Carbothiomide	Potassium channel agonist	Alopecia
KC 516	Chugai	Unknown	Potassium channel agonist	Alopecia
LGD-1331	Ligand	Unknown	Non-steroidal antiandrogen	Alopecia, hirsutism, acne, BPH

of men ages 40–70 have some degree of erectile dysfunction, with 15% suffering from complete erectile dysfunction (118).

Premature ejaculation is another widespread male sexual disorder. It is defined as the persistent or recurrent ejaculation of semen with minimal sexual stimulation before, on, or shortly after vaginal penetration, and before the person or his partner desires it (119). Although less publicized than erectile dysfunction, premature ejaculation is believed to be the most common male sexual disorder. The prevalence rate.is estimated at 30–40% of "normal" men (120).

Female sexual dysfunction encompasses a wide range of little understood disorders. These include the following: hypoactive sexual desire disorder. sexual arousal disorder, orgasmic disorder, and sexual pain disorders. The overall prevalence of female sexual disorders is estimated at 30–50% (121, 122). The U.S. National Health and Social Life Survey of women ages 18–59 reported a prevalence rate of 43% (123). Until recently, most of the female sexual disorders were considered part of the natural aging process, and the subject gained very little attention from researchers and the public. The only available treatment was counseling. This has changed recently, as a result of the soaring interest in sexual disorders and lifestyle drugs in general, that was fueled by the phenomenal success of Viagra. It is now widely recognized that female sexual dysfunction is

Table 9.8 Currently Approved Drugs for Erectile Dysfunction

Generic Name	Brand Name	Originator	Dosage Form	Dosage Strength
Sildenafil citrate	Viagra	Pfizer	Oral tablet	25, 50, 100 mg
Alprostadil	Muse	Vivus	Urethral suppository	125,250,500,1000 μg
Alprostadil	Caverject	Upjohn	Intracavernosal injection	5, 10, 20, 40 μg
Yohimbine	Aphrodyne	N/A[a]	Oral tablet	5.4 mg 3 times a day

[a]Yhimbine and Yohimbe were used in folk medicine as aphrodisiacs for a long time.

an important health issue that affects the quality of life of millions of women.

6.1 Clinical Use

6.1.1 Current Drugs. Several pharmacological agents are available for the treatment of erectile dysfunction with different etiologies, which makes the condition treatable in the vast majority of sufferers. Table 9.8 lists the pharmacotherapeutic agents currently approved for erectile dysfunction.

The guidelines for management of erectile dysfunction issued by the First International Consultation on Erectile Dysfunction recommends involving the patient in selecting the treatment of his preference after explaining the benefits, risks, and costs involved with each treatment option (124). The non-invasiveness and convenience of oral medication makes it the first choice by the vast majority of patients unless contraindicated. Sildenafil is currently the most prescribed treatment for erectile dysfunction. It is effective as a single dose and is recommended to be taken approximately 1 h before sexual activity, but can be taken anywhere from 30 min to 4 h before sexual activity. The overall efficacy of sildenafil, defined as percentage of successful attempts at sexual intercourse, is approximately 70% (125).

The leading intracavernosal agent is alprostadil. It is indicated for the treatment of erectile dysfunction caused by neurogenic, vasculogenic, psychogenic, or mixed etiology. Erection occurs in 5–10 min after intracavernosal injection of alprostadil. In clinical practice, the dose is individualized and titrated slowly to the lowest possible effective dose to avoid prolonged erection and priapism (erection that lasts for more than 6 h), which can be potentially serious side effects. The target is an erection that lasts for approximately 1 h. Intracavernosal alprostadil is still the most effective treatment, although its use is limited by the side effects and the inconvenience of self-injection and rapid onset of action, which results in an unnatural erection. More than 90% of alprostadil intracavernosal injections result in successful sexual intercourse (126). Transurethral alprostadil is a micro-suppository that is inserted into the stem of the urethra using an applicator. Although it is a more convenient route of administration, its overall efficacy is about 50% (126, 127).

The vasoactive amines phentolamine and papaverine are occasionally used as intracavernosal therapy, usually in combination with alprostadil, although their use for erectile dysfunction is off-label. Moxisylyte is another vasoactive agent used as intracavernosal therapy. The drug is approved in several European countries, but is not approved in the United States. The advantages over alprostadil are that with moxisylyte, sexual stimulation is still required to achieve full erection and that detumescence occurs on ejaculation.

Yohimbine is a moderately effective and well-tolerated oral agent for erectile dysfunction, yet it has not been adequately evaluated in well-designed placebo-controlled studies, although two meta-analyses of the few randomized placebo-controlled studies demonstrated its advantage over placebo (128–130). It is more effective in patients with erectile dysfunction of psychological etiology (131, 132). Yohimbine is a registered drug in the United States. The label indications are sympatholytic and mydriatic, but the label also states "impotence has been successfully treated with yohimbine in male patients with vascular or diabetic origins and psychogenic origins (18mg/day)" (93). One of the disadvantages of yohimbine is its daily dosing schedule, which in addition to being inconvenient, may

contribute to the low efficacy observed in some of the clinical studies. A single on-demand dose has not been evaluated, although it has been successfully used by some clinicians and seems to be at least as effective as the standard daily dose (128, 132). Moreover, it has been suggested that tolerance develops after chronic administration of yohimbine, which further diminishes the efficacy of a daily dose (128). Because of the lack of conclusive clinical data on yohimbine, the American Urological Association, in its guidelines on the treatment of organic erectile dysfunction issued in 1996, concluded that "based on the data to date, yohimbine does not appear to be effective for organic erectile dysfunction, and thus should not be recommended as treatment for the standard patient" (133).

Topical anesthetics are the only approved treatment for premature ejaculation. Few uncontrolled clinical studies reported positive results (119, 134). However, the decades-old therapy has not been evaluated in controlled clinical trials. Oral pharmacotherapy has been used off-label and is gaining more popularity with the increased public interest in oral therapies for sexual disorders. Some of the drugs that have been historically used are adrenergic antagonists and y-amino butyric acid (GABA). Selective serotonin reuptake inhibitors, namely paroxetine and fluoxetine, are currently the most commonly used oral agents for premature ejaculation.

There are no approved treatments for female sexual dysfunction, although the subject is currently enjoying vast interest. At present, the main off-label treatments are the vasoactive agents developed for erectile dysfunction. Clinical studies are underway to support their use.

The following sections will focus on approved treatments for erectile dysfunction.

6.1.2 Adverse Effects. Sildenafil is well tolerated in patients with normal cardiovascular function. Most of the side effects reported in the clinical trials at a higher rate than placebo are mild and are related to the drug's vasodilatory effect. They include headache, flushing, and nasal congestion. Other frequent side effects include abnormal vision (impairment of color discrimination) and dyspepsia. The former is a result of PDE6 inhibition and the latter is caused by the inhibition of PDE5 in the lower esophageal sphincter. Prolonged erection has been reported but is less frequent than with intracavernosal therapy. Priapism was reported in few patients, but the risk rate is extremely low. Potentially serious cardiovascular effects including decreased blood pressure caused by systemic vasodilatation and decreased cardiac output may occur in patients with preexisting cardiovascular risk factors. Sexual activity is an added risk factor in those patients. In addition, sildenafil potentiates the hypotensive effects of nitrates and is contraindicated in patients who are using organic nitrates. Most of reported deaths with sildenafil were caused by the concomitant treatment with nitrates. Some reported deaths occurred in patients with preexisting cardiovascular risk during or after sexual activity.

The main side effects of alprostadil are local. For intracavernosal injection, these include the following: penile pain after injection, prolonged erection, priapism, painful erection, penile fibrosis, and injection site hematoma. Priapism and prolonged erection are more serious side effects with alprostadil than with sildenafil. The local side effects after intraurethral administration are usually milder and include penile pain, urethral discomfort, and urethral bleeding/spotting and prolonged erection. Systemic side effects include hypotension (intraurethral administration only), headache, dizziness, and upper respiratory tract infections.

Yohimbine is well tolerated at the oral doses used for erectile dysfunction. The main side effects are nausea, dizziness, and nervousness. Headache and skin flushing have also been reported. Yohimbine has no significant effect on β-adrenergic receptors, and its effect on blood pressure has not been adequately evaluated. Common side effects after parenteral administration include sweating, nausea, and vomiting. Yohimbine penetrates the blood-brain barrier and can produce a complex pattern of responses (93). The central effects include anti-diuresis, a general picture of central excitation including elevated blood pressure and heart rate, increased motor activity, irritability, and tremor.

6.1.3 Pharmacokinetics

6.1.3.1 Sildenafil. Sildenafil is rapidly absorbed after oral administration. The peak plasma concentration is reached after 30–120 min of dosing, but the absorption rate is delayed if taken after high fat meals. The absolute oral bioavailability is approximately 40%. The mean steady-state volume of distribution is 105 L, which indicates distribution into tissues. Sildenafil and its major metabolite are approximately 96% bound to plasma proteins.

Sildenafil is eliminated mainly by hepatic metabolism. The main metabolizing enzyme is cytochrome P450 3A4. The major circulating metabolite is N-desmethyl sildenafil, which is further metabolized. N-desmethyl sildenafil retains sildenafil's selectivity profile and approximately 50% of its activity. Both sildenafil and its major metabolite have terminal half-lives of 4 h. The elimination half-life of sildenafil is 3–5 h. Most of the administered dose is excreted in the feces as metabolites with only 13% excreted in the urine.

6.1.3.2 Alprostadil. Intraurethral PGE, (alprostadil) is absorbed from the urethra and distributes to the erectile tissue (cavernosal smooth muscles) by communicating blood vessels between the corpus spongiosum and the corpora cavernosa. The transurethral absorption is biphasic with an initial rapid phase followed by slower absorption. Approximately 80% of the administered dose is absorbed within 10 min and about 20% of the total dose reaches the cavernosal tissue.

Alprostadil binds to plasma proteins, primarily albumin and to a lesser extent to a-globulin IV-4 fraction. There is no evidence of tissue binding or accumulation.

Alprostadil is rapidly metabolized locally within the corpus cavernosum and in the urethra by enzymatic oxidation of the 15-hydroxyl group to 15-keto-PGE,. 15-Keto-PGE, has only 1–2% of the biological activity of PGE,. 15-Keto-PGE, is rapidly reduced to the inactive metabolite 13,14-dihydro,15-keto-PGE,, which is the most abundant metabolite in plasma. DHK-PGE, is further metabolized to other inactive metabolites. The half-life of alprostadil after intracavernosal administration is 5–10 min (135). Only a small fraction of the dose reaches the systemic circulation after intracavernosal or intraurethral administration. The systemic metabolism of alprostadil is also very rapid. Eighty percent of the circulating alprostadil is metabolized in one pass through the lungs; thus, plasma levels are low and become undetectable within 1 h of intraurethral or intracavernosal administration. The main pathways for systemic metabolism are β- and ω-oxidation. The metabolites are excreted primarily by the kidneys. Ninety percent of the intravenously administered alprostadil appears in urine as metabolites within 24 h after administration. The remaining 10% is excreted in the feces.

6.1.3.3 Yohimbine. Yohimbine absorption after oral administration is highly variable. Both rapid and slow absorption have been reported (136,137). Peak plasma levels occurred at 10–45 min post-dosing in one study and 36–120 min with an average of 1.1 h in another study. The oral bioavailability is generally low and highly variable. It ranges from 4% to 87%. Yohimbine is highly metabolized, with only 1% of the administered dose excreted unchanged in urine. The mean elimination half-life is 0.63–1.5 h. Clearance is highly variable as well, with a mean value of 11 mL/min/kg. Yohimbine has a steady-state volume of distribution of approximately 32 L, and is highly bound to plasma proteins (around 80%). It has been postulated that an active metabolite with a longer duration of action is at least partially responsible for yohimbine pharmacological activity (138, 139). Two metabolites have been identified: 10-hydroxy-yohimbine and 11-hydroxy-yohimbine. Both the parent drug and the two metabolites are distributed to the cerebrospinal fluid. ll-Hydroxy-yohimbine was shown to have α2-adrenoreceptor antagonist activity in *vitro*. It has a long half-life of approximately 9.6 h and is present at a much higher plasma concentration than yohimbine after oral administration of the parent drug (137).

6.2 Physiology and Pharmacology

6.2.1 Physiology of Erection. The penis is composed of three bodies: a pair of corpora cavernosa on the dorsal side and a corpus spongiosum on the ventral side. The role of the corpus spongiosum is to protect and support the urethra, while the two corpora cavernosa

are the parts that provide structure to the penis in the erect state. The cavernosal bodies consist of a network of vascular sinuses supplied by the terminal branches of the cavernosal arteries. The vascular sinuses are supported by smooth muscles that are normally contracted when the penis is in the flaccid state. Factors that mediate cavernosal smooth muscle contraction, and therefore promote penile flaccidity, include the following (140): α-adrenoreceptors, endothelin, angiotensin, and thromboxane A_2.

Erection occurs as a result of increased pressure in the corpora cavernosa, which translates into penile rigidity. The pressure increase is caused by three synergistic processes: (*1*) relaxation of the smooth muscles of the corpora cavernosa; (2)increase in arterial blood flow to the penis; and (*3*) restriction of the venous blood flow out of the penis. Both central and peripheral mechanisms contribute to the process of penile erection. At the central level, the psychological component of penile erection is controlled by the hypothalamic and limbic systems (140). At the peripheral level, both sympathetic and parasympathetic pathways as well as several mediators are involved. Psychogenic and local stimulation results in the release of neurotransmitters from the cavernosal nerve terminals and smooth muscle endothelium. Factors that mediate the corpus cavernosum relaxation include nitric oxide (NO), vasoactive intestinal peptide (VIP), calcitonin gene-related peptide (CGRP), and prostaglandin.

6.2.2 Role of Nitric Oxide. The NO pathway is the best understood and is believed to be the most important pathway for penile erection. NO is released from nonadrenergic noncholinergic (NANC) nerves in the corpus cavernosum and from the endothelium that lines the cavernosal vascular sinuses and blood vessels (140). Nitric oxide synthase catalyses NO formation from the precursors L-arginine and molecular oxygen (141). NO production in the vascular endothelium is stimulated by muscarinic acetylcholinergicreceptors. The released NO diffuses into smooth muscles and interacts with guanylate cyclase, which catalyses the formation of cyclic guanosine monophosphate (cGMP) from guanosine triphosphate. The resulting increase in cGMP activates several processes that lead to smooth muscle relaxation. The increased arterial inflow to the corpora cavernosa as a result of smooth muscle relaxation leads to an increase in intracavernosal pressure and volume and thus increased penis length and rigidity. The increase in intercavernosal pressure results in compression of the subtunical venules and hence reduction of the venous outflow, which further increases penile rigidity (140, 142). cGMP activity is terminated by hydrolysis by cGMP specific type-5 phosphodiesterase (PDE).

6.2.3 Other Mediators. It is believed that VIP-, CGRP-, and prostaglandin-mediated pathways contribute to smooth muscle relaxation by increasing intracellular cAMP concentration in the corpora cavernosa (140). Increased cAMP results in phosphorylation and dephosphorylation of the actin-smooth-muscle-myosin cascade that causes smooth muscle relaxation. Prostaglandin E (PGE) causes the elevation of cAMP through a G-protein–coupled mechanism and activation of adenylyl cyclase. In addition, PGE reduces the adrenoreceptor mediated vasoconstriction by inhibiting norepinephrine release through prejunctional receptors on norepinephrine containing neurons.

6.2.4 Causes of Erectile Dysfunction. Erectile dysfunction can result from neurological, vascular, hormonal, or psychological factors, or from a combination of two or more of these factors. Neurological causes include spinal cord injury, multiple sclerosis, or any other condition that impedes the transmission of the neural signal generated by psychogenic stimulation. Vascular causes include arterial insufficiency, which results in low arterial pressure delivered to the penis, and venous insufficiency, in which excessive venous outflow occurs because of inadequate compression of the subtunical venules. Erectile dysfunction of hormonal origin results from inadequate androgenic stimulation of the sexual center in the anterior hypothalamus, which lowers libido and hence erection quality. In psychogenic erectile dysfunction, sexually inhibiting psychological issues can impede

stimulatory signals to the penis, and anxiety can cause a sympathetic discharge, which favors flaccidity (142). It is usually difficult to distinguish primary psychogenic erectile dysfunction because many men with organic erectile dysfunction have a psychological response to their condition.

6.2.5 Mechanisms of Action. Most erectile dysfunction pharmacotherapies act by either inhibiting the contractile system (e.g., α-adrenoreceptor antagonists), or by stimulating or enhancing the vasodilatory system (e.g., prostaglandin E_1, NO donors, PDE inhibitors). These mechanisms can work regardless of the origin of the disorder.

***6.2.5.1* Sildenafil.** Sildenafil is a selective PDE5 inhibitor. PDE5 is the cGMP-specific PDE and is the predominant form of the isoenzyme in the corpora cavernosa. By inhibiting the hydrolysis of cGMP that is generated through the NO pathway, sildenafil enhances the cavernosal smooth muscle relaxation. For sildenafil to exert its effect, it requires intact NO-relaxing nerve fibers and an intact cavernosal epithelium. Sildenafil is not effective in patients with vascular disease where NO production is impaired, and has no effect in the absence of sexual stimulation.

Sildenafil has high selectivity for PDE5. It has 80- to more than 8500-fold selectivity to PDE5 versus PDE1, PDE2, PDE3, and PDE4 (125). PDE6, the isoenzyme found in the retina, is closely related to PDE5. The selectivity of sildenafil to PDE6 is one-tenth of that to PDE5 (143). This explains the visual abnormalities observed with higher plasma levels of sildenafil. In addition to the cavernosal tissue, PDE5 is also found in lower concentrations in other human tissues including platelets, vascular smooth muscles, and skeletal muscles. The inhibition of PDE5 in these tissues is believed to be responsible for the vascular side effects of sildenafil.

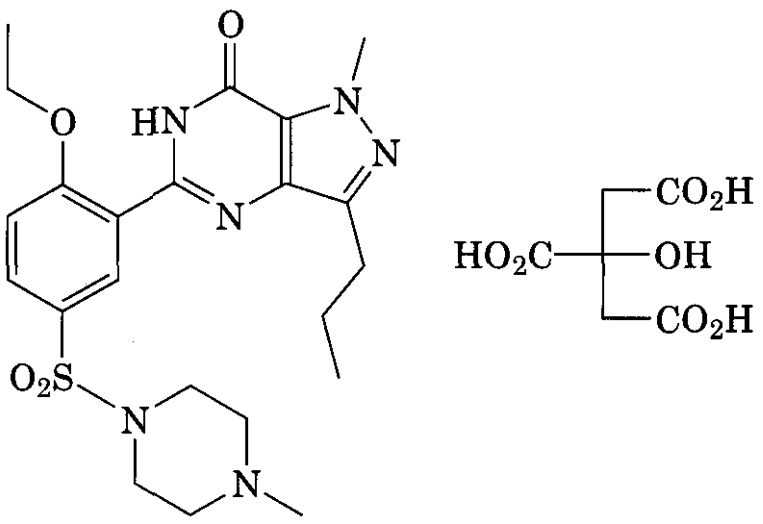
***6.2.5.2* Alprostadil.** Alprostadil is chemically identical to the naturally occurring form of prostaglandin E_1 and acts similar to the endogenous PGE. It induces erection by relaxation of the cavernosal smooth muscle and dilation of cavernosal arteries, which leads to increased arterial inflow and decreased venous outflow. Alprostadil has various systemic effects including vasodilatation, inhibition of platelet aggregation, and stimulation of intestinal and uterine smooth muscles.

***6.2.5.3* Yohimbine.** Yohimbine is an α-adrenoreceptor antagonist, with high selectivity to the presynaptic α2-adrenergic receptors. It is generally believed that yohimbine exerts its erectogenic effect by antagonizing the adrenergic inhibitory tone that suppresses erection. The mechanism of action of yohimbine on some sexual functions seems to be central, i.e., by inhibiting the presynaptic a2 receptors in the brain (129). Published data from animal experiments support the postulation that yohimbine's effects on sexual arousal and ejaculation occur through a central mechanism, because the drug can induce arousal even after genital anesthesia (144) and it reverses clonidine-induced inhibition of ejaculation (145,146). However, the mechanism of action of yohimbine on erectile function was not fully understood and its site of action was not conclusively identified until it was demonstrated few years ago that functional a2 receptors are expressed in the human corpus cavernosum (147). It is now believed that yohimbine exerts its erectogenic action by blocking post-synaptic a2 receptors in the corpus cavernosum and thus inhibits the contractility of the cavernosal tissue (128). In addition to its α-adrenergic effects, yohimbine exerts a stimulatory action on the mood and may increase anxiety. This effect is at least partially attributed to yohimbine's central serotonergic activity (148), although it has not been adequately studied.

***6.2.5.4* Other Agents.** Phentolamine is a nonselective α1- and α2-adrenoreceptor antagonist. It is a weak erectogenic agent when used alone as an intracavernosal injection. Phentolamine is usually used in combination with alprostadil and/or papaverine.

6.3 Chemistry and Structure-Activity Relationships

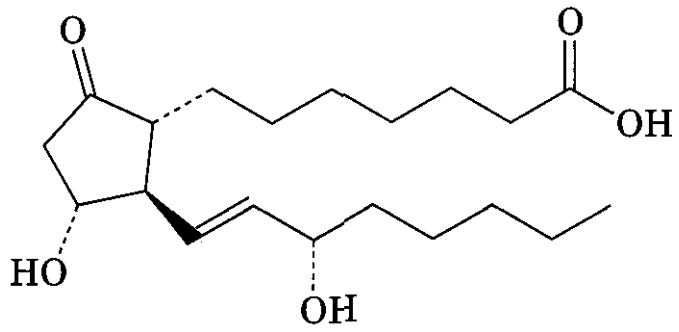
Sildenafil (**6**) is a pyrazolopyrimidinonederivative. The pyazolopyrimidinone ring is essential for PDE5 inhibitory activity. It seems that this ring structure mimics the guanosine base of cGMP (149). Another ring system that has been shown to produce potent and selective PDE5 inhibitory activity is imidazoquinazoli-



(6) Sildenafil Citrate

none (150). In the sildenafil series, the n-propyl substitution on position 3 of the pyazolopyrimidinone ring gives a more potent compound compared with a 3-methyl analog. Intramolecular hydrogen bonding of sildenafil and its analogs is important for biological activity by maintaining co-planarity between the phenyl and purine ring (151). The 2'-alkoxy moiety on the phenyl group serves this purpose by providing the oxygen lone pair for hydrogen bonding with the pyrimidinone NH (149). The alkoxy moiety also provides the requirement for a small lipophilic substituent, which is important for activity. Replacement of the alkoxy moiety with a hydrogen reduced PDE5 affinity by 200-fold (149). Hydroxy, nitro, and sulphonamide derivatives also had lower affinity to PDE5. It has been demonstrated that an open chain 2'-alkoxyl group serves the lipophilicity requirement better than a fused ring system. An ether ring fused into the phenyl moiety, although increasing the degree of co-planarity, largely reduced the PDE5 inhibitory activity (151). An N-acylamido substitution at the 5'-position of the phenyl ring was shown to enhance PDE5 inhibitory activity (152). Sildenafil analogs with such a substituent were one to three times more potent than sildenafil, based on in *vitro* PDE5 inhibitory activity. The activity increased with increasing the chain length of the N-acylamido moiety. This substitution, however, decreased the analogs' selectivity to PDE5 over PDE6 (152). The sulfonamide substituent on position 5' enhances the aqueous solubility of sildenafil and also increases its affinity to cGMP (149). Introduction of a carboxylic acid group to the 5'-sulfonamide moiety of the phenyl ring greatly enhanced the in *vitro* PDE5 activity (153), possibly caused by mimicking the phosphate group of cGMP.

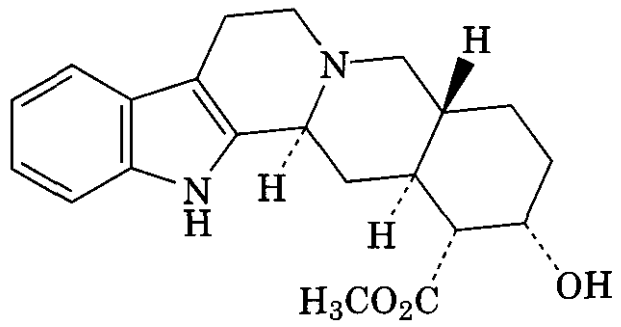
Alprostadil (7) is a synthetic form of prostaglandin E_1 (PGE_1). It is chemically identical



(7) Alprostadil

to the naturally occurring PGE_1. Prostaglandin E_1 is an acidic lipid that is synthesized by mammalian tissues from fatty acid precursors.

Yohimbine (8) is a natural alkaloid found in Rubaceae and related trees, mainly, from the



(8) Yohimbine

bark of the African tree *Pasinystalia yohimbe*, and is also found in Rauwolfia Serpentina. It is an indolalkylamine alkaloid with structural similarity to reserpine. Chemically, it is known as 17α-hydroxy-yohimban-16α-carboxylic acid methylester. The commercial product contains the hydrochloride salt of yohimbine.

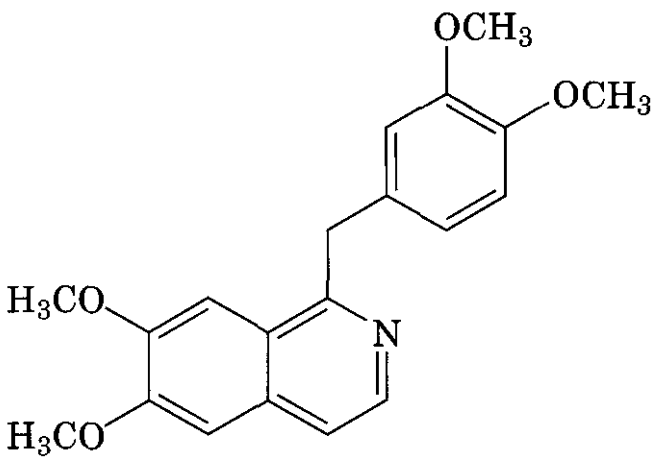
6.4 History

The use of drugs for the treatment of impotence and other sexual disorders is very old. Yohimbine has been used in folk medicine by men and women for various forms of sexual dysfunction, mainly as an aphrodisiac for over a century. In the United States, yohimbine hydrochloride has been marketed and prescribed by doctors for more than 75 years (154). Be-

cause the mainstream medical use of yohimbine pre-dated the Food and Drug Act, the drug did not go through the formal FDA review and approval process, and hence, there are no well-controlled clinical trials with a sufficient number of patients to allow for conclusive results on its efficacy and long-term safety. The lack of conclusive clinical data and the daily dosing schedule that has been practiced for decades, rather than a more convenient on-demand use, are the main reasons for the low level of interest in yohimbine by medical practitioners. Needless to say, pharmaceutical companies lack the interest in conducting expensive large-scale clinical trials of yohimbine, because the ancient drug is nonpatentable.

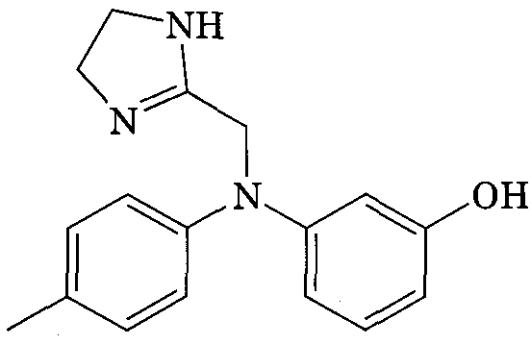
Because of the absence of clinically proven safe and effective pharmacotherapy for erectile dysfunction, psychotherapy was until recently the core treatment for most patients, except for a few cases where a surgically correctable cause could be identified. The results of psychotherapy have been disappointing (155), and often times, the goal of psychotherapy was to help men accept their sexual dysfunction rather than to correct it. The widespread belief that erection failure is primarily of psychological origin, and hence the acceptance of psychotherapy as the primary treatment, continued until the early 1980s.

In 1982, a new era for erectile dysfunction started when Virag (156) showed that penile injection of a vasoactive amine—papaverine (9)—could result in erection without sexual



(9) Papaverine

stimulation. In 1983, Brindley (157) reported the clinical efficacy of cavernosal a-blockade in the treatment of erectile dysfunction. As a result of the extensive research on the mechanism of erection that followed Virag's and Brindley's pioneer work, it was established that underlying organic causes are responsible for approximately 80% of persistent erectile dysfunction cases (142). This resulted in changing the primary treatment from psychotherapy to pharmacotherapy and stimulated further research in this area. Intracavernosal injections of papaverine and phentolamine **(10)** became a common treatment for erectile



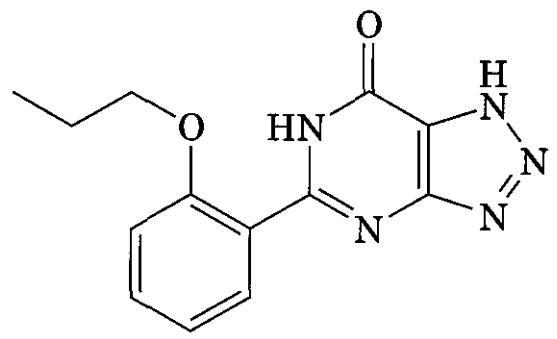
(10) Phentolamine

dysfunction in addition to vacuum/constriction devices. As a result of the growing interest and increased understanding of the condition, the U.S. National Institute of Health (NIH) advocated the use of the term erectile dysfunction (ED) rather than impotence in 1992 (158).

The first pharmacological agent for ED to be approved by the FDA was intracavernosal alprostadil. It was approved in June 1995 for the treatment of erectile dysfunction caused by neurogenic, vasculogenic, psychogenic, or mixed etiology. Despite its clinical efficacy, the high cost, and more importantly, fear of self injection limited the use of intracavernosal therapy. The search for a less invasive therapy led to the development of a novel transurethral dosage form of alprostadil, which gained FDA approval in 1997. This was still far from the ideal therapy. The main disadvantages of alprostadil therapy are loss of spontaneity, because erection occurs even without sexual stimulation, and rapid onset of action, which does not allow for discreet administration and also contributes to the loss of spontaneity.

In 1985, the story of sildenafil discovery (159) started when two chemists at Pfizer's site in Kent, U.K. proposed to look for antihypertensive and antianginal compounds that

would work by inhibiting phosphodiesterase (PDE) and thus promoting the vasodilator action of cGMP. The project started with the usual literature review to identify a chemical starting point. Zaprinast (11), a compound



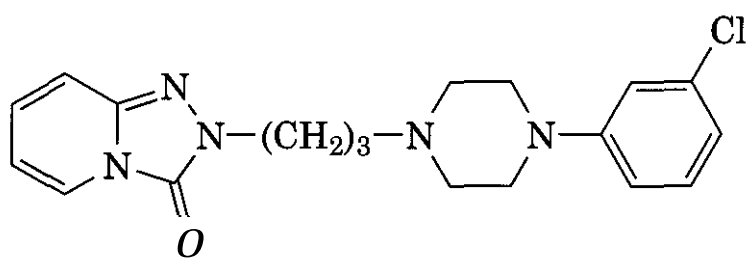
(11) Zaprinast

that was developed as an antiallergic by May and Baker (later part of Rhone-Poulenc Rorer, which is now part of Aventis), was selected. Zaprinast, which was never commercialized, was not selective enough to PDE; however, it provided a satisfactory starting point for the Pfizer team. In their effort to modify the Zaprinast structure to make it more selective and more potent, Pfizer chemists explored other ring systems and varied the side-chain substitutions. A total of about 1600 compounds were synthesized. In 1989, sildenafil (UK 92480) was identified as a promising candidate based on its in *vitro* selectivity to PDE and its potency (159). Sildenafil had a more than 500-fold affinity to PDE than the starting compound (149). Phase I clinical trials of sildenafil started in 1991. In 1992, the results of a limited phase II clinical study in patients with severe coronary heart disease were disappointing, but at the same time, the first report of sildenafil's effect on erectile function came from a parallel high dose phase I study. The "side effect" of erection caught the team's interest; however, the decision to change the direction of the sildenafil development program from cardiovascular to erectile dysfunction came only after long deliberations (159). The discovery of nitric oxide and the understanding of its role as a signaling molecule in the early 1990s helped Pfizer's researchers to understand the mechanism of sildenafil's effect on erection and therefore helped in making that decision. The first phase II clinical trial for erectile dysfunction started in 1994 and included 12 men with ED. Ten of 12 patients showed improvement. The next trial, which took place in 1994 and 1995, was an outpatient trial. This was followed by a large open-label multi-center clinical trial in which 225 patients were assessed for over 32 weeks. Eighty-eight percent of patients reported improvement, and over 90% expressed interest in continuing the treatment. In total, the drug was tested in about 4000 men, ages 19–87, who suffered from erectile dysfunction of organic, psychogenic, or mixed origin, or with no identified etiology (117). In all of the studies, sildenafil exhibited superior efficacy over placebo. The NDA was submitted to the FDA in September 1997, and sildenafil was approved in March 1998 after a fast track review.

6.5 Future Trends

A few dozen potential products are in clinical development for male and/or female sexual disorders. Several of these potential products are novel delivery systems of already marketed compounds, such as two topical delivery systems of alprostadil developed by NexMed and Macro Chem, and a lyophilized liposomal delivery system for urethral administration of alprostadil, developed by Harvard Scientific.

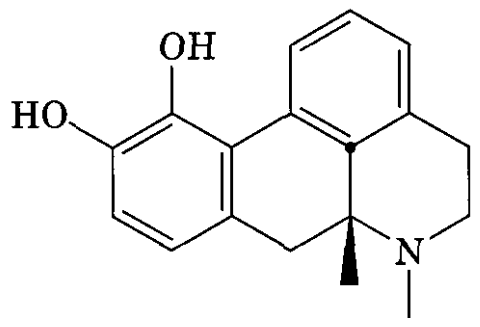
New oral agents that target central and peripheral pathways of erection are expected to be introduced in the next few years. Some of the older off-label agents are also in late phases of clinical development or under FDA review. An injectable formulation of phentolamine is being developed by Novartis, and Senetek is developing a combination of vasoactive intestinal peptide (VIP) and phentolamine in an autoinjector. Trazodone is another older drug marketed as an antidepressant and is in clinical development for erectile dysfunction. Trazodone (12) is a centrally acting serotoninergic agonist with peripheral sympatholytic activity. Studies indicate that it



(12) Trazodone

is mostly effective in patients with psychogenic etiology (160). Other serotonin agonists are being tested by Vivus for premature ejaculation and other sexual disorders.

Apomorphine (13)is a dopaminergic agonist that acts at the paraventricular and supraoptic nuclei in the brain, but has low oral bioavailability. TAP Pharmaceuticals is developing a sublingual formulation of apomorphine for the treatment of erectile dysfunction. Recently, TAP withdrew its NDA application of the sublingual preparation after the FDA advisory panel rejected the use of 4 mg of apomorphine in ED treatment because of safety concerns. The company hopes to resubmit the 2-mg NDA application in October 2002. Nastec is testing an intranasal formulation of apomorphine (1mg) for erectile dysfunction. The lower dose will help reduce the side effects.



(13) Apomorphine

In addition, several new PDE5 inhibitors are in various phases of clinical development. The closest to marketing are ICOS/Eli Lilly's IC-351 (Cialis) and Bayer's vardenafil. Both drugs are expected to be approved by the FDA in 2002. Other novel compounds include didesmethylsibutramine (by Sepracor), which is a single isomer of an active metabolite of sibutramine—a norepinephrine and dopamine reuptake inhibitor--and nitric oxide NMI-870 (NitroMed), which is a nitric oxide–enhanced compound of yohimbine for specific delivery of NO to target tissue.

Melanotan II or PT-141 (Palatin Technologies) is a synthetic analog of melanocyte-stimulating hormone (161). The peptide is currently under development as an intranasal formulation for both male and female sexual dysfunction. It is a non-selective melanocortin receptor agonist, which in animals regulates sexual behavior including penile erection, sexual motivation, and in female rats, the secretion of sexual attractants from the preputial gland. Melanotan, which was originally discovered and synthesized by researchers at the University of Arizona, started as a suntanning agent. Spontaneous erection reported in a tanning clinical study led to the initiation of a joined development program between University of Arizona and Palatin Technologies. Results from phase IIA studies were very positive, with response rate greater than 80% and good safety profile (162). Phase III trials for erectile dysfunction are expected to start in 2003. Topical testosterone (gel formulations from Solvay and Cellegy, and a patch from Watson) is being tested for reduced sexual desire in women. Bupropion was shown to improve sexual desire in a pilot clinical study, and the indication is likely to be pursued.

7 SMOKING CESSATION AGENTS

Although smoking cessation is a lifestyle decision, the ability to quit is usually hindered by nicotine chemical dependence. According to the Surgeon General report on smoking in 2000 (163), "Tobacco dependence is in fact best viewed as a chronic disease with remission and relapse. Even though both minimal and intensive interventions increase smoking cessation, most people who quit smoking with the aid of such interventions will eventually relapse." It is estimated that 24.7% of all adults who lived in the United States in 1997 were smokers (164). This is only slightly lower than the 25.5% prevalence in 1994 (165). Statistics show that over 70% of smokers express a strong desire to quit and about 20% actually try to stop smoking. However, only 3–5% of smokers who attempt to quit smoking on their own achieve a successful abstinence after 1 year (165, 166). The agency for Health Care Policy and Research (AHCPR) in its Clinical Practice Guidelines on Smoking Cessation released in April 1996 (The U.S. Public Health Service practice guidelines) (167) state that every patient attempting to stop smoking should be treated with pharmacotherapy. Pharmacological intervention is often part of a multicomponent therapy that also includes one or more non-pharmacological components, mainly psychosocial therapy (behav-

ioral therapy). The AHCPR found that smoking cessation interventions by healthcare providers doubled the success rate of smoking cessation (167). When multiple types of providers (e.g., clinicians and psychotherapists) deliver these interventions, the likelihood of smoking cessation increases by a factor of four. The panel also found that nicotine replacement therapy (gum and patches) is efficacious regardless of the use of adjuvant treatment (psychosocial interventions), but its efficacy is increased when used with adjuvant interventions.

Treating tobacco dependence is one of the most cost-effective preventive measures. It is estimated that smoking cessation is more cost-effective than several common clinical preventive services such as screening for cervical, breast, and colon cancer, treatment of mild-to-moderate high blood pressure, and treatment of high cholesterol (163).

7.1 Clinical Use

7.1.1 Current Drugs. The FDA-approved smoking cessation medications include nicotine replacement therapy (NRT) available in several delivery systems and one non-nicotine drug. Only nicotine gum and nicotine patches are available over-the-counter. Table 9.9 lists smoking cessation medications that are approved for clinical use in the United States. The dose of NRT varies depending on the delivery system and the current usage level of tobacco by the patient. Bupropion therapy is usually started 1 week before quitting smoking as 150-mg slow-release tablets twice a day. The usual length of treatment of smoking cessation therapy is 6–12 weeks. Because all the approved smoking cessation agents are almost equally effective and safe (168), the choice of treatment usually depends on the patient preference. Bupropion is particularly useful for smokers who had unsuccessful attempts with NRT. It is also useful for smokers who are concerned about possible weight gain after quitting. Bupropion was shown to attenuate weight gain after smoking cessation; however, the benefits are not sustainable once the treatment is discontinued (169,170). Although the OTC labels recommend against using nicotine gum or patches in combination with other nicotine products, there is evidence that combining a nicotine patch with 2-mg gum *ad libitum* for high craving instances may be advantageous (171). The results, however, were not sustainable in clinical trials after 12 months (172, 173). Using the nasal spray along with the patch for severe craving also increased the success rate (174). The combination of bupropion and nicotine patch was found to be more effective than either treatment alone, although the difference between the combination and bupropion alone was not statistically significant (175, 176).

7.1.2 Adverse Effects. The main adverse effect of nicotine is psychoactive substance dependence. Acute and chronic tolerance to nicotine effects on the brain function and activity lead to increased nicotine consumption through smoking (177, 178). Acute tolerance to nicotine can develop in less than 1 h, but develops at different rates for different physiological effects. Because of the highly addictive effects of nicotine, smoking cessation results in nicotine withdrawal syndrome. The symptoms can start as early as 2 h after the last nicotine consumption and reach a peak in 24–48 h after smoking cessation. The symptoms include, in addition to tobacco craving, depression, insomnia, irritability, nervousness, restlessness, difficulty concentrating, anxiety, drowsiness, sleep disturbances, decreased heart rate, and increased appetite (179, 180). Side effects of excess nicotine consumption include nausea, vomiting, abdominal pain, diarrhea, flushing, dizziness, disturbed hearing and vision, weakness, confusion, and palpitation.

Side effects specific to nicotine delivery systems are mainly caused by local irritation at the site of administration. For example, nausea, indigestion, sore gums, and mouth ulceration may occur when using the gum. The patches may cause skin irritation, which is characterized by erythema, pruritus, edema, and rarely, vesicles. The nasal spray may cause irritation of the nasal mucosa, sneezing, coughing, and lacrimation, although tolerance to these effects develops rapidly with continued use. Side effects from the inhaler include mild mouth and throat irritation and coughing (181).

Table 9.9 Current Smoking Cessation Agents on the U.S. Market

Generic Name	Brand Name	Delivery System	Originator	Strength	Daily Dose	Current Regulatory Status
Bupropion hydrochloride	Zyban	SR tablet	Glaxo Wellcome	150 mg	300 mg	Prescription
Nicotine	Habitrol	Transdermal system	Novartis	21, 14, 7 mg	1 patch	OTC
Nicotine	Nicotrol	Transdermal system	Cygnus/Pharmacia	15 mg	1 patch	OTC
Nicotine	Nicotrol inhaler	Inhalation system	Parke-Davis	10 mg/Cartridge (4 mg delivered)	6–16 Cartridges	Prescription
Nicotine	Nicotrol NS	Nasal spray	Parke-Davis	10 mg/mL (0.5 mg/spray)	Up to 40 mg (80 sprays)	Prescription
Nicotine polacrilex	Nicorette	Nicotine-polacrilex gum	Lakeside Pharmaceuticals (Merrell Dow)	2 mg, 4 mg	Up to 24 pieces	OTC

Bupropion is a well-tolerated antidepressant. It is non-sedating and lacks the cardiovascular and anticholinergic side effects of tricyclic antidepressants. Bupropion's most commonly observed adverse effects are insomnia and dry mouth. The drug is also known to be associated with a low rate of seizures; however, no seizure incidents were reported in the smoking cessation clinical trials. Other less frequently occurring side effects include nervous system disturbances (mainly tremor) and skin rashes (170, 182).

7.1.3 Pharmacokinetics. Nicotine is readily available by inhalation after tobacco smoking. Inhalation is the fastest route for nicotine absorption and results in the highest bioavailability. The bioavailability from cigarettes in smokers who inhale is about 90% (183). These smokers attain a maximum blood level almost instantaneously. Nicotine, a small lipophilic molecule, is also rapidly absorbed through skin and the mucous membranes. It is a basic compound, and therefore, is absorbed in a pH-dependent manner. Absorption through the oral mucosa seems to be slower than the nasal route (possibly because of the pH effect). Cigarette smoke is mildly acidic (pH 5.3) and, therefore, only little absorption occurs in the buccal cavity (about 10%). However, in the alkaline smoke of pipes and cigars (pH 8.5), nicotine is absorbed rapidly through the oral mucosa (183). Nicotine penetrates the blood-brain barrier and reaches the central nervous system rapidly, with maximum brain concentration reached within a few seconds after cigarette smoking (182). In general, absorption from nicotine-replacement drug delivery systems is controlled by the release rate from the vehicle. The nasal spray provides the fastest release and hence fastest absorption of nicotine, and therefore has the advantage of rapid alleviation of nicotine withdrawal symptoms. The maximum nicotine concentration is reached in 4–15 min after administration of nicotine nasal spray, which is still slower than the almost instantaneous peak after smoking tobacco. Nicotine absorption from the nicotine polacrilex gum is slower because of a slower release rate from the delivery system and slower absorption through the oral mucosa. When used properly, each piece of the gum provides continued absorption for approximately 30 min. Although the inhaler has the advantage of mimicking the act of smoking, absorption rate is comparable with Nicorette gum and slower than that from the nasal spray. Nicotine absorption from the "inhaler" occurs through the buccal mucosa rather than the lungs. Nicotine patches, extended release transdermal delivery systems, maintains the most sustained blood level and therefore requires less frequent dosing.

Nicotine bioavailability also varies with the delivery system. The reported bioavailability from the nasal spray and transdermal patch is 53% and 82%, respectively **(93,** 184). In nicotine polacrilex gum, nicotine is bound to an ion exchange resin and is released only by chewing. Nicotine bioavailability, therefore, is dependent on the vigor, rapidity, and duration of chewing. Of the 10 mg in each cartridge of a nicotine inhaler, only 4 mg is actually delivered from the device to the oral mucosa and is available for absorption.

Nicotine has low plasma protein binding (<5%) and a large volume of distribution (2–3 L/kg). It is eliminated mainly by hepatic metabolism, although some metabolism occurs in the lungs and kidneys. The main metabolites are cotinine (15% of the dose) and trans-3-hydroxycotinine (45% of the dose). Only 10% of the absorbed dose is excreted unchanged in urine. In healthy adult smokers, nicotine has an apparent elimination half-life of 1–2 h and the average plasma clearance is 1.2 h (93).

Bupropion oral bioavailability in humans has not been determined because the drug was never administered intravenously to humans. The relative oral bioavailability in rats and dogs ranges between 5% and 20%. In healthy volunteers, peak plasma concentration is reached approximately 3 h after administration of the sustained release tablet (93). Based on *in vitro* protein binding data, bupropion is 84% bound to plasma proteins. It is widely distributed to tissues and has an apparent steady-state volume of distribution (V_{ss}/F) of approximately 2000 L. Bupropion is extensively metabolized by oxidation and reduction to at least six metabolites with only 0.5% of a bupropion oral dose excreted unchanged in urine. The major metabolite in urine is a glycine conjugate of metachlorobenzoic acid,

which is formed by oxidation of the bupropion side-chain followed by glycine conjugation. Three active metabolites have been identified: hydroxybupropion and the two amino-alcohol isomers threohydrobupropion and erythrohydrobupropion (185). These metabolites are formed through hydroxylation of the *tert*-butyl group of bupropion and/or reduction of the carbonyl group. The potency of the active metabolites has not been assessed in humans. However it is suggested that hydroxybupropion has a primarily noradrenergic effect, whereas threohydrobupropion has some dopaminergic activity and minor noradrenergic effect (186). The CSF concentrations of hydroxybupropion and erythrohydrobupropion are 6 times greater than the parent drug, whereas threohydrobupropion CSF concentration is 40 times that of the parent drug (185). In *vitro* data suggest that cytochrome P450IIB6 (CYP2B6) is the principal enzyme involved in the formation of hydroxybupropion, whereas P450 isoenzymes are not involved in the formation of threohydrobupropion. The mean apparent clearance (Cl/F) of bupropion ranges between 135 and 209 L/h. The mean apparent elimination half-life of bupropion after administration of the sustained release tablet is approximately 21 h. The elimination half-life from the immediate release formulation is 11–14 h. The main elimination half-lives of the active metabolites are 20, 37, and 33 h for hydroxybupropion, threohydrobupropion, and erythrohydrobupropion, respectively (93).

7.2 Physiology and Pharmacology

7.2.1 Pharmacological Action of Nicotine. Nicotine binds selectively to the nicotinic receptors that are present in the adrenal medulla, brain, autonomic ganglia, and neuromuscular junctions. It causes the release of several neurotransmitters and hormones such as acetylcholine, norepinephrine, dopamine, serotonin, arginine vasopressin, β-endorphin, adrenocorticotropic hormone, and cortisol (187). This neuro-regulatory effect of nicotine is dose-dependent and occurs as plasma nicotine level rises when tobacco is smoked. The neurotransmitters released in the brain mediate the behavior modulating effects and positive reinforcing effects of nicotine and other habit-forming drugs.

*7.2.1.*1 ***Neurological Basis of Nicotine Dependence.*** In the brain, nicotine exerts a multitude of psychological and behavioral effects. At low doses, it exerts a predominantly stimulating effect, which occurs in the cortex through locus ceruleus and is mediated by norepinephrine. At high doses, a dopaminergic reward effect predominates (184, 188). The reward and positive reinforcement effects of nicotine are responsible for the drug-seeking behavior in tobacco smokers. A common effect of many drugs of abuse, as well as natural rewards (e.g., food, sex), is the elevation of extracellular dopamine levels (189). The mesolimbic-dopaminergic neurons in the midbrain are thought to be the final common pathway for reward, and the reward effect occurs as a result of the elevated dopamine level. Although there have been some recent challenges to the dopamine reward theory (190–193), it is still unequivocally agreed that dopamine plays a crucial role in the reward system and drug-seeking habit formation. An alternative view to the classical dopaminergic reward theory is that the dopaminergic-neuron activation functions as a learning signal (189, 194), and that the neuroadaptive changes (up-regulation) of the dopaminergic neurons with the chronic use of tobacco (or other drugs of abuse) might result in the generation of a deficit state that enhances drug craving and hence drug-seeking behavior (189). Other neurotransmitters such as norepinephrine and 5-HT have been implicated in the reward and positive reinforcement effects of nicotine.

Nicotine is an agonist to the neuronal nicotinic acetylcholine receptors (nAChR). These receptors are the likely site at which nicotine exerts its central actions. Evidence supports the involvement of the central nAChR in neurotransmitter release (195), and a large body of evidence indicates that dopamine release from dopaminergic neurons is mediated by activation of the brain nAChRs (196). It has also been shown that both nicotinic receptors and muscarinic receptors in the ventral tegmental area (VTA) activate the dopaminergic neurons and thus play a role in the reward effect of nicotine (197). In addition, animal data sug-

gest an involvement of the 5-HT (2C) receptor in mediating the mesolimbic-dopaminergic system (198).

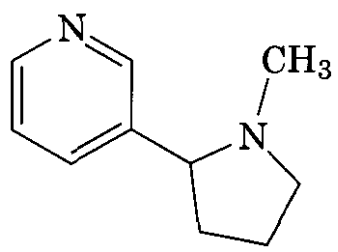
These positive reinforcement effects of nicotine include anxiolytic effect, antinociceptive/analgesic effects, enhanced vigilance, and improved cognitive function. Tolerance to many of these effects occurs rapidly, leading to substance dependence (199, 200). In animal studies, a single pretreatment with nicotine results in acute tolerance to the subsequent dose (201,202). The role of norepinephrine in the positive reinforcement effects of nicotine is not clear, but it has been suggested that nicotine's effects on concentration and attention are mediated by a noradrenergic mechanism (182). The noradrenergic system is also implicated in mediating the effects of nicotine withdrawal. 5-HT receptors in the dorsal raphe nucleus (DRN) are implicated in the anxiolytic effect of nicotine (203). Changes to these receptors mediate the development of tolerance to the anxiolytic effect and hence the anxiogenic response during nicotine withdrawal (203). The antinociceptive/analgesic effect of nicotine is believed to be mediated by a cholinergic pathway through the neuronal nicotinic acetylcholine receptors (204, 205). 5-HT receptors are also implicated in the antinociceptive effect of nicotine, possibly through an interaction between the nicotinic and serotonergic systems (206). Acetylcholine is known to play a crucial role in nicotine's effects on the cognitive function.

7.2.1.2 **Peripheral Pharmacological Actions of Nicotine.** Nicotine effects on the cardiovascular system include tachycardia and peripheral vasoconstriction, which leads to elevated blood pressure. Because the cardiovascular effects are mainly caused by elevated levels of catecholamines and cortisol, tolerance to these effects does not occur. Other pharmacological actions of nicotine include increased gastrointestinal motility caused by parasympathetic ganglionic stimulation and skeletal muscle contraction caused by the effect on nicotinic receptors in the neuromuscular junction (184).

7.2.2 Bupropion Mechanism of Action. Bupropion is a monocyclic non-MAO inhibitor antidepressant with both dopaminergic and noradrenergic activities. It is a weak inhibitor of dopamine, norepinephrine, and serotonin reuptake (207), but it has a greater effect on the neuronal reuptake of catecholamines than of serotonin. Some studies suggest that bupropion is entirely selective for catecholamines and is devoid of any serotonergic activity (185). Bupropion's effectiveness as a smoking cessation agent is not related to its antidepressant effect. The drug is equally effective in smokers with current depression, past depression, or no depression (168). The mechanism by which bupropion enhances the ability for smoking cessation is believed to be related to its dopaminergic and noradrenergic activity (186). In addition, it has been shown recently that bupropion is a potent inhibitor of central nAChRs and that it blocks nicotine activation of these receptors in a dose-dependent noncompetitive fashion (208). This suggests that the nicotinic antagonist effect contributes to bupropion mechanism of action in smoking cessation.

7.3 Chemistry and Structure-Activity Relationships

Nicotine (14) [*S*-3-(1-methyl-2-pyrrolidinyl) pyridine] is the main alkaloid in tobacco. It is a tertiary amine composed of a pyridine and a



(14) Nicotine

pyrrolidine ring. Nicotine exists in two isomeric forms, but tobacco contains only the levorotatory isomer, which is the more pharmacologically active form.

7.3.1 Nicotinic Acetylcholine Receptors. Nicotinic acetylcholine receptors are ligand-gated ion channels whose opening is controlled by acetylcholine and nicotine agonists (196,209,210). They are transmembrane protein structures. Each receptor consists of five subunits. The structure is inserted in the plasma membrane with an aqueous channel in

the center. The subunits have common general structure that comprises the following:

- A large extracellular N-terminal that forms the bulk of the acetylcholine-binding site
- Four putative transmembrane hydrophobic regions that form the channel
- An intracellular loop joining the third and fourth transmembrane domains: this is a very important structure for the regulation of receptor function
- An extracellular C-terminus

In the CNS, the nAChRs are located mainly at the presynaptic nerve terminals where they modulate the synaptic activity by regulating the neurotransmitter release. Multiple populations of nAChRs exist, with a largely diverse subunit composition.

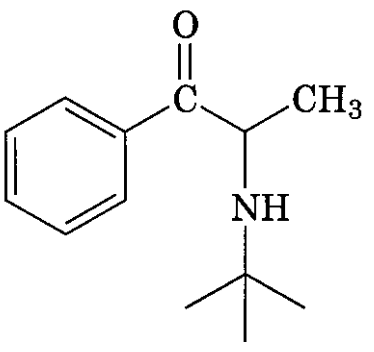
The nAChR subunits are grouped into two main types: ligand-binding subunits (a subunits) and structural subunits (β subunits). Several types of the a and β subunits have been identified. The subunits that are present in the neuronal nAChRs are $\alpha 2$–$\alpha 9$ and $\beta 2$–$\beta 4$. The pharmacological properties of the nAChR subtypes largely depend on their subunit composition. For example, the analgesic effect is believed to be mediated by the $\alpha 4\beta 2$ subtype, whereas the dopamine release from dopaminergic neurons in the brain is controlled in part by another $\alpha 4$-containing subtype and possibly by an $\alpha 6$-containing subtype.

Since the discovery of the neuronal nAChRs, there has been a rapidly growing interest in designing drugs that are selective to neuronal nAChR subtypes and hence can specifically target diseases or clinical conditions such as smoking addiction, pain, anxiety, attention deficit, Alzheimer disease, Parkinson disease, and other cognitive and neurodegenerative disorders.

7.3.2 Structural Requirements for nAChR Ligands. Schmitt (211) summarized the general structural requirements for binding at the $\alpha 4\beta 2$ and a7 receptors (the most abundant nAChRs subtypes in the CNS) as follows.

1. A cationic center, preferably a basic or quaternized nitrogen, is required. In nicotine, this requirement is satisfied by the pyrrolidine nitrogen.
2. A hydrogen bond acceptor (HBA) and/or π-electron rich moiety is favored. This is satisfied by a less basic nitrogen such as the pyridine nitrogen of nicotine or a carbonyl oxygen (e.g., the carbonyl oxygen of acetylcholine).
3. The receptor favors a relative separation between the cationic center and the H-bond acceptor or π-electron moiety of 4–8 Å.
4. The receptors exhibit the tendency toward stereospecific interaction.
5. The receptor may prefer some degree of cation-HBA/π coplanarity.

7.3.3 SAR of Bupropion and Its Analogs. Bupropion (**15**), also known as amfebutamone, is 1-(3-chlorophenyl)-2-[(1,1-dimethylethyl)amino]-1-propane. It is a trimethylated monocyclic phenylaminoketone and is structurally unrelated to the tricyclic antidepressants or MAO inhibitors. Commercially available bupropion is a racemic mixture. The two enantiomers were synthesized and assayed for their potencies as inhibitors of biogenic amine uptake into nerve endings obtained from mouse brain (212). No significant difference was found. However, the relative pharmacological activities and pharmacokinetics of the two enantiomers have not been studied. Bupropion has a novel chemical structure among antidepressants. The absence of polycyclic rings and presence of more common functional groups usually found on tranquilizers contribute to the lack of marked side effects



(15) Bupropion

usually seen with polycyclic antidepressants (213). Bupropion was a designed antidepressant. A series of compounds that included aminoketones and aminoalcohols was designed and screened for antidepressant activity by Glaxo chemists. Structure-activity relationships of a series of bupropion analogs were investigated (213). The strong electron withdrawing effect of the chloro substituent on the aromatic ring in bupropion is believed to be responsible for the lack of CNS stimulant effect. The a-ketone moiety contributes to the metabolic fate of the drug. It prevents the formation of a chloro-monoarylalkylamine metabolite, which would possibly be a CNS stimulant. The use of a tertiary butyl substituent as the alkyl group on the nitrogen atom was designed to diminish the N-dealkylation and hence prevent the formation of metabolites with sympathomimetic side effects. The *meta*-orientation of the two substituents on the aromatic ring was also a designed feature. The ortho position could have high steric hindrance, and the para position would result in facile displacement and para-hydroxylation, which in turn would result in rapid elimination of the drug.

7.4 History

Early research on smoking cessation therapy started in the 1930s. The first experimental medication was lobeline, an alkaloid with physiological actions similar to nicotine. Lobeline and other early non-nicotine drugs failed to show any benefits beyond those seen with placebo (170). As a result of poor efficacy of these experimental drugs, researchers started focusing on nicotine. The first U.S. Surgeon General's report on smoking, which was released in 1964 (214), stimulated the research efforts on nicotine's pharmacological and physiological effects and its role as the main addictive substance in tobacco. The first generation of smoking cessation medication was nicotine polacrilex gum. It was approved by the U.S. Food and Drug Administration in 1984. The second generation was the nicotine transdermal delivery system or patches, the first of which was approved in 1991. Three other patches followed by 1992. Nicotine nasal spray, approved in 1996, and the nicotine inhaler, approved in 1997, were the third generation.

Although NRT has consistently demonstrated better efficacy than placebo, the success rate is still relatively low (30% at the end of treatment and 20% 6–12 months later) (170). This, in addition to increased knowledge on the neurochemical basis of nicotine dependence, renewed the interest in non-nicotine therapy for treatment of nicotine dependence. Clinical trials on clonidine as an experimental non-nicotine therapy in the 1980s led to an interesting and unexpected finding of a strong association between nicotine dependence and depression. Although the study excluded smokers with evidence of depression, analysis of the participants' data showed that 60% had an episode of major depression in the past. Furthermore, depressed mood was one of the symptoms reported frequently by participants in the first week after quitting (215). These findings suggested that antidepressants might be useful in smoking cessation therapy. Researchers started looking into marketed antidepressants for the indication. Ferry et al. (216) selected the antidepressant bupropion because of its dopaminergic activity, because it has been long believed that the reward system is activated through a dopaminergic mechanism. Bupropion, already marketed under the brand names Wellbutrin and Wellbutrin.SR tablets, was first launched in the United States as an antidepressant in 1986, but was voluntarily withdrawn the following year because of concerns over seizure side effects. Glaxo Wellcome was subsequently able to demonstrate to the FDA that the level of seizures in bulimic patients was acceptable, and therefore, the antidepressant was relaunched in 1989. The outcome of the first open-label smoking cessation clinical trial was very positive. It was then followed by three double-blind placebo-controlled studies in non-depressed smokers (a dose–response study, nicotine patch comparative study, and a long-term maintenance trial). The results from these studies demonstrated significant increase in quit rate over placebo for the 150- and 300-mg doses (182). This subsequently led to U.S. Food and Drug Administration approval of bupropion sustained release tablets

as the first non-nicotine treatment for nicotine dependence in 1997. Glaxo Wellcome launched the product under the brand name Zyban for the new indication.

7.5 Current and Future Trends

Because of the limited success of NRT, smoking cessation research efforts are focusing more on non-nicotine medications. Several antidepressant and anxiolytic agents, as well as adrenergic and noradrenergic agents, were tested recently or are in clinical trials for this indication (170). There is emerging evidence that antidepressants and anxiolytics may be particularly helpful for subpopulations of smokers (i.e., those with mood disturbances). With the current trends in the smokers' population, anxiolytic and antidepressants will become increasingly important.

It is speculated that the following emerging trends will have an effect on the development of smoking cessation therapies.

- Smoking is becoming more recognized as drug dependence, and therefore, smoking cessation therapy will be viewed not only as a cancer and cardiovascular disease preventive measure, but also as a treatment for a psychological disorder (drug dependence).
- Current trends indicate that the population of smokers is changing. Future smokers are likely to be those who are more poor and uneducated as well as those with psychiatric/alcohol/drug abuse disorders and/or heavy nicotine dependence (217).
- With the high cost-efficacy of smoking cessation therapy, it is expected to eventually become reimbursable (163,217).
- More government regulations against tobacco products and tobacco advertisement. Efforts of the government agencies will be targeted at preventing the onset of smoking, especially in young people, and protection of nonsmokers (163).
- Newer NRTs (nicotine inhaler and nasal spray) will be switched to OTC market.
- More use of NRTs and non-nicotine therapy combinations to enhance the outcome.

In addition, with the increased knowledge of the neurochemistry of substance dependence, it is anticipated that the next generation of smoking cessation agents will be novel compounds designed to target neurotransmitters and receptors that mediate the reward system responsible for habit formation or to target the neuroadaptive mechanism responsible for substance dependence and withdrawal symptoms. Several compounds based on these novel mechanisms are in the pipelines of pharmaceutical companies. Nicotine replacement is still an active area of research. Several novel transmucosal drug delivery systems and improved second-generation transdermal patches are in clinical development. Oral nicotine combined with a bioavailability enhancer is also undergoing clinical trials (218). An antismoking vaccine being developed by Xenova (219) has shown successful results in clinical trials. Table 9.10 lists some of the compounds in various stages of development as antismoking therapeutic agents (116, 220).

8 SUNSCREENS

Most people are at least occasionally exposed to the sun for extended periods of time, either as a result of their lifestyle and recreational activities or as a normal part of their jobs. The main adverse effects to exposure to the sunlight are sunburn, photoaging, and skin cancer. Skin cancer is the most common type of cancer, accounting for almost 40% of all malignancies (221). According to the American Cancer Society, overl.3 million people will be diagnosed with nonmelanoma skin cancer in the United States each year, and 53,600 people will be diagnosed with skin melanoma (221, 222). Approximately 9600 people will die from various types of skin cancer, with the majority dying from malignant melanoma. Unprotected long-term exposure to sunlight is blamed for about 90% of skin cancer cases. Ultraviolet radiation (UVR) is believed to be responsible for most of the harmful effects of sunlight.

The UV spectrum is divided into three bands: UVA, UVB, and UVC. The UVA band is the longest wavelength. It ranges from 320 to 400 nm. It is further divided into two subsets: UVA I (340–400 nm) and UVA II (320–340

Table 9.10 Compounds in Development for Smoking Cessation or Tobacco Addiction Prevention

Generic Name/Laboratory Code	Originator	Chemical Class	Mechanism of Action
Methoxsalen (with oral nicotine)	ICN/Toronto University	Furocoumarin	Cytochrome P450 2A6 inhibitor, inhibits first pass effect of oral nicotine
Mecamylamine (transdermal in combination with nicotine)	Elan	Bicycloheptanamine	Nicotine antagonist[a]
NS-2359	NeuroSearch (Denmark)	Unknown	Mixed monoamine reuptake inhibitor
GW 320659 (1555U88)	GlaxoSmithkline	Phenyl morpholinol	Catecholamine selective reuptake inhibitor
LY 354740	Lilly	Bicyclohexane dicarboxylic acid	Group II metabotropic glutamate receptor agonist (glutamate release inhibitor)
CP 526555	Pfizer	Unknown	Nicotinic partial agonist
SR 141716	Sanofi-Synthelabo	Unknown	Central cannabinoid receptor antagonist
Cotinine (NIH 10498)	LecTec	Pyrrolidinyl pyridine (nicotine metabolite)	Nicotinic partial agonist
GW 468816	GlaxoSmithkline	Unknown	Glycine receptor antagonist
BP-897	Bioproject (France)	Naphthalene carboxamide derivative	Dopamine D3 receptor agonist
TA-NIC	Xenova/ImmuLogic	Nicotine conjugated to a carrier protein	Nicotine vaccine (prophylaxis)
Nabi-NicVax	Nabi	Derivatized nicotine on a carrier protein	Nicotine vaccine (prophylaxis)
Hydroxy bupropion	Sepracor	Phenyl aminoketone (R-isomer of bupropion)	Noradrenaline reuptake inhibitor
CMI 477	Millennium	azabicycloheptane	Nicotinic partial agonist
LY 426965	Lilly	Piperazinyl phenylbutanone	5HT 1A antagonist
SSR 591813	Sanofi-Synthelabo	Unknown	Nicotinic partial agonist

[a]Previously marketed as a hypertension treatment, but discontinued as in 1996.

nm). UVA is more abundant in the solar spectrum than UVB (15–20 times more than UVB) (223) but is less potent in causing erythema or sunburn (223). Because sunburn is the most visible UV-induced skin damage, UVB used to be blamed for most of the destructive effects of UVR. However, it is now believed that UVA, particularly UVA II, is at least as damaging as UVB. It has been shown that UVA II can cause the same molecular effects (i.e., direct DNA damage) as UVB (224). UVA penetrates deeper into the skin and is believed to cause more damage to the dermis layer (225). Moreover, UVA is the band where most of the pho-

Table 9.11 Sunscreen Agents Approved for OTC Marketing in the United States

Sunscreen agent	Class	Structure	Absorbance range (nm)	Approved concentration
Arninobenzoic acid (ABA)	ABA	(16)	260–313	Up to 15
Avobenzone	Dibenzoylmethane	(22)	320–400	Up to 3
Cinoxate	Cinnamates	(27)	270–328	Up to 3
Dioxybenzone (benzophenone-8)	Benzophenones	(28)	260–380	Up to 3
Homosalate	Salicylates	(25)	295–315	Up to 15
Menthyl anthranilate	Anthranilates	(29)	260–380	Up to 5%
Octocrylene	Cinnamates	(20)	250–360	Up to 10
Octyl methoxycinnamate (ethylhexyl-*p*-methoxy-cinnamate)	Cinnamates	(18)	290–320	Up to 7.5
Octyl salicylate (2-ethylhexyl salicylate)	Salicylates	(21)	280–320	Up to 5
Oxybenzone (benzophenone-3)	Benzophenones	(17)	270–350	Up to 6
Padimate O	Aminobenzoate	(24)	290–315	Up to 8
Phenylbenzimidazole sulfonic acid	Miscellaneous	(23)	290–320	Up to 4%
Sulisobenzone (benzophenone-4)	Benzophenones	(19)	260–375	Up to 10
Titanium dioxide	Physical agent	TiO_2	290–700	Up to 25%
Triethanolamine salicylate (trolamine salicylate)	Salicylates	(26)	260–320	Up to 12
Zinc oxide	Physical agent	ZnO	290–700	Up to 25%

tosensitizing chemicals exert their effect, especially above 360 nm (in the UVA I range).

The UVB band, which ranges from 290 to 320 nm, is the most potent radiation in producing erythema. It is also considered the radiation primarily responsible for skin cancer, although recent evidence shows that UVA is also carcinogenic. The intensity of UVB radiation is higher in the late morning and early afternoon. On the positive side, UVB is the radiation responsible for vitamin D_3 synthesis in skin.

UVC is the shortest wavelength of the UVA radiation (200–290 nm). It is also known as the germicidal radiation. Most of UVC is screened out by the ozone layer before it reaches the earth's surface. If it reaches the skin, W C can cause some erythema; however, most of it is absorbed by the stratum corneum.

8.1 Clinical Use

8.1.1 Current Drugs. Most sunscreens currently in use are compounds that have high absorbance or reflectance throughout the entire W B range, part of the UVA range, and in some instances infrared wavelengths. They are divided into two main groups: chemical (organic) and physical (particulate) agents. Physical sunscreening is the only way to block radiation across the entire spectrum (UVB, W A , visible, and infrared) (226). However, in practice, combined chemical sunscreens or combinations of physical and chemical agents provide higher levels of protection than physical sunscreening alone, because of the limited concentration of physical sunscreens that can be incorporated in a formulation without causing visible whitening when applied to the skin. Table 9.11 lists the compounds considered by the FDA as safe and effective sunscreens for the OTC market. The lists of approved sunscreens in Europe and Australia are much longer, mainly because of the simpler approval process for new sunscreens that is implemented in those countries. Examples

Table 9.12 Examples of Sunscreening Agents Approved for Use in Europe and Australia, but Not Available in the United States

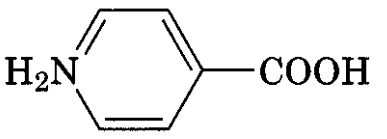
Sunscreen agent	Class	European Union	Australia
Isoamyl methoxycinamate	Cinnamate		✓
3-methylbenzylidene camphor	Camphor	✓	
4-methylbenzylidene camphor	Camphor	✓	✓
Octyl triazone	Phenol		✓
Bis-ethylhexyloxyphenol methoxyphenyl triazine	Phenol	✓	
Drometrizole trisiloxane	Phenol	✓	
Ecamsule	Camphor		✓

of the sunscreening agents that are not approved for use in the United States but are approved by the regulatory agencies in the European Union and Australia are listed in Table 9.12. Comparison of sunscreening agents available in Europe, the United States, Australia, and Japan can be found in Hayden et al. (227) and Steinberg (228).

In addition to the 16 sunscreens approved in the United States, there are 2 non-sunscreen topical agents that are approved for the treatment of conditions related to photo-induced skin damage. These are tretinoin and hydroquinone. Both are prescription products. Tretinoin is approved as an adjunctive treatment for "mitigation of fine wrinkles, mottled hyperpigmentation, and tactile roughness of facial skin." Hydroquinone has been marketed for decades for bleaching of hyperpigmented skin conditions.

The following section will only discuss sunscreen agents.

8.1.2 **Side Effects.** Most common adverse reaction to sunscreening agents are local. Sunscreens can induce allergic, irritant, or phototoxic dermatitis. Sunscreens are frequently blamed for allergic contact dermatitis and contact urticaria. However, distinguishing sunscreen-induced dermatitis from a solar-induced condition can be difficult (229). Moreover, the allergic reactions are, in many cases, caused by fragrances, preservatives, and other ingredients in the vehicle. Both aminobenzoic acid derivatives and cinnamates are known to cause skin sensitization. However, aminobenzoic acid derivatives have lower sensitization potential than the parent compound, ABA (16). The latter was eliminated



(16) Aminobenzoic acid

from most sunscreen formulations because of frequent sensitization reactions. Photoallergic reactions to benzophenones have been reported (230). Some drugs including thiazide diuretics, sulfonamides, and some local anesthetics such as benzocaine and lidocaine can induce cross-sensitivity to aminobenzoic acid derivatives. Physical sunscreens are extremely safe and non-sensitizing. The only reported side effect to some physical sunscreens is skin occlusion. Titanium dioxide has been shown to be photochemically active in vitro, but no such reaction has been shown in *vivo* (231).

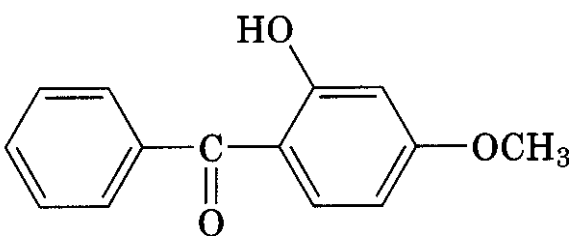
Several studies indicate that UVR irradiation of organic sunscreens may induce changes in the chemical structures of those compounds (232). Some of the degradation products have been shown to be capable of damaging cell components including DNA (233). This raises concerns over the photoirritancy, photosensitization, and photomutagenicity potential of sunscreens, especially that significant amounts of these photodegradation products may be absorbed through skin (232). Reports on the carcinogenicity of sunscreens are controversial. In vitro photogenotoxicity studies showed that, after UV irradiation, sunscreens may attack the DNA to produce mutation or cell death (233–236), possibly through the formation of free radicals (234). However, there are no in vivo data to support these conclusions. Furthermore, sev-

eral acute and chronic *in vivo* studies demonstrated that sunscreens protect from skin damage and either prevent or delay carcinogenesis (237). For a comprehensive reference on sunscreens toxicity, the authors recommend that the reader refers to Hayden et al. (232)

8.1.3 Absorption and Disposition. Because sunscreens are usually applied on a daily basis, and occasionally, over very large areas of the body, there are concerns over the systemic absorption and toxicity of these compounds. Unfortunately, very little information is available on the pharmacokinetics and long-term biological effects of sunscreens, although there is evidence that significant systemic absorption can occur (238, 239). Skin penetration of sunscreening agents depends on several factors, including the compound's molecular weight, lipophilicity, and other physicochemical properties, as well as the composition and properties of the vehicle in which it is applied to the skin (240,241).

There are few published data on the percutaneous absorption of sunscreens through human skin (239, 242–248). Most of the information came from *in vitro* penetration studies or by estimation from the amount recovered in the stratum corneum after tape stripping. The rationale for using the latter method (often referred to as the "reservoir technique") is based on the finding by Treffel and Gabard (248) that a linear relationship exists between the drug concentration in the stratum corneum and its *in vivo* percutaneous absorption.

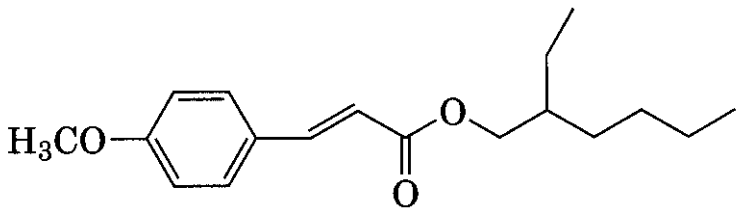
Very few systemic absorption data have been reported. Hayden et al. (238) reported that 1–2% of a topical dose of benzophenone-3 (oxybenzone) (17) was excreted in the urine



(17) Oxybenzone (Benzophenone-3)

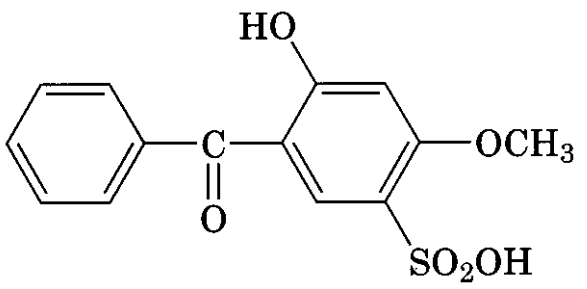
over a period of 48 h after a single application of a sunscreen product (238). High subject variability was reported in that study. Arancibia et al. (249) reported that 1.6–9.6% of p-aminobenzoic acid (16) was recovered in the urine of six volunteers after application of three different formulations containing the sunscreen. No significant difference was found between the formulations. In an earlier study, Feldman and Maibach (250) reported that 28% of a topical dose of p-aminobenzoic acid was recovered in the urine over 5 days after application of the sunscreen as a solution in acetone. It is possible the organic solvent used in the latter study caused the considerably higher systemic absorption.

In vitro absorption studies (239, 251, 252) showed small but significant penetration of several organic sunscreens through the entire thickness of human skin. For example, 0.03% (251) and 0.4% (252) of octyl methoxycinnamate (18) were reported to be detected in



(18) Octyl methoxycinnamate

the receptor fluid in two different *in vitro* penetration studies. Less than 0.1% penetration through the human skin was reported for benzophenone-4 (19) (252). The data on benzo-

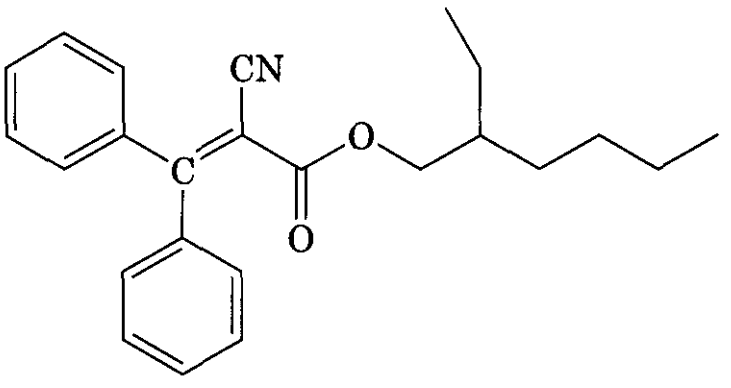


(19) Sulizobenzone (Benzophenone-4)

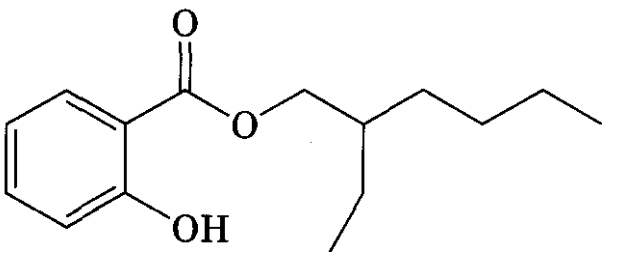
phenone-3 (oxybenzone) (17) varied from 1% (251) to approximately 10% (239) of the applied dose. The higher percentages (5.8–10%) were reported from a study that used several commercial products.

Penetration and binding of organic sunscreens into the stratum corneum has been evaluated as a property that affects their resistance to removal by water or perspiration. The amount of sunscreen retained in the stra-

tum corneum has been suggested to be directly related to the sun protection factor and duration of action (248). Aminobenzoates were reported to penetrate into the horny layer of the stratum corneum and become bound to the proteins in the epidermis by hydrogen bonding (253). This prolongs the duration of protection by aminobenzoates beyond the time of their presence on the skin surface. For organic sunscreens that do not penetrate into the stratum corneum, the duration of action depends on the length of their presence on the skin surface. Highly lipophilic sunscreens (e.g., octyl methoxycinnamate) have been shown to have higher affinity to the stratum corneum than water-soluble sunscreens (e.g., sulisobenzone) (252). However, penetration of lipophilic sunscreens to deeper tissue and hence their systemic absorption seems to be limited (240). In an in vitro penetration study, the percentage of applied dose of octyl methoxycinnamate (**18**), sulisobenzone (**19**), and octocrylene (**20**) that was retained in the stratum corneum after 16-h exposure ranged from 4% for benzophenone-4 to approximately 10% for octyl methoxycinnamate and octocrylene (251). In the same study, 8.5% of the applied dose of benzophenone-3 was retained in the stratum corneum. In an in *vivo* penetration study using skin-stripping method, 4% of benzophenone-3 was found in the stratum corneum after 30 min of application (254). The formulation dependence of stratum corneum penetration of three sunscreens [benzophenone-3 (**17**), octyl methoxycinnamate (**18**), and octyl salicylate (**21**)] was demonstrated by Treffel and Gabard (248,255).



(20) Octocrylene



(21) Octyl salicylate

The absorption of ZnO from intact skin after topical application is non-detectable. The data on TiO_2 are controversial. Earlier studies suggested that a very small amount of titanium dioxide may penetrate the skin, but it is unlikely that this would have any biological significance (237). However, a recent in viuo human study, in which skin punch biopsies were collected after application of titanium dioxide, (256) showed that this sunscreen is solely deposited on the outermost surface of the stratum corneum and does not penetrate into the deeper stratum corneum layers, the epidermis or the dermis regardless of the surface properties of the particles (256).

Very little is known about the distribution and metabolism of sunscreens in humans, but animal studies showed that benzophenone-3 undergoes substantial dermal metabolism and protein binding in rats (257, 258), and is excreted in both urine and feces. Excretion of several sunscreening agents (including benzophenone-3 and octyl methoxycinnamate) in human breast milk after normal use has been reported (259).

8.2 Physiology and Pharmacology

UV radiation has both acute and delayed adverse effects on the human skin. The acute effects are inflammation and sunburn, and the delayed or chronic effects are primarily photoaging and photocarcinogenesis.

The susceptibility of individuals to the harmful effects of UV radiation depends on several factors: (1) skin pigmentation, (2) type and amount of radiation, (3) skin hydration, (4) thickness of the stratum corneum and epidermis, and (5) the distribution and concentration of peripheral blood vessels (260).

8.2.1 Acute Effects of UVR. The acute effects of UVR exposure are sunburn/erythema and immediate pigment darkening (IPD). Sunburn is a superficial skin burn with a mild inflammatory reaction, the main manifestation of which is erythema. The symptoms may also include tenderness, pain, and edema. It is usually a first-degree burn, although occasionally with extensive UVR exposure, a second-degree burn that involves a thicker layer of skin may develop. The main local symptom of a second-degree burn is the development of vesicles (blisters). In addition, systemic symptoms such as fever, weakness, and shock may occur.

Erythema is the most common effect of UVR exposure. The exact mechanism of how UVR induces skin erythema is not fully understood; however, it is believed that a number of mediators are involved in the inflammatory reaction (260). These include histamine, lysosomal enzymes, kinins, and at least one prostaglandin. These mediators produce vasodilatation, which is manifested in erythema. As the UV radiation penetrates into the epidermis, another inflammatory reaction involving a lymphocytic infiltrate develops. Swelling of the endothelium and leakage of red blood cells also occurs. The inflammatory effect of UVB radiation reaches its maximum in 12–24 h after exposure (260).

Microscopic changes in the skin in response to UV radiation can be detected as early as 30 min after exposure (261). Epidermal changes include intracellular edema, vacuolization, and swelling of melanocytes and the development of characteristic "sunburn cells." Sunburn cells, which were first described by Daniels et al. (262), are photodamaged cells in the process of undergoing apoptotic cell death (224). They are dyskeratotic keratinocytes with pyknotic nuclei and homogeneous eosinophilic cytoplasm that develop in proportion to the amount of UVB exposure. Sunburn cell formation reaches a maximum at 18–24 h after exposure and resolves in 3–7 days. Changes in the dermis include, initially, interstitial edema and endothelial cell swelling, and later, perivenular edema, degranulation and loss of mast cells, decrease in the number of Langerhans cells, neutrophil infiltration, and erythrocyte extravasation. The dermal reaction peaks at 24 h after exposure and resolves in 3–5 days. The later phase of microscopic changes include hyperkeratosis, acanthosis (epidermal thickening), disorganization and misalignment of keratinocytes, dermal vascular ectasia, and mononuclear perivascular infiltration (261).

Erythema is primarily mediated by UVB, and its intensity is proportional to the dose of UVB radiation. The reaction resolves in 3–5 days and initiates increased melanogenesis, which reaches a maximum in 2- to 3-week period.

Immediate pigment darkening (IPD) is another acute effect of UVR exposure. IPD, which is marked in some individuals and undetectable in others, is sometimes considered as the initial phase of erythema (261). It is mediated by UVA and lasts about 13–30 min. The maximum wavelength for IPD is 340 nm (263). IPD is thought to be a result of photooxidation process (224). The relative contribution of UVA to sunburn and other acute effects of UVR is estimated at 18–20% (225).

8.2.2 Chronic Effects of UVR

8.2.2.1 Adaptive Responses. There are two adaptive responses to the exposure to UV radiation: thickening of the stratum corneum and skin tanning (melanogenesis). Stratum corneum thickening is mediated by UVB, whereas skin tanning can be induced by UVB and UVA (225, 261).

Thickening of the stratum corneum occurs as an adaptive response to prolonged UVB exposure. The stratum corneum proliferates through increased synthesis of keratin by basal keratinocytes.

Skin tanning, or delayed pigment darkening, results from the production of melanin and serves as a protective mechanism against further UVR-induced damage. Skin tanning is mainly induced by UVB, with a wavelength spectrum similar to that of erythema (224). UVB induces melanogenesis by enhancing the binding of circulating melanocyte-stimulating hormone (MSH) to melanocytes. This leads to melanocyte proliferation, dendritic arborization, and melanin production. The melanin is then distributed to the surrounded keratinocytes (261). Melanin absorbs, reflects and scatters UV light and is a free radical trap. UVA

induces a delayed tanning reaction. This melanogenesis reaction involves oxidation of preexisting melanin followed by new and increased synthesis of melanin. It is also accompanied by increased population density of melanocytes with increased production of melanosomes and increased melanization of the epidermis (225).

8.2.2.2 ***Photoaging.*** Premature photoaging is one of the long-term effects of exposure to sunlight (264). It is induced by prolonged exposure to all portions of the solar spectrum including W A , UVB, and infrared (261). The incidence and severity of photoaging is believed to be proportional to the cumulative dose of UVR (237). There is evidence that chronic exposure to suberythemal doses of UVA produces changes in human skin indicative of photoaging (265–267).

The common manifestations of skin photoaging are dryness, roughness, irregular pigmentation, actinic keratosis, wrinkling, elastosis, inelasticity, and sebaceous hyperplasia (264). Although the physical appearance of photoaging is similar to normal aging, there are histological and biochemical differences that distinguish each condition from the other (261). In addition, photoaging can be slowed down and even reversed by reduction of UVR exposure (including using sunscreens), or by other treatments such as retinoic acid, whereas normal aging is irreversible (268). The typical macroscopic appearance of photoaged skin results mainly from break down of the skin elastic fibers (elastosis) caused by long-term exposure to UV light. The exact mechanism is not fully understood. Other changes associated with photoaging include cracking, telangiectasia (spider veins), ecchymoses (subcutaneous hemorrhagic lesions), and hyperpigmentation. Solar radiation also decreases the regenerative capacity of the skin fibroblasts and keratocytes and therefore accelerates skin aging (261).

8.2.2.3 ***Carcinogenesis.*** Skin cancer is the most serious adverse effect of UVR. There are three common types of skin cancer: melanoma, basal cell carcinoma (BSC), and squamous cell carcinoma (SCC). BSC and SCC are known collectively as non-melanoma skin cancer (NMSC). BSC is the most common skin cancer, but it has the lowest mortality rate (269). Malignant melanoma is the most serious type of skin cancer.

Although the link between skin cancer and exposure to sunlight is very strong, the susceptibility of individuals to the carcinogenic effect of UV radiation varies depending on several factors including skin pigmentation, age, sex, and phenotype. Particularly, factors such as fair skin, blue eyes, red or fair hair, and inability to tan have been linked to increased risk of NMSC in several studies (270–276).

Both chronic human studies and animal studies proved the causal relationship between cumulative UVR exposure and skin cancer (277), particularly non-melanoma skin cancer (NMSC). The link between the two is also evident from the fact that NMSC is most common on the head, neck, arms, and hands (278, 279). In particular, the correlation between UVR exposure and SCC seems to be very strong. Cutaneous SCC of the head and neck occurs almost exclusively on areas receiving maximal exposure (269,280). The link between BCC and cumulative exposure to UVR is not as evident (270, 281). Although BCC occurs on the face, head, and neck, unlike SCC, its distribution does not correspond well with the areas that receive maximum sun exposure (269). Case-control studies indicated that cumulative sun exposure is the most important risk factor for SCC, whereas inability to tan was the most important risk factor for BCC (270,282,283). Subsequently, it has been suggested that, for BSC, intermittent sun exposure, particularly in the childhood, may be more important than cumulative exposure (282).

The data on a causal relationship between cumulative W R exposure and malignant melanoma is not conclusive, but there is strong evidence that intermittent intense sunlight exposure, particularly severe sunburn in childhood, is a major risk factor for development of malignant melanoma (284–286). There is scientific evidence that UVB can induce melanocyte proliferation in both exposed and covered areas of the body (287), which explains why malignant melanoma sometimes occurs in unexposed parts of the body.

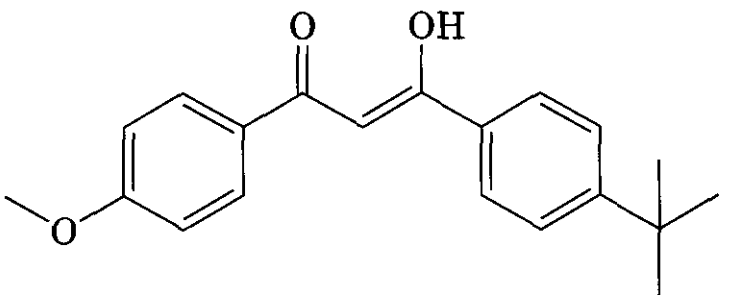
Both W A and UVB can induce DNA damage, and photocarcinogenesis has been re-

ported following repeated UVA exposure in rodents (288). In addition, UVA is believed to augment the development of UVB-induced non-melanoma skin cancer (287).

The first step in UVR-induced skin cancer is UVR-initiated DNA mutation, which causes the transformation of the normal cells to malignant cells. For UVR to initiate a biological reaction, it has to be absorbed by endogenous molecules (chromophores). UVB is absorbed directly by the DNA, and therefore can directly induce DNA mutation (224), in the form of thymine dimer formation (289). Some protein components may also ad as chromophores for UVB (224). UVA is absorbed by the reduced forms of the co-enzymes nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH), tryptophan, riboflavin, and melanin (224, 290). UVA-induced DNA damage is believed to be mediated by oxygen reactive species that are released after the absorption of UVA by those endogenous chromophores and results in photooxidation of selected bases (224,290,291). The induced damage may take the form of single-strand breaks, induction of thymine dimers, or DNA-protein crosslinks (290,291). In addition, UVA II may be directly absorbed by the DNA similar to UVB (224).

It is believed that UVR exposure causes skin immune suppression. Both UVB and UVA have been implicated (292). A possible mechanism is UVA-induced lipid peroxidation, which results in the migration of immune-mediating cells from the epidermis and therefore leads to skin immune suppression (224). It has been suggested that skin immune suppression plays a role in carcinogenesis. However, the relationship between the two is not well understood yet (293).

8.2.3 Mechanisms of Action of Sunscreens. Sunscreens delay the induction of sunburn by absorbing or reflecting a portion of the UVR reaching the epidermis. Organic sunscreens are aromatic compounds that absorb light energy in the UV region, and therefore reduce the amount of UVR reaching the stratum corneum. A benzene ring has the ability to transform high energy UVR into harmless long wave radiation above the 380-nm range, which is emitted from the skin as heat (294). The conversion of UVR to longer wavelength occurs through resonance delocalization. Most chemical sunscreens absorb up to 95% of the UVB spectrum, but do not absorb in the UVA range. Avobenzone (22) is the only organic



(22) Avobenzone

sunscreen that provides a high degree of UVA protection. Benzophenones and anthranilates provide partial protection in the UVA range. Sunscreens are almost always used in combinations to broaden the absorbance spectrum and increase the SPF value of the product.

Little is known regarding the quantitative ability of sunscreens to prevent UVR-induced adverse effects other than sunburn. For example, there is no information on the threshold or dose-response for UVR-induced immunosuppression and DNA damage (237), but there is strong evidence that regular use of Sunscreens reduces the incidence of precancerous lesions. A large population study in Australia proved the effectiveness of sunscreens in reducing the incidence of solar keratosis (291). Solar keratosis is a known precursor for squamous cell carcinoma and an established risk factor for basal cell carcinoma and melanoma.

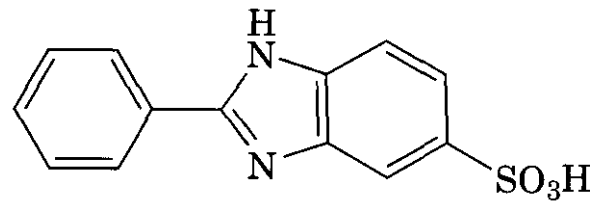
It has been suggested that sunscreens augment the defense mechanism against oxidative damage by UV-generated free radicals. This defense mechanism is mediated by thioredoxin reductase in the human keratocytes, which reduces superoxide anion radicals through hydrogen peroxide to water (295). However, the oxygen radicals concentrations generated by UVA and UVB radiations, even below the minimal erythemal dose, are high enough to cause considerable deactivation of thioredoxin reductase. Sunscreens have been shown to protect the thioredoxin reductase against both UVA and UVB in human skin of types I and II. The same sunscreen, however,

failed to protect the enzyme in more pigmented skin (types III and IV).

8.2.4 Sun Protection Factor. Sunscreens were originally developed to prevent sunburn and minimize erythema (224). The efficacy of sunscreen products is rated by their sun protection factor (SPF), which is defined as the ratio of UV energy required to produce a minimal erythemal dose (MED) on protected skin to the UV energy required to produce MED on unprotected skin. It is calculated as the ratio of time of W exposure necessary to produce minimal erythema in sunscreen protected skin to that time in unprotected skin. This SPF measures the effect of UVB only and does not account for the W A effect. Few methods have been proposed for a W A protection factor, including an *in vivo* pigment darkening method, and an *in* vitro critical wavelength test proposed to the FDA by the Cosmetic, Toiletry and Fragrance Association (CTFA). However, there is still no generally acceptable method for UVA testing, and the FDA has not yet reached a conclusion. It is also noteworthy that the SPF is not a measure of other photodamage protective properties of sunscreens (e.g., protection against skin cancer and immunologic protection). However, it is generally agreed that a higher SPF sunscreen will provide better protection against UVB-induced damage. There have been several attempts at determining the immune protection factor of sunscreens (293,296). All these studies seemed to indicate some immune protection of sunscreens, but with no correlation with the SPF. It seems that the extent of immune protection is much less than erythema protection, which suggests that the wavelengths that cause immune suppression are different from those that cause erythema (293).

8.3 Chemistry and Structure-Activity Relationships

The chemical sunscreens approved for OTC use in the United States are classified into seven classes: aminobenzoates (previously known as PABA and PABA derivatives), salicylates, cinnamates, benzophenones, anthranilates, dibenzoylmethane derivatives, and one miscellaneous chemical (phenyl-benzimidazole sulfonic acid, 23). In addition, there are several camphor derivatives that are marketed as sunscreens in Europe, but none



(23) Phenylbenzimidazole Sulfonic Acid

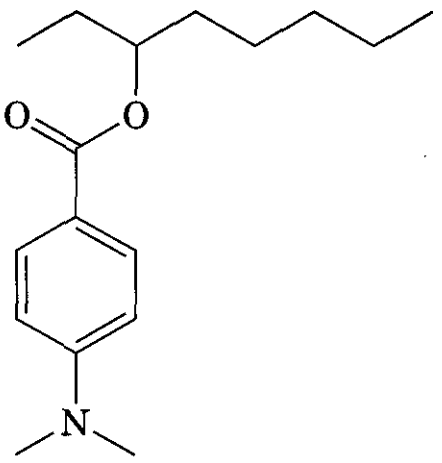
are approved in the United States. The minimal structural requirements for absorbance in the UVB and W A regions are an aromatic ring with an electron-releasing group and an electron-accepting group either in the ortho- or para-position from each other. This structural arrangement facilitates resonance delocalization. The more easily the compound resonates, the lower the required quantum energy for the electron transition, and hence the longer the maximum absorbance wavelength, because λ is inversely proportional to the energy. In addition, the spectral properties of sunscreens may be affected by dielectric effects, solvent–solute interactions, and pH of the vehicle (232). An excellent analysis of the structure-activity relationships of sunscreen chemicals has been reported by Shaath (297). The following discussion is largely based on his findings.

8.3.1 Aminobenzoates. In aminobenzoic acid (16), the parent compound of this class, the presence of the amino group and carboxylic acid group in the para-position from one another allows for the electron transition responsible for absorption in the UVB region. However, the very position of the two groups results in a number of properties that are undesirable for a sunscreening agent. These include the following (297):

- Susceptibility of the free amine to oxidation
- Intermolecular hydrogen bonding resulting in a crystalline physical state: crystalline sunscreens are difficult to incorporate in topical products and may result in an unacceptable product if the sunscreen agent is not solubilized properly

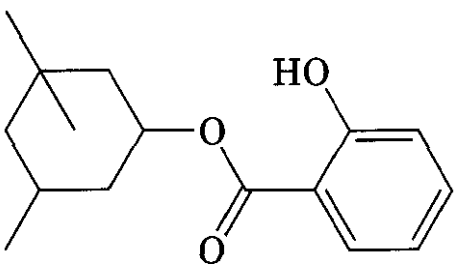
- High aqueous solubility because of excessive hydrogen bonding with the solvent and therefore shorter retention time on the skin, especially with perspiration
- A dramatic solvent effect, which shifts the λ_{max} from 293 nm in nonpolar solvents to 266 nm in polar solvents: this influences the efficacy of sunscreen products

Because of the structural limitations of ABA, several sunscreening chemicals based on ABA structure were developed with attempts to capitalize on the strengths and eliminate the drawbacks. There are four compounds that successfully addressed the problems of ABA. These are *N,N*-dimethyl PABA ethyl ester, *N,N*-dimethyl PABA butyl ester, *N,N*-dimethyl PABA amyl ester (padimate A), and *N,N*-dimethyl PABA octyl ester (padimate O). Padimate O (24) has the most desirable properties in this class. The molar extinction coefficient of padimate O is 28,400 in polar solvents, more than twice that of ABA, and one of the highest values among all chemical sunscreens. The solvent effect shift is much smaller than that of ABA (from 300 nm in nonpolar solvents to 316 nm in polar solvents). Moreover, eliminating the intermolecular hydrogen bonding, and the subsequent decrease in intermolecular association, led to the formation of a liquid instead of crystalline solid, which is a major advantage for formulating sunscreen products (297).

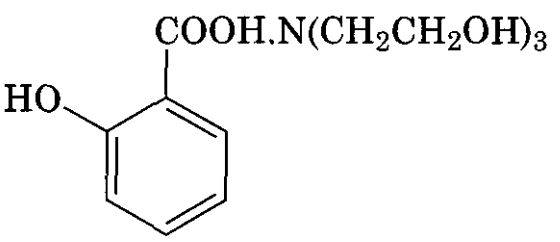


(24) Padimate O

8.3.2 Salicylates. Salicylates are *ortho*-hydroxybenzoic acid esters. The presence of the hydroxyl group in the ortho- position allows for intramolecular hydrogen bonding, which lowers the energy requirements for the compound to be prompted to the excited state. This results in a UV absorbance in the 300-nm range, compared with 270 nm for the *para*-hydroxybenzoates (parabens). Furthermore, the ortho-disubstitution seems to stabilize these agents against solvent effect. This is also attributed to intermolecular hydrogen bonding (298). On the negative side, the ortho-substitution causes crowding and strain on the molecule, which results in deviation from planarity and hence a low extinction coefficient. Although salicylates are weak UV absorbers, they are very popular sunscreens because of their excellent safety record and their physicochemical properties that are favorable for cosmetic formulations. They are water-insoluble liquids and excellent solubilizers for other insoluble cosmetic ingredients and sunscreens such as benzophenones. 2-Ethylhexyl salicylate (21) (also known as octyl salicylate) is the most commonly used sunscreening salicylate. The 2-ethylhexyl moiety is a common substituent in other popular sunscreens (2-ethylhexyl p-methoxycinnamate, 2-ethylhexyl dimethyl p-aminobenzoate, and 2-ethylhexyl-2-cyano-3,3-diphenyl acrylate). The moiety ensures the compounds' insolubility in water, and makes them useful for water-proof sunscreen formulations (297). Other salicylates that are approved for OTC marketing in the United States are homosalate (**25**) and triethanolamine salicylate (26).

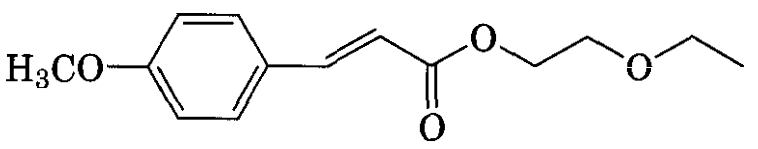


(25) Homosalate



(26) TEA Salicylate

8.3.3 Cinnamates. Cinnamates have an unsaturated bond conjugated both to the aromatic ring and to the carbonyl group, which allows the electron delocalization to occur throughout the molecule. This results in a UV absorbance at about 305 nm and high molecular extinction coefficient, which makes this group strong UV filters. One of the best designed cinnamates is 2-ethylhexyl p-methoxycinnamate (octyl-p-methoxycinnamate).It has an electron releasing methoxy group in the para- position, which further facilitates the electron delocalization process. The 2-ethylhexyl group makes it practically insoluble in water. In addition to octyl-p-methoxy cinnamate, cinnoxate (27) is also included in the

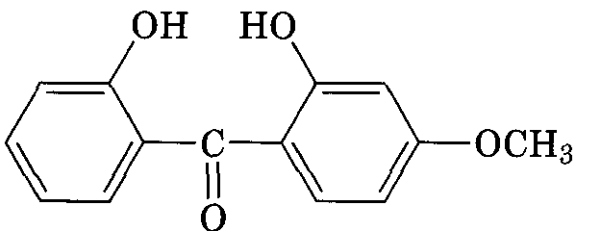


(27) Cinoxate

sunscreens monograph. One of the drawbacks of cinnamates is their moderate photostability caused by cis-trans isomerism (297).

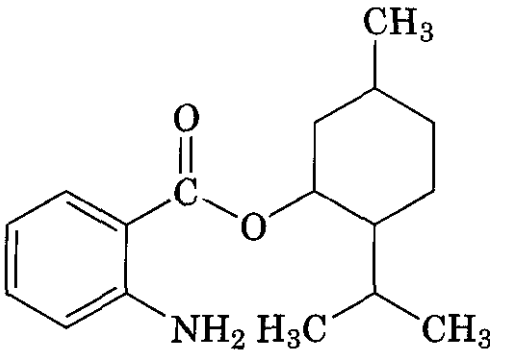
8.3.4 Benzophenones. Benzophenones are aromatic ketones. In this group, the electron donating substituent is located in the para-position to the carbonyl group. The carbonyl group acts as the electron-acceptinggroup and participates in the resonance delocalization process. Aromatic ketones resonate easier than esters and therefore absorb at a longer wavelength (λ_{max} > 320 nm), which puts them slightly into the UVA region. The main problems with benzophenones are as follows (297):

- They are crystalline solids that are difficult to solubilize in cosmetic formulations
- Some benzophenones are susceptible to large shifts in their UV maxima because of solvent effect
- Unlike esters, which are rapidly hydrolyzed in the body, benzophenones maybe potentially toxic if absorbed through the skin: statistically, more frequent allergic reactions have been reported with oxybenzone than with PABA (299). Three benzophenones are listed in the sunscreens monograph: dioxybenzone (**28**), oxybenzone (**17**), and sulisobenzone (**19**).



(28) Dioxybenzone (Benzophenone-8)

8.3.5 Anthranilates. Anthranilates are *ortho*-aminobenzoates. There is only one anthranilate compound approved as a sunscreening agent in the United States, and two anthranilates in Europe. The ortho- positioning of the amino group results in intramolecular hydrogen bonding and greatly enhances the resonance delocalization. The result is a λ_{max} of 336 nm for menthylanthranilate compared with 298 nm for PABA (the para- substituted amino benzoate). Similar to the case with salicylates, the molar extinction coefficient of anthranilates is much lower than that of aminobenzoates because of the steric crowding that causes the compound to deviate from planarity. Also, similar to salicylates, anthranilates are stable and do not exhibit significant solvent effect (297,232).



(29) Menthyl anthranilate

8.3.6 Dibenzoylmethanes. Avobenzone (**22**) is the only member of this class that is approved for use in the United States There are two other dibenzoylmethanes that are approved in Europe in addition to avobenzone. Dibenzoylmethanes are substituted diketones, with a UV spectrum well into the UVA region. This unique spectrum among W filters is a result of the keto-enol tautomerism. The W A absorbance of these compounds is

mainly caused by the enol form, which has a λ_{max} greater than 345 nm, whereas the keto form exhibits a λ_{max} of about 260 nm. The presence of the hydroxyl group in an ortho-position to the carbonyl group shifts the equilibrium of the keto-enol tautomerism toward the enol form and away from the keto. Dibenzoylmethanes have a high molar extinction coefficient, but their main drawback is their low photostability and susceptibility to photoisomerization, which results in an irreversible loss of activity. The keto form is more susceptible to photoisomerization than the enol (297). Dibenzoylmethanes are relatively stable in polar solvents (alcohols), but unstable in nonpolar solvents (300).

8.3.7 Physical (Particulate) Sunscreens. Zinc oxide and titanium dioxide are the two metal oxides approved as sunscreens in the United States. Microfine particles of these metal oxides, with a mean particle size of 0.2 μm or less, and a narrow particle size distribution, are used in physical sunscreen products. The average particle size is below the optimal light scattering size, which allows visible light to be transmitted and therefore renders the product virtually invisible on the skin (237, 290). Zinc oxide is primarily a UV absorber, whereas titanium dioxide is predominantly a reflector, although it does absorb UV light both in the UVB and UVA region. Zinc oxide strongly absorbs UVR from about 380 nm all the way through the UVB and into the UVC region, and therefore, was commonly referred to as UV block. Its UVA protection is superior to that of titanium dioxide, mainly because of the stronger absorbance. The efficacy of particulate sunscreens depends largely on the refractive index, particle size, particle morphology, dispersion in the vehicle, and film thickness (231). Microparticulate sunscreens are frequently coated with silicones and/or alumina to aid their dispersibility.

8.4 History

8.4.1 Emergence of Sunscreens. The first commercial sunscreen product appeared in the United States in 1928. It was an emulsion of benzyl salicylate and benzyl cinnamate. In the early 1930s, a 10% solution of phenyl salicylate was developed and marketed in Australia. In 1935, quinine oleate and quinine bisulfate were marketed in the United States. *p*-Aminobenzoic acid (PABA) was patented in 1943, and was for many years the leading organic sunscreen. Several aminobenzoates (PABA derivatives) were introduced subsequently. PABA derivatives remained the primary sunscreen ingredients during the 1960s and 1970s. The first reference to particulate sunscreens appeared in 1947 (301) in an article titled "Zinc Oxide in Face Powders." The article discussed the absorption spectra of metal oxides, including zinc oxide and titanium dioxide. In the same article, the author predicted the value of zinc oxide as a broad spectrum UV block, based on its absorption spectrum. The first report on titanium dioxide as a sunscreening agent appeared in 1952 (302). However, the first commercial use of microparticulate metal oxides as sunscreens was in 1989 with the introduction of a titanium dioxide product. Zinc oxide was introduced in 1991 (303).

The US military contributed to the introduction of new sunscreens as well as increasing the public awareness and widespread use of sunscreening agents. The first reported use of red petrolatum was by the U.S. military during World War II (304). They also used other agents such as glycerol-PABA and several salicylate derivatives. Moreover, the U.S. military issued the first specifications on sunscreens in 1951, in which it listed approved sunscreens and their recommended concentrations (297). However, most of the sunscreen compounds and sunscreen research originated from the cosmetic and personal care industry. The SPF testing, as a means of quantifying the efficacy of sunscreens, started in the 1960s, and was initially known as Light Protection Factor (305).

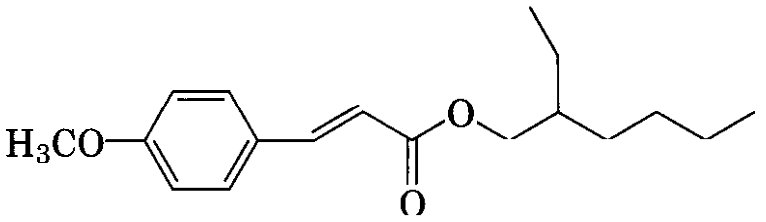
8.4.2 History of FDA Regulations. Although the FDA did not start regulating sunscreens as OTC drugs until 1978, it has in fact, considered them drugs as early as 1940. This position concerning the legal classification of sunscreens came in a trade correspondence to the cosmetic industry (306), in which the FDA stated: "Articles which refer to sunburn or any other disease condition are drugs under

Section 201(g), but articles which are represented exclusively for the production of an even tan will be regarded as cosmetics under Section 201(i)." In 1976, in another correspondence to the cosmetic industry, the FDA stated "We have concluded that a product containing a sunscreen ingredient . . . even when labeled solely as a tanning aid, is both intended and understood to be a preventative of sunburn, and is therefore a drug" (307). On August 25, 1978, the FDA published the first tentative monograph on sunscreens (308). The monograph contained 21 UV filters and their acceptable usage levels. However, the FDA did little to regulate sunscreen-containing products manufactured by the cosmetic industry until the mid-1980s. Until then, cosmetic products containing sunscreens continued to be marketed as cosmetics, unregulated, as long as they were correctly labeled as cosmetics and did not make clear therapeutic claims. In 1985, the FDA reiterated its previous position on sunscreen classification in a memorandum of meeting with the cosmetic industry (307), and in 1987, it launched a major regulatory campaign against cosmetic products with antiaging claims, many of which contained sunscreens. In warning letters to the marketers of these products, the FDA cited them as drugs under Section 201(g) of the Food and Drug Act. Moreover, those products that contained sunscreens unapproved by the FDA were cited as "new" drugs within the meaning of Section 201(p) (307).

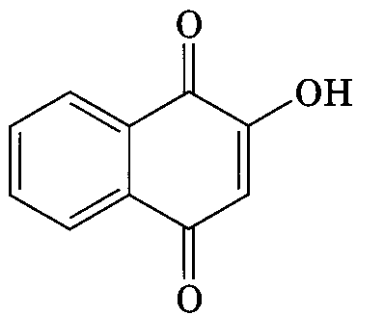
In May 1993, the FDA issued the tentative final monograph for OTC drug sunscreen products (309), which included 20 sunscreen actives. In the monograph, the FDA redefined cosmetic products that contains any sunscreen, use the term sunscreen, claim an SPF, or make any therapeutic claim about sun protection as drugs. Since then, the FDA has made it clear that any sunscreen agent not eligible under the OTC Drug Review process must go through an NDA to be approved for use in the United States. In 1994, the FDA published a proposed amendment to the tentative monograph (310) announcing its intention to delete five sunscreens for lack of interest from the industry. The agency proposed to keep only the 15 actives for which USP monographs existed or were being developed. The deleted agents were hardly used in any sunscreen product in the United States.

During the late 1980s and early 1990s, sunscreen products started moving away from being purely beach and recreational products to being part of daily skin care. This occurred largely as a result of a public education campaign that started in the 1980s because of the increased scientific evidence on the correlation between skin cancer and long-term exposure to UVR. In 1989, UVA protection started to become an issue in the United States. Until then, the tentative monograph contained no broad-spectrum UVA filters and no mention of UVA. In early 1993, Roche petitioned to the FDA to include avobenzone, a broad spectrum UVA filter that it developed in 1971 and had been widely used in Europe since 1981. However, when the FDA issued the tentative final monograph for OTC sunscreens in May 1993, avobenzone was not included. In the following years, the FDA came under pressure from Roche, the CTFA, and the American Academy of Dermatology to recognize avobenzone as a safe and effective (Category I) sunscreen. In September 1997, the agency amended the tentative final monograph to include avobenzone as a Category I OTC sunscreen (311). Before this amendment, the FDA had approved a single product containing avobenzone (Shade UVA Guard from Schering-Plough). The approval of Schering-Plough's product came after an NDA. Zinc oxide, which was until then considered Category III (available data are insufficient to classify as safe and effective and further testing is required), was also added to the tentative final monograph in October 1998 (312).

On May 21,1999, the FDA issued the final monograph of OTC sunscreen products (313). The final monograph contained the 16 agents listed in Table 9.11. Those are the agents that currently have USP monographs. The FDA kept two other agents under consideration in case monographs are developed in the future. The two agents are diethanolamine methoxycinnamate (30) and lawsone (31) with dihydroxyacetone. The final monograph also contained new regulations for SPF testing and labeling. The FDA gave sunscreen makers 2 years to comply with the new regulations. The



(30) DEA Methoxycinnamate



(31) Lawsone

effective date was later extended to December 31, 2002, in response to a petition submitted by the CTFA (314).

8.5 Current and Future Trends

It is evident from the number of new cases of skin cancer that there is a need for new sunscreens with better protection. However, the cost and time required for introducing new sunscreen agents through an NDA is prohibitive. Therefore, a large portion of the current research in the field focuses on novel drug delivery systems to prolong the duration of the sunscreening effect, enhance the water resistance (substantivity) of the products, enhance their SPF, or decrease their irritation potential. Another important area is improving the aesthetics of sunscreen products, particularly physical sunscreens.

Several new sunscreening compounds have been introduced in Europe, Australia, and Japan in the past few years. Two of the most interesting new agents are bis-ethylhexyloxyphenol methoxyphenol triazine (BEMT) and methylene bis-benzotriazolyl tetramethylbutylphenol (MBBT). BEMT is a potent broad-spectrum UV-absorber that covers the full UVA and UVB spectrum. In contrast to avobenzone (the only broad spectrum UVA filter available in the United States), BEMT has a good photostability (315). MMBT (316) represents a new class of broad-spectrum UV filter (organic microparticulates). The product consists of microfine organic particles that are dispersed in the aqueous phase of sunscreen emulsions (315, 316). It is equally protective against UVA and UVB radiation (UVA/UVB protection ratio = 1.0), and is extremely photostable. The safety and lack of genotoxicity of these two agents have been demonstrated both ***in vitro*** and ***in vivo***. The European Unions Scientific Committee on Cosmetic Products and Non-Food Products Intended for Consumers (SCCNFP) has approved the two compounds at concentrations up to 10%. In September 2000, Ciba Specialty Chemicals has petitioned with the FDA to add BEMT and MMBT to the OTC sunscreen monograph (315).

Alternative approaches for photodamage repair and protection is an emerging trend in sun protection. Antioxidants (particularly vitamin E) are common ingredients in commercially available sunscreens. It has been previously shown that topical application of vitamin E inhibits UVR-induced cellular damage, edema, and erythema (317). In the past few years, there has been rising interest in scientific research to prove the efficacy of antioxidants as chemopreventive agents (318, 319). Recent work demonstrated evidence of DNA photoprotection of a-tocopherol (a vitamin E compound) (317). In 2001, an oral antioxidant (astaxanthin) was promoted as sun protectant pill (320). The FDA approved the marketing of astaxanthin as dietary supplement in 1999, but the product did not undergo the review for sun protection claims. Astaxanthin is a carotenoid obtained from micro algae and is over 500 times more potent antioxidant than vitamin E. Other natural products that gained considerable attention as potential chemopreventive agents are tea polyphenols, resveratrol (a natural product from grapes), ginger compounds, curcumin, diallyl sulfide (a compound from garlic), and several plant flavonoids, such as apigenin, catechin, silymarin, and genistein (318, 319, 321, 322). A number of these compounds are found in skin care products, with no cancer prevention claims. Chemoprevention has a large potential to become a viable approach for skin cancer prevention. However, well-designed case-control clinical studies are needed to prove its efficacy (321).

With regard to FDA regulations, there remain few areas of concern. The main ones are: the determination of a standardized UVA testing methodology (with in *vivo* relevance), and the testing and labeling of sunscreen drug products with SPF values above 30. The FDA is concerned that high SPF number may encourage consumers to extend their exposure to the sun (314).

9 FUTURE TRENDS FOR OTC AND LIFESTYLE DRUGS

What will the future of OTC look like? Back in the 1990s, Rx-to-OTC switches were deemed to be the driving force for growth in the OTC industry. We did see rapid growth in the mid-1990s, but the number of switches has dwindled substantially near the end of the 20th century (323). The so-called "easy ones" have been switched. Pharmaceutical companies need to be creative with future switches. Several seemingly promising switches such as the statins and acyclovir have been denied by the Advisory committee (35). Refusal by the FDA to switch a product in the past does not mean there is no opportunity for future switch. It simply means the sponsor will need to develop more data. Most switches need to go through a few passes in front of the Advisory Committee before they are eventually switched. Some of the therapeutic categories such as hair growth and smoking cessation that have not been incorporated into the monograph system by the OTC review panel were eventually switched. The reason why these OTC products existed before the OTC review strongly suggests that those are unmet consumer needs. They got switched when the right actives came along and the right studies were done to convince the FDA that self-medications of these products are possible (33). The timing and regulatory environment has to be right. By the same analogy, some of the previously denied Rx-to-OTC switch candidates may be resurrected as more safety data are accumulated. The fact that healthcare providers would like to control cost will lead to more citizen petitions as in the case of non-sedative antihistamines. When these candidates will be switched will depend on how the politics play out. The innovator companies said that the FDA has no authority to force them to switch. However, the same picture may not hold in a country like the U.K. The MCA has been aggressively promoting switches.in recent years, and they want to accelerate them in the next few years to control costs (48).

No matter what the future of Rx-to-OTC switch will be, one thing for sure is that future switches will be driven by science and an evidence-based medicine approach as in the past. Even though most of the OTC products are for acute use, there are already a few products such as hair growth products and smoking cessation product for chronic use. The desire of the baby boomers to maintain their vigor and the desire of healthcare providers to control cost favors preventive OTC products, which will continue to challenge the chronic use barrier. The switch of these kinds of products will be enhanced by the advance of diagnostic products (324).

The future of the OTC industry will also be different because of dietary supplements and cosmeceuticals. The boundary between them and OTC products will be blurred (325). The FDA has already issued draft guidance for registering herbal drug products as either OTC or prescription products (326). In countries such as Germany and Japan, herbal and dietary supplements have been registered as drug products for many years. The current lax regulatory environment may not favor the extra efforts of registering an herbal product as a drug product. However, it may become a worthy attempt as the quality standard demanded on the dietary supplement industry becomes tighter. Here again, the science will dictate the success of the conversion. Similar changes may occur with cosmeceuticals. The FDA will undoubtedly work out a proper procedure for how to regulate the so-called cosmeceuticals. Some of them may first become Rx before they will be switched to OTC.

For a long time, the FDA has not required any adverse event reporting for OTC monograph products. However, this will be the focus of the agency and the pharmaceutical companies to place a high priority on adverse event reporting. This not only represents responsible marketing but also can be used to enhance public relationships.

The recent merger of pharmaceutical companies and the demand for double-digit growth have put the OTC divisions of the consolidated companies in a very demanding position. What benefits besides growth does the OTC division bring to the table? Perhaps one important function the OTC division serves is for lifestyle management through Rx-to-OTC switch. The other benefit is their direct-to-consumer advertising expertise. The prescription businesses are rapidly catching up in this area.

The advance of communication technology will also significantly alter the landscape of the OTC industries. There are already companies that provide data-logging devices that allow transfer of diagnostic data between patients at home and the physician's office (327). Right now, the technology is limited to immobile patients. The advance of handheld wireless devices and cellular phones will make transfer of medical information much easier for mobile OTC consumers. With the advance of better internet security, personal data may be readily portable **(328).** It is not hard to image that consumers can carry a credit card-like device that has all his/her medical history and possible drug–drug interactions stored on the device.

Pharmaceutical companies and government used to the two major drivers for Rx-to-OTC switches and they will continue to be the major drivers for the switches. The former conducts switches for lifestyle management of prescription drugs while the latter promotes switches to reduce national prescription costs. In future, manage care providers will also be a major driver to petition for switches as in the case of second-generation antihistamines.

The growth in the OTC industry will come not only from additional switches but also from a lot of "think outside the box" approaches. One thing for sure, the industry cannot keep marketing additional flavors to grow their business. The business model will have to change!

What is the future for lifestyle drugs? The future is very promising despite the strong resistance of the healthcare providers to pay for lifestyle drugs simply because the consumers want them to feel and look better. With the advances in our understanding of causes of diseases and disorder and genomic research, pharmaceutical scientists can now create drugs to target receptors that were previously unknown. To maintain double-digit growth, the pharmaceutical industry cannot just focus on unmet consumer needs in the area of life-threatening conditions such as cancer, asthma, anti-infective diseases, and cardiovascular diseases. They also need to capitalize on unmet consumer needs in the area of lifestyle enhancement to the aging consumers. The growth in the market for lifestyle drugs will likely be faster than that for traditional life-threatening diseases. The allowance of direct-to-consumer advertising creates a favorable environment to market lifestyle prescription drugs to informed consumers. The advances in genomic research will theoretically allow for more precise targeting, thereby minimizing unwanted side effects. This will facilitate the acceptance of lifestyle drugs and possible switch to OTC because of their lower side effects. Several lifestyle products such as hair growth and smoking cessation have already been switched and became quite successful. The issue of quality of life is as important for treating life-threatening diseases especially in developing countries. Our stressful lifestyle will in turn cause many more lifestyle diseases such as mild depression, anxiety, ulcer, and other psychosomatic diseases. In fact, the opportunities for lifestyle drugs in treating psychosomatic diseases are endless considering the extensive classifications in the Diagnostic and Statistical Manual of Mental Disorders **(329).**

Pharmaceutical companies will need to increase the awareness of the consumers about their lifestyle products, but they need to be very careful in advertising them. They need to cultivate a positive image of responsible marketing. Roche is already doing that in advertising their product Xenical, which is targeted for a special group of diabetic patients that has a medical need to lose weight. The company will also need to tailor their advertising to create psychological ties with the patients and ensure compliance. All lifestyle products have dedicated websites to educate and provide product information to consumers. Careful

consumer marketing will be needed to identify how to best position and advertise the products.

The lifestyle drugs are here to stay and their definition will continue to change. Their market will continue to grow as the baby boomers age.

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CHAPTER TEN

Radiopaques

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Contents

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1 INTRODUCTION

Radiocontrast agents delineate body organs and tissues against their immediate environment during fluoroscopic or roentgenographic examination. They function by renderingthe spaces or cavities occupied or the surfaces to which they adhere either lucent or opaque in contrast to their immediate surrounding tissues, in the path of X-rays, and are accordingly classified as negative- and positive-contrast agents (1, 2). Positive-contrast agents absorb X-rays and produce a darker shadow on the fluoroscopic screen and lighter or whiter shadows on the X-ray film, of the organ to be visualized against the surrounding tissues (2). Radiopaques fall in this category. Negative-contrast media are more transparent than either water or body tissue to X-rays, and give a lighter shadow on the fluoroscopic screen and a darker or blacker shadow on the X-ray film. Fats, lipoid substances, and gases such as air, oxygen, nitrogen, carbon dioxide, helium, nitrous oxide, and xenon, absorbing less X-ray radiation than the body tissue, belong to this category.

Radiopaques have in common the property of opacity to X-ray radiation and constitute many substances that, although dissimilar in chemical form, structure, and pharmacological properties, consist of elements of high atomic number. To understand the unique dependency of radiopacity on atomic number, a brief discussion of the properties of X-rays is necessary.

2 PROPERTIES OF X-RAYS

In 1895 W. C. Roentgen discovered X-rays, also known as Roentgen rays (3, 4). Within a month of the discovery, medical examinations were being made using the newly discovered mysterious rays. X-rays are penetrating electromagnetic radiations with quantum energies of a few thousand to several million electron volts (eV), generated by transitions of either bound electrons within an atom or free electrons between two positive energy levels in the field of an atomic nucleus (5). The former is the atomic transition and consists of the filling of a hole or vacancy in an inner shell by an electron from an outer shell. The difference in binding energies between the two shells involved is emitted as electromagnetic radiation in the form of monochromatic (i.e., monoenergetic) X-rays, typical of the element and the transition. Transitions of free electrons between energy states give rise to continuous X-rays; these are produced when electrons accelerated to high kinetic energy are allowed to impinge on a metal target. The slowing down of fast electrons in the vicinity of the target nucleus is essentially an electron transition between two positive energy states in the field of a target nucleus and leads to the formation of *bremsstrahlung* ("braking radiation"), composed of polychromic X-rays.

X-rays for medical use, produced by impinging fast electrons on a tungsten target in an X-ray tube, are polychromic. The diagnostic X-rays have an energy range of 30 to 100 kVp (kilovolts at the peak) but higher energies (120–140 kVp) may be used in computed tomography (see Section 6.6.1). The energies of X-rays produced in this manner depend on the kinetic energy of the accelerated electrons (5).

Table 10.1 Physical Characteristics of Some Radiopaque Elements

Element	Atomic Number Z	Density (g/cm^3)	K-Edge at (keV)	Total Cross Section for X-rays of Several Energies," (cm^2/g)			
				40 keV	60 keV	80 keV	100 keV
F	9	1.11	—	0.278	0.1912	0.1630	0.1497
Ca	20	1.55	4.038	1.792	0.6487	0.3630	0.2564
Ti	22	4.45	4.965	2.174	0.7543	0.4009	0.2706
Fe	26	7.86	7.112	3.559	1.174	0.5806	0.3643
Br	35	3.12	13.474	8.061	2.607	1.200	0.6849
Y	39	4.45	17.080	10.74	3.479	1.587	0.890
I	53	4.93	33.169	22.40	7.696	3.549	1.955
Ba	56	3.50	37.441	24.53	8.627	3.997	2.197
Gd	64	4.45	50.240	6.876	12.19	5.727	3.164
Yb	70	6.98	61.332	9.112	3.161	7.010	3.987
Ta	73	16.60	67.414	10.25	3.581	7.482	4.231
W	74	19.30	69.524	10.73	3.686	7.788	4.432
Pt	78	21.37	78.395	12.35	4.254	8.778	4.894
Au	79	19.37	80.723	12.81	4.442	2.111	5.116
Pb	82	11.32	88.006	14.05	4.893	2.335	5.461
Bi	83	9.80	90.527	14.98	5.267	2.520	5.808
Th	90	11.70	109.64	18.36	6.450	3.106	1.786

"Taken from Ref. 8.

In traversing through matter, X-rays are attenuated by coherent (Rayleigh) and incoherent (Compton) scattering and are absorbed by the photoelectric process (6, 7). X-rays of energy below 100 keV are mainly absorbed by the photoelectric process with a cross section (i.e., the probability for absorption) proportional to $Z^5/E^{7/2}$ (6), where E is the X-ray energy and Z is the atomic number of the absorber. Energy and wavelength of X-rays are related by the formula $\lambda = 12.40/E$, where λ is in angstroms and E in kiloelectron volts (8). As X-ray energy is increased, absorption by the photoelectric process diminishes, and Compton scattering becomes important. At energies above 1.02 MeV, the X-rays are predominantly attenuated by pair production, creating electron-positron pairs; this process does not play a role in the attenuation of medical diagnostic X-rays.

The attenuation of a collimated beam of monoenergetic X-rays is an exponential function of the depth of penetration (7). The relation may be expressed as

$$I = I_0 e^{-\mu d} \qquad (10.1)$$

where I_0 is the intensity of the initial X-ray beam, I is the intensity of the transmitted X-ray beam, e is the base of natural logarithm, μ is the mass absorption (or attenuation) coefficient, and d is the mass of the material penetrated. The attenuation may also be expressed using linear attenuation coefficient (μ/ρ), where p represents the density, and x is the thickness or the "mass-thickness" of the material penetrated, equal to ρd, as shown in Equation 10.1a in the following:

$$I = I_0 e^{-(\mu/\rho)x} \qquad (10.1a)$$

The mass absorption coefficient is a function of the X-ray energy and the atomic number Z of the absorber and is equal to the total cross section, expressed in cm^2/g, of the interactions mentioned above. In Table 10.1 are listed the atomic number, density, K-edge, and total cross section of elements that either have been used or have potential use in contrast media. The total cross sections given are calculated for the absorption of monochromatic X-rays of 40, 60, 80, and 100 keV in energy (8); for the absorption of polychromic X-rays of the same energy designation, the values differ somewhat. The total cross section decreases monotonically as the X-ray energy is increased. At the K and L absorption edges, sharp discontinuities appear, and the total cross sections jump to higher values as a result of enhanced absorption.

In roentgenography, the energy of X-rays is optimized according to the depth of penetration desired, as determined by the size and density of the object under examination (1, 9–11). Depending on the operational energy range, a large fraction of the polychromatic X-rays may fall in the region where absorption by the radiopaque element is small or may fall in the region of enhanced absorption near the K-edge. The fact that iodine has a higher total cross section for 40 keV X-rays than that of tantalum or tungsten, for example, is attributed to the proximity of the X-ray energy to the K-edge, which occurs at 33.169 keV. Increasing the iodine content of an iodinated contrast medium from 28.7 to 37.5% doubles the contrast of the radiographic image at an X-ray energy of 70 kVp but increases the contrast by only 60% at 90 kVp (9).

Johns and Yaffe (12) used the energy and mass dependency of the linear attenuation coefficient upon X-ray energy to characterize normal and neoplastic breast tissues. Carroll et al. (13, 14) showed that there are differences in the linear attenuation coefficients between normal and selected cancerous breast tissues and that the contrast increases as the beam energy in the energy range of 14 to 18 keV of monochromatic X-rays decreases. These monochromatic X-rays were generated at the National Synchrotron Light Source at the Brookhaven National Laboratory.

Substances in medical radiography are classified into five categories (2) according to their opacity to X-rays:

1. Radiolucent: gases.
2. Moderately radiolucent: fatty tissues.
3. Intermediate: connective tissues, muscle, blood, cartilage, cholesterol stones, uric acid stones.
4. Moderately radiopaque: bone, calcium salts.
5. Very radiopaque: heavy metals and their salts.

3 CLASSIFICATION OF RADIOPAQUES

The basic characteristics desirable for a contrast medium are (1)satisfactory radiopacity (related to atomic number, material density, and concentration), (2)stability, (3) pharmacological inertness, and (4) minimum sensitizing properties (15). Radiopaques constitute heavy metals and their salts, iodized oils, organic iodine compounds, and miscellaneous agents, such as iodinated particulate suspensions. Among these, the iodinated organic compounds offer a wide range of diverse radiopaque agents for clinical diagnosis in roentgenography, and the heavy metal gadolinium chelates can enhance the image contrast in computed tomography and in nuclear magnetic resonance imaging.

4 HEAVY METALS AND THEIR SALTS

4.1 Heavy Metals

Heavy metals with their high atomic numbers have satisfactory radiopacity and are potentially useful radiopaques. Powdered metals such as tantalum, gold, and lead have been used for experimental bronchography in dogs (16–18). Tantalum elicits no unfavorable tissue reaction and is widely used in surgery (19–22). The cost of its application as a radiopaque is minimal. Inhaled tantalum dust induces no acute or chronic inflammatory response in the airways or pulmonary tissue (23). Ingested tantalum powder produces no untoward effects on the gastrointestinal (GI) tract and is excreted. Injected tantalum powder is engulfed by the Kupffer cells without visible damage or pericellular reaction (24). The toxicity of tantalum powder is extremely low, given that as much as 8000 mg/kg can be administered orally without systemic toxicity (17, 22).

Nadel et al. (17, 25) were the first to use tantalum dust for bronchography in canine and human lungs. Tantalum dust, with an average particle size of 2.5 μm diameter, has been administered by insufflations in bronchography, esophagography (25), and gastrography (26). Tantalum dust adheres tenaciously to bronchial and esophagal mucosa, to yield bronchograms and esophagograms of excellent detail over a prolonged period (17, 23, 25, 27). Experimental double-contrast cystography has also been carried out (28). An aerosol preparation containing 2.4% tantalum dust (particle size, 2–50 μm), 5% lecithin (as surfactant), and 55% dichlorodifluoromethane has been introduced to simplify the preparation for the roentgenographic procedure

and to shorten the preparation for examination (29). Commercial tantalum powders are nonporous (30) and must be fractionated before use in bronchography (31). Because of its high density and high atomic number, tantalum compares favorably with other contrast agents and yields superior bronchograms to those obtained with propylidone (15d) (17). Tantalum provides equal attenuation to an X-ray beam with 1/5 to 1/10 as much as the amount required of barium or iodine and about 1/20 the amount required of iodized oils (17, 18). The metal is removed from the bronchi within a few days by the ciliary activity and by coughing (17, 23, 32) and from the lung in a much shorter time (23). The clearance of tantalum is slower, however, than that of barium sulfate under similar conditions (23); this may be attributed to its high density, which slows the transport of particles by the flow of body fluid. The clearance half-time ranges from 105 to 817 days when determined with the use of radioactive ^{182}Ta (32).

Powdered tantalum (average particle size: 3 μm in diameter) has been given intravenously as suspensions to dogs and rabbits for splenohepatography (24). Although good splenohepatograms have been obtained, the aggregation of tantalum powder has caused fatal pulmonary embolism in dogs. The usefulness of tantalum dust for bronchography has been reviewed (33, 34).

Link et al. (35) proposed the use of tantalum and tungsten to add radiopacity to hydrogel embolic agents for embolotherapy and studied the emboli formed by coagulation under high pressure of a 1:1 or 2:1 mixture of tungsten particles (1–10 μm) and a liquid hydrogel (polyacrylonitrile copolymer) in a rabbit model.

4.2 Heavy Metal Salts

Soluble heavy metal salts are in general highly toxic because of the presence of free metal ions, although many insoluble ones including oxides have been used in roentgenography (36–42). Barium sulfate has been successfully used as a contrast agent for visualization of the alimentary tract for the past 70 years and has not been supplanted by iodinated contrast media (43). The ferrites are experimental radiopaque agents because the magnetism of this class of iron oxide allows the movement of the ingested material to be controlled by an external magnetic field (44, 45). Ultrasmall superparamagnetic ferrite particles have clinical use for contrast enhancement in magnetic resonance imaging (MRI).

4.2.1 Barium Sulfate. Bachem and Günther (46) introduced barium sulfate in 1910 to replace insoluble and toxic bismuth salts for clinical visualization of the alimentary tract. Its nontoxicity, effectiveness, and low cost have made barium sulfate the most widely used radiocontrast agent for gastrointestinal roentgenographic examination since that time (47, 48). Dihlmann and Hering (49) described its usage up to 1993. Patton (50) reviewed the history of barium sulfate as a contrast medium and noted its commercial use in the early days as a flour adulterant because of its nontoxicity and lower cost.

Colloidal barium sulfate preparations are available from numerous commercial sources. Information about their exact composition has not been freely available because of proprietary interest (47, 51). Preparations may differ in their effectiveness for coating mucosal surfaces as determined by (1) particle size, (2) ionic charge on suspended particles, (3) pH, (4) resistance to flocculation, (5) suspension aid, and (6) viscosity or osmotic toxicity (52).

The particle size of barium sulfate may vary from a fraction of a micron to several microns or more (48). Ultrafine grain size by itself may give inferior visualization of the gastric mucosa but the particles can be more easily held in suspension. On the other hand, particles larger than 1 μm may offer better contrast, provided that they can be made to stay in suspension. An electron micrograph of barium sulfate particles of less than 1 μm in diameter shows that they are of irregular shape (53). The influence of microcrystalline shape on the coating property of the suspension is not well studied.

The viscosity of barium sulfate suspension is determined by the quantity and size of particles and can be modified by added peptizing agents (52). A thick paste of barium sulfate in water can be peptized or thinned by the addition of sodium citrate and sorbitol. The function of the citrate is to stabilize the colloidal

preparation and that of the sorbitol is to enhance that function. The use of a polybasic acid and sugar alcohol combination can lead to the incorporation of so much barium sulfate in a liquid suspension as to obtain a preparation with a specific gravity close to 3 (54, 55). Other polybasic acids such as tartaric acid and ethylenediaminetetraacetic acid (edetic acid) may also be used.

Particles in unprotected barium sulfate suspensions have a tendency to aggregate, resulting in flocculation. Such suspensions may be stabilized by the use of a dispersingagent or by placing an electric charge on the particle (52). The surface of barium sulfate particles is either positively or negatively charged, depending on the residual lattice ions present. It is also affected by the nature of the material added to coat the particles. The coated particles are uniformly charged and flow easily on account of the like charge on their surface, which resists aggregation and increases the colloidal stability.

Additives for coating barium sulfate particles include methylcellulose (47, 52, 53, 56–61), carboxymethylcellulose (53, 54), hydroxypropylcellulose (53, 54, 62), chondroitin sulfate (63), heparin (52), and sodium dextran sulfate (52). Dispersing agents used for stabilization and peptization of the colloidal suspension include agar (48, 54, 55), acacia (47, 56), alginates (54, 56, 59, 64, 65), alginic acid and propylene glycol mixtures (66), anionic galactanes (66), bentonite (47, 60), casein (47), erythrobic acid (58, 59, 67, 68), gelatin (47, 60), glycerol (60, 69), gum arabic (53, 55, 62), hexametaphosphate (57, 58, 62), lecithin (47, 65), mannitol (69), pectins (47, 54, 55, 62), polyalkylarene sulfonates (66), polyethylene glycol 400 (64, 69, 70), polymetaphosphate (71), nonionic poly(oxyethylene)glycol stearate (65), polysorbate 80 (47, 69), poly(vinyl alcohol) (54, 55, 70), poly(vinylpyrrolidone) (54, 55, 62, 71), pyrophosphate (55), sodium ascorbate (58, 59, 67–69), sodium dioctylsulfosuccinate (69), sodium lauryl sulfoacetate (64, 69), sorbic acid (64, 69), sorbitol (54, 55, 64, 69), and trisodium citrate hydrate (55, 64, 66, 69). Classification of these agents by their function is only superficial, given that many have dual functions.

The mobility of the particles depends on the pH of the suspension, the ionic strength, the age, and the method of preparation. Adherence of barium sulfate particles to mucosal membrane is pH dependent because the pH affects not only the electrokinetic charge on the barium sulfate particles but also the charge on the mucosal lining, which is composed of glycoprotein mucopolysaccharideand is capable of carrying charges (52). Coating of mucosal surfaces by commercial barium sulfate preparations has been studied by Schwartz et al. (72), who found that an optimal viscosity is important for satisfactory coating; at low viscosities the coating is too thin, and at high viscosities the preparation is too viscid for use. According to Fisher (43), 0.01 g of barium sulfate/cm^2 of surface area is needed to visualize mucosa.

Many different preparations of colloidal barium sulfate for roentgenography are available commercially. The so-called barium meals are not limited to liquid suspensions; they also appear as tablets (73). Barium sulfate suspensions containing an effervescent agent have been introduced for use in double-contrast studies (74, 75). Barium sulfate may also be coated with Fe_2O_3, MgO, and Al_2O_3 (63,761. The coated material has good dispersibility and very low viscosity in acidic media. Other barium preparations are also useful. A barium titanate suspension was compared with barium sulfate (72). An emulsified mixture containing castor oil and barium sulfate can visualize colon fistula more economically than can iodinated contrast media (77).

Barium sulfate is used not only in roentgenographic examination of the GI tract (51) but also in inhalation bronchography (61). Bullowa and Gottlieb (78) did early experiments on dogs in 1920. Barium sulfate was used for bronchography in infants and children (79). According to Clement (61), satisfactory bronchograms can be obtained with less barium sulfate if the bronchial surface is previously exposed to methylcellulose. Methylcellulose improves the bronchogram by forming a viscous film on the bronchial mucosa to which barium sulfate easily adheres, thus reducing considerably the amount of contrast agent required for an examination.

When used in bronchography, barium sulfate is nonallergenic and nontoxic. It induces only a mild, benign, foreign body reaction and causes no pulmonary fibrosis or bronchial spasms. It is cleared from the normal lung by ciliary action, coughing, and phagocytosis at a rate similar to that of tantalum (61). When the bronchial surface is precoated with methylcellulose, the clearance of barium sulfate is usually complete within 24–48 h (61). Any residual amount that may remain trapped in the alveoli is usually located within the macrophages in tiny intra-alveolar granuloma. When used in roentgenography of the GI tract, barium sulfate has the inherent danger of inspissation and impaction when water is reabsorbed, particularly in the colon (48, 80, 81).

4.2.2 Bismuth Subnitrate. Bismuth subnitrate was the first contrast agent used clinically to visualize the alimentary tract (36). It was found to be toxic in humans through the reduction from nitrate to nitrite; as a result, bismuth subcarbonate was substituted (82). When the toxic action was traced to the metal itself, the use of bismuth salts was discontinued.

4.2.3 Ferrites. Novel materials such as ferrites were introduced as experimental radiocontrast agents (44, 45). Ferrites are iron oxide (Fe_2O_3) in solid solution with one or more metallic oxides. They possess about 80% of the radiopacity of barium sulfate and a higher percentage if oxides of high atomic number are incorporated. The ferrites are magnetic, a property that allows their movement within the body to be controlled by an external magnet. Because of their magnetic property, ultrasmall superparamagnetic iron oxide particles are studied in animals for accumulation in lymph nodes, to develop a target-specific contrast agent for magnetic resonance imaging (MRI) (83).

Frei et al. (44) reported the use of magnesium ferrite as a contrast material for X-ray diagnosis. Sugimoto et al. (45) studied the suitability of four ferrites containing Mg, Zn, Cu, Ni, and Mn as radiopaques with respect to toxicity, solubility in gastric juice, X-ray absorption, and effective magnetic field strength necessary for controlling the ferrite powder. These ferrites can be prepared to yield low solubility in acid or stimulated gastric juice. They are nonallergenic and have no acute toxicity (84). On prolonged daily oral administration (30 days), the ferrites cause a slight decrease in body weight, hematocrit, and the enzyme glutamic-pyruvic transaminase in rats and mice (85). The median lethal dose (LD_{50}) for rats and mice is >20 and >10 g/kg, respectively (85). When fed to experimental animals, the ferrites are cleared entirely from the body within 1 week, as shown in absorption studies using ^{55}Fe-, ^{54}Mn-, or ^{65}Zn-labeled ferrites (84). Only soft ferrites, which do not coalesce in the absence of the applied magnetic field, are used as contrast media. Satisfactory roentgenograms of the esophagus, stomach, bronchus, and small intestine have been obtained with these ferrites (45). The particle size of the ferrite powder may range from less than 1 micron to several microns in diameter, and a stable suspension of the particles may be obtained using stabilizing and peptizing agents (86–91) similar to those mentioned in Section 4.2.1.

4.2.4 Metal Chelates. Chelating agents capable of combining with metals of high atomic number are potentially useful contrast media (15, 37, 38). Chelation may significantly reduce the toxicity of metal ions; the toxicity of Ca, Ni, Co, and Pb salts of ethylenediaminetetraacetic acid (EDTA or edetic acid) is many times lower than the toxicity of the metal ions. On the other hand, Cr, Cu, and Hg edetates and their metal ions have comparable toxicity, as indicated by the LD_{50} (7-day mortality) values. Because the binding between metal ions and the ligands is reversible, the sequestered metal ions in the latter group of chelates may have become free in *vivo*.

In addition to edetic acid, multidentate chelating agents such as 1,2-diaminocyclohexane-N,N'-tetraacetic acid, diethylenetriaminepentaacetic acid (DTPA), *N,N'*(2-hydroxycyclohexyl)ethylene-diaminediacetic acid, 2-hydroxycyclohexylethylenediaminetriacetic acid, and β-hydroxyethylethylene diaminetriacetic acid have been used to sequester heavy metal ions for roentgenographic purposes (37). Solutions (8%) of Bi-DTPA and Pb-EDTA were used in experimental bronchogra-

phy and angiography in dogs (37) but the margin of safety between a useful dose and the minimum lethal dose is so narrow as to render them hazardous for clinical trials. Zwicker et al. (92) compared iodinated and noniodinated contrast media, that is, iopromide (2.94 molar iodine, 370 mg I/mL) with gadolinium (as 0.5 molar Gd-DPTA, 78 mg Gd/mL) and ytterbium (0.5 molar Yb-DPTA, 86 mg Yb/mL) in computed tomography of aorta and liver in dogs. At equimolar concentrations, Gd-DTPA and Yb-DTPA are superior to iodine in maximum contrast enhancement of the aorta and the liver. but iodine at 2.94 molar concentration exceeds these enhancement values of metal chelates at 0.5 molar concentrations. The lanthanide chelates, because of their high atomic number and approximately 40% greater efficiency in attenuating X-rays of 120 kV than iodine at equivalent mass concentration, are useful agents for contrast enhancement in computed tomography (CT) (93, 94). Schmitz et al. (94) found in a rabbit model that the neutral macrocyclic chelate gadobutrol is a more effective contrast agent than iopromide for CT at lower doses of the imaging atom. These agents may be indicated in patients with known previous allergic reactions to iodinated contrast media.

The high atomic number and the large magnetic moment of gadolinium have made its compounds potentially useful for both CT and nuclear magnetic resonance imaging (MRI). The effect of gadolinium chelates on the relaxation behavior of water protons provides the contrast in the MRI of human tissues (95). The mechanisms for contrast enhancement by Gd-chelates in CT and in MRI are very different, although the imaging results are very similar. A brief description of MRI is given below as a basis for discussion of the development and use of potential ferrites and gadolinium contrast-enhancing agents.

4.2.5 Ultrasmall Ferrites, Gd-Chelates, and MRI. Nuclear MRI is dependent on the use of superparamagnetic and paramagnetic materials to enhance the contrast in tissue discrimination and is both complex and difficult. The patient is not exposed to X-ray radiation while undergoing MRI. Unlike the radiocontrast media attenuating the X-rays, these agents facilitate the relaxation of water-protons and are not visualized directly in the MRI image (96, 97). In MRI, the patient is placed within a uniform magnetic field; the water-protons in the body of the patient will line up, giving a net nuclear macroscopic magnetization along the long axis of the patient. If one applies an external radiofrequency pulse to induce magnetic resonance, the nuclear magnetization of the individual protons will relax them back to their original position in an exponential manner, called spin-lattice relaxation, with a time constant T1. Relaxation also occurs in the transverse axis, called spin-spin relaxation, with a time constant T2. The MR contrast agents that facilitate the relaxation will increase or decrease the signal intensity accordingly. For diagnostic purposes, relaxation data acquisition after a pulse sequence is computer controlled and the data are reconstructed and displayed as an image, to show the contrast enhancement between normal and diseased tissues. According to Carr (98), the MR images are dependent on pulse sequences, proton density, T1, and T2. Saturation-recovery sequences rely on proton density, inversion-recovery sequences on proton density and T1, and spin-echo sequences on proton density and T2.

The contrast media for MRI, according-to their effect on image enhancement, are categorized as (1) relaxivity-based T1 agents and (2) susceptibility-based T2 agents (96, 97). The term $1/T_i$, where i = 1, 2, represents the rate of relaxation of solvent nuclei. Relaxivity (R_i) is obtained as the slope of relaxation rate per 1 mM of paramagnetic species, in the unit of $mM^{-1}\,s^{-1}$. It represents the efficiency with which the contrast agent enhances the proton relaxation rate of water and is defined as follows (94):

$$R_i = [(1/T_i)_p - (1/T_i)_0]/[\mathrm{C}] \quad (i = 1, 2)$$

where the suffixes p and 0 indicate with and without paramagnetic species, respectively. Susceptibility effect refers to the relaxation of T2, attributed to the moving spins relaxed by the fluctuating field. A paramagnetic metal complex can interact strongly or weakly with water molecules, depending on whether these

water molecules are covalently bonded or hydrogen bonded to the metal complex, sometimes referred to as "inner-sphere" relaxation, or whether the water molecules are passing through the field by translational diffusion, often referred to as "outer-sphere" relaxation (95, 96). A shortening of the T1 and T2 relaxation times will lead, respectively, to either a positive (brightening) or a negative (darkening) effect in image intensity. Metal ions are toxic and need to be sequestered as chelates with very small dissociation constant for safe use as contrast media. Chelation decreases the relaxivity.

Ultrasmall ferrite particles and lanthanide chelates are potential contrast media in MR imaging. Ferromagnetic, superparamagnetic, and paramagnetic compounds affect 1/T1 and 1/T2 differently (99). Ferromagnetic and superparamagnetic iron oxide particles enhance 1/T2 more than 1/T1 and have a greater transverse relaxivity/longitudinal relaxivity ratio ($R2/R1 \gg 1$) than paramagnetic substances ($R2/R1 \approx 1$) (99). The iron oxide particles, when prepared by different methods, differ in particle size, polycrystalline state, core concentration, and the dextran coating. The recognition of these iron oxide particles by macrophages and their distribution in the body are determined by their size and surface properties (100). The ultrasmall particles can migrate across capillary endothelium upon intravenous administration and are taken up by the reticuloendothelial system, including the liver, spleen, lymph nodes, and bone marrow. Ferrite particles AMI 25 are about 80 nm in diameter, have a very short blood half-time of 6 min, and are phagocytosed by Kupffer cells in the liver, resulting in contrast enhancement between normal liver and tumor metastasis (101, 102). The ultrasmall superparamagnetic iron oxide particles (USPIO), obtained by size fractionation of AMI 25, have a mean diameter of 11 nm and a plasma half-time of 81 min and tend to accumulate in lymph nodes (83,103). USPIOs have a molecular weight of approximately 700 kDa by gel filtration, comparable to that of ferritin (11 nm; molecular weight, 440–600 kDa) (103). AMI 227 is a nanoparticulate iron oxide with a mean size of 18 nm (17–21 nm) and an outer coating of low molecular weight T10 dextran (104, 105). BMS 180549 (Squibb Diagnostics, Princeton, NJ) is similar to AMI 227 in size but has an iron core of 4.3–6.0 nm in diameter and a blood half-life of greater than 200 min, and is clinically useful for differentiating reactive from tumor-bearing lymph nodes (106, 107). The maximum dose is 1.7 mg Fe/kg or 120 mg Fe for a 70-kg person, which is small compared to the body iron store. Intravenous or interstitial administration of superparamagnetic particles is relatively free of untoward side effects, but clumping of the particles and microembolization of the AMI 25 particles may lead to back pain and fever (108). The compound is nontoxic and readily metabolized in the body. Its cellular uptake and trafficking is by receptor-mediated endocytosis to the terminal lysosome (109). Ultrasmall iron oxide particles can be targeted to asialoglycoprotein receptor in MR receptor imaging (103). An oral formulation of magnetic ferrite particles was also tested for enhancing visualization of the GI tract in MR imaging (110).

Gadolinium has seven unpaired 4f electrons and a large magnetic moment and is highly paramagnetic. The free gadolinium ions are toxic. When administered intravenously, the ions combine with endogenous metal-binding sites and endogenously available counter ions, such as phosphate, carbonate, and hydroxide, to form insoluble complexes that are very slowly excreted. Sequestering the metal ions by chelation with organic chelating agents decreases the toxicity. Gd-DTPA meglumine salt, a chelate of gadolinium with diethylenetriaminepentaacetic acid (DTPA), was reported to have a median lethal dose (LD_{50}) of 10 mmol/kg, compared to that of gadolinium chloride ($GdCl_3$) with an LD_{50} value of 0.5 mmol/kg in the rat when administered intravenously (110). Gd-DTPA has a thermodynamic stability constant of $10^{22.46}$ at 25°C (94). Gd-chelates enhance contrast in MRI. The signal response of gadolinium is not dose dependent but biphasic; that is, the signal intensity increases initially and then decreases with an increase in Gd concentration (112). Paramagnetic gadolinium-chelated complexes for MRI (113) can be categorized, based on their chemical structures, into two classes: (1) those sequestered by linear ligands (EDTA, DTPA, etc.) and (2) those

sequestered by macrocyclic ligands (polyaza polycarboxylic acids, etc.) as follows:

1. Gadolinium-chelates with linear ligands
   - o Gadobenate dimeglumine (Gd-BOPTA)
   - o Gadodiamide (Gd-DTPA-BMA)
   - o Gadopenamide
   - o Gadopentetate dimeglumine (Gd-DTPA)
   - o Gadoversetamide
   - • Gadoxetic acid
2. Gadolinium-chelates with macrocyclic ligands
   - • Gadobutrol
   - • Gadoteric acid (Gd-DOTA)
   - • Gadoteridol (Gd-HP-DO3A)

Tweedle et al. (114) studied the biodistribution of gadodiamide, gadopentetate, gadoterate, and gadoteridol, labeled with radioactive gadolinium-153 in both mice and rats. Gadodiamide and gadopentetate have linear ligands, and gadoterate and gadoteridol have macrocyclic ligands. Gadodiamide and gadoteridol are nonionic, whereas gadopentetate and gadoterate are ionic. These authors found that the radioactivity of the linear complexes, compared with the macrocyclic complexes, remained longer in the bone and liver of the animals. The order of their residual Gd concentration at long residence times from the lowest to the highest, was gadoteridol = gadoterate ≤ gadopentetate ≪ gadodiamide. The macrocyclic chelates of gadolinium have higher thermodynamic stability constants and tend to be more stable and do not undergo decomplexation in *vivo*. Gd-DTPA is rapidly excreted through the kidneys. Dean et al. (115) listed the rank of tissue distribution of Gd-DTPA in the rat 2 min after intravenous injection of the contrast agent. McLachlan et al. (116) showed the mean distribution half-life of the injected gadoteridol in human volunteers to be 0.20 ± 0.04 h and the mean elimination half-life 1.57 ± 0.08 h. More than 94% of the contrast agent was excreted in the urine in 24 h. The elimination half-life and the distribution half-life of gadoteridol were independent of the dose used. Staks et al. (117) studied the pharmacokinetics of gadobutrol in humans at different dose levels, up to the maximum dose level at 0.5 mmol/kg body weight, and showed that the contrast agent distributed predominantly in the extracellular fluid space, with a half-life in plasma of approximately 1.5 h. More than 95% of the injected dose was excreted by glomerular filtration within 12 h. No metabolite was detected.

Paramagnetic contrast agents that are distributed throughout the extracellular space and rapidly excreted by the kidneys have limited value for perfusion and organ function studies in MRI. Brasch et al. (118,119) showed that human serum albumin covalently bound to 5–18 Gd-DTPA molecules were useful for blood-pool enhancement but these macromolecules were slowly excreted. These authors also quantified capillary permeability of experimental mammary adenocarcinoma in female rats with albumin-(Gd-DTPA)$_{n}$ (120), and identified and differentiated pulmonary diseases with polylysine-(Gd-DTPA)$_{n}$ (121). Paramagnetic dextran (i.e., dextran linked to Gd-DTPA by diaminohexane through carbamate and carboxymethyl groups) was prepared as a potential MRI contrast agent (122). Liposomes containing Gd-HP-D03A or amphipathetic agents were evaluated for use in liver MR imaging (123, 124). Gadozelite, a gadolinium zeolite complex, has been listed for oral use (113). New derivatives of linear and macrocyclic polyaza and polyamino polycarboxylic acids have been synthesized as ligands for potential MRI contrast agents. Among them are Gd-DTPA-bis(amide) complexes, 15- to 17-membered macrocyclics with three pendent carboxymethyl groups, and others (125–131). Ranganathan et al. (132) designed and synthesized for testing biochemical processes a series of multimeric MRI agents, with molecular weight of 1–5 kDa and with a varying number of hydrated water molecules in the inner sphere of the gadolinium atom to modulate the reflexivity.

4.2.6 **Tantalum Oxide.** Although tantalum oxide is less radiopaque than tantalum powder by a factor of 0.5 per volume or 0.8 per mass, its chemical and biologic inertness and toxicity are very similar to those of tantalum (40). The technical advantages and safety in handling and storage of the oxide make it superior for use in roentgenography to metallic tantalum,

which presents an explosion hazard. The oxide is formed as β-Ta_2O_5, with a particle size of about 1 mm or less, by burning metallic tantalum powder in air. It is used in bronchography and esophagography by topical application. When injected intravenously in dogs and rabbits in experimental hepatosplenography, the oxide forms microscopic emboli in the liver and kidney. Injected particles of the oxide are engulfed by the reticuloendothelial cells without appreciable cellular or pericellular reaction.

4.2.7 Thorium Dioxide. The use of thorium dioxide as a contrast agent began in 1928 to 1929 (133); it has been used under the name of Thorotrast (Fellows-Testager Division, Detroit, MI) for angiography and cerebral arteriography. Thorotrast is a colloidal suspension of 25% by weight of thorium dioxide, of particle size less than 0.15 pm diameter, prepared by oxidation of thorium oxalate at 550°C (134). The high atomic number, high density, and the lack of acute toxicity make thorium dioxide an ideal radiopaque for use in large quantities in roentgenography (135). Thorium dioxide gives excellent contrast and has proved to be valuable in studies in which other contrast media would fail.

The drawback of using thorium dioxide as a contrast agent is its radioactivity and indefinite retention in the body (136,137). Thorium-232 is the longest-lived parent radionuclide in the thorium series (138). A considerable amount of work has been done regarding the disposition and fate of thorium dioxide after its systemic and local use in humans for roentgenography (139–147). The dioxide when administered intravenously is engulfed by the phagocytic cells of the reticuloendothelial system and is permanently localized in these cells (139). The long-term effects of radiation are fibrosis and neoplastic growth in the liver and spleen and fibrosis of their efferent lymph nodes (39, 146). Localization of thorium and its daughter nuclides in the bone may result in leukemia and other blood dyscrasias (144). Locally injected thorium dioxide, if in contact with epithelium for long periods, induces carcinoma (39, 148).

When colloidal thorium dioxide was applied within the lumen of the GI tract, no deleterious late effects were observed (39). The continuous shedding of the intestinal epithelium can remove the deposited dioxide and excrete it together with the intestinal content.

Because of the radiation effects of thorium, the use of thorium dioxide is limited only to the procedures approved by the U.S. Food and Drug Administration, such as hepatolienography in patients with metastatic cancer (39).

4.2.8 Other Metallic Salts. In addition to the heavy metal salts mentioned above, barium titanate and barium metatitanate have been used for visualization of the hypopharynx, esophagus, stomach, and small intestine (41, 149). Barium metatitanate is used as a powder with grains measuring from 0.8 to 1.0 pm. It is insoluble in water and has a density of 6 compared to that of barium sulfate with a density of 4.5. In addition, the metatitanate adheres better than barium sulfate to the intestinal mucosa, a property considered favorable for visualization. Powdered calcium tungstate can also be used to opacify airways but was found ineffective for filling airways smaller than 2 mm in diameter (42).

5 IODIZED OILS

Radiopaque iodine atoms can be introduced into vegetable oils to form iodized oils by reaction with hydroiodic acid. This converts unsaturated fatty acid groups into iodinated saturated ones, such as linoleic acid to diiodostearic acid and oleic acid to monoiodostearic acid (150). Commercial preparations of iodized oils are glyceryl esters (Lipiodol) and ethyl esters (Ethiodol) of iodinated fatty acids of poppyseed oil. Lipiodol and Ethiodol contain approximately 38–42 and 37% of organically bound iodine, respectively. Ethyl diiodostearate (45.0% I) and ethyl triiodostearate (55.2% I) have higher iodine content than that of Ethiodol and are available as emulsions (151). The iodized oils are yellow or amber and decompose with liberation of iodine upon exposure to air or light. Ethiodol has a lower viscosity than that of Lipiodol. The toxicity or tolerance of iodized oils is determined by their viscosity (150).

Iodinated oils are emulsified for injection with surfactants such as polyoxyethylene sor-

bitan monooleate (Tween-80) and sorbitan monooleate (Span-80) (152), polyethylene glycol-400 stearate (153,1541, soya lecithin (155), or ethylene oxide and castor oil (156), and stabilized with polyhydric alcohols such as glycerol and glucose. The toxicity of iodized oils administered intralymphatically is between one and two times that administered intravenously (150, 157). Ethiodol has an average lethal dose in the dog of 3.62 mL/kg for intralymphatic injection compared to 1.58 mL/kg for intravenous injection (157). Iodized oils are injected slowly into the peripheral lymphatic; an 8-mL dose is usually given over a period of 40–80 min at a pressure of 0.4 atm. High injection speeds and pressure may cause rupture of the lymph trunk and extravasation.

Intralymphatically injected oil contrast medium may remain in the lymph nodes for months or even for more than 1 year (150). The excess of that retained in the lymphatic system will enter the systemic veins through the thoracic duct or by way of lymphaticovenous communications and eventually pass into the pulmonary artery and its branches, to be distributed in the lung capillaries. Experiments in the dog showed that 50% of the injected Ethiodol was found in the lung and about 23% in the nodes at 3 days post-lymphography. The concentration of iodized oil in the nodes remained essentially the same but that in the lung had decreased to about 13% of the dose at 17 days postinjection (150, 158).

Metabolic studies with ^{131}I-labeled Ethiodol indicated that the iodized oil was rapidly deiodinated by enzymes in tissues with the iodine appearing as inorganic iodide, which was excreted by the kidney. In humans, no more than 0.5% of the injected iodized oil was found in the blood at any one time, and the urinary excretion was less than 2.5% of the dose per day (159). The most serious side effect of the iodized oils is pulmonary or systemic embolization and granuloma formation (160), which is related to the particle size of the oily drops (150, 157), but Kupffer cells can actively capture and phagocytize the iodized oil droplets (161).

Iodized oil is used in lymphography (150, 157), intra-arterial hepatography (154), intravenous hepatosplenography (162), and hysterosalpingography (160), and also used for treatment of hepatic tumors by chemembolization (161). In the latter the iodized oil additionally exerts a synergistic effect by activating the immune system in the liver. The oily contrast medium allows the detection of small avascular masses in the liver or spleen by computed tomography, which is superior to both contrast angiography with water-soluble contrast media and scanning with radiopharmaceuticals; the former is too transient for tomographic imaging and the latter is inadequate in resolution (162). Miyamoto et al. (160) found that the water-soluble nonionic iodinated contrast agent iotrolan has none of these side effects.

Lipiodol, when injected intra-arterially, can diagnose malignant hepatic tumors (163). Lipiodol as a carrier for anticancer drugs, such as neocarzinostatin, mitomycin, aclarubicin, and doxorubicin, has been administered to patients with hepatomas (164). In hysterosalpingography both oil and aqueous contrast media can reliably provide information about uterine-tubal anatomy, although Ethiodol gives a sharper image and the added advantage of a high conception rate (165–167). After oil contrast medium hysterosalpingography, one-third of the infertile women had normal pregnancies and childbirths (167, 168). An in *vitro* study showed that a potential mechanism for fertility enhancement might be attributed to the inhibition of peritoneal lymphocyte and macrophage function by the oil contrast medium. The advantages of Ethiodol versus diatrizoate, ioxaglate, and iohexol in hysterosalpingography have been varied (167).

Kishi et al. (169) evaluated the acute toxicity of Lipiodol infusion into the hepatic arteries (HAI) of beagles and found the influence of Lipiodol HAI to be dose dependent. The infused Lipiodol first passed through an arterioportal communication and distributed through the hepatic sinusoids to pulmonary capillaries and thence into systemic blood circulation. The circulation and embolization of oil droplets were found in the renal tubular cells of supracapsular cortex, the choroid plexus, the vascular endothelium, and the pancreatic duct epithelium, showing a process of intracellular collection of Lipiodol from the systemic blood circulation and of further metabolism-provoking cellular reactions.

6 ORGANIC IODINE COMPOUNDS

Organic iodine compounds make up the largest group of radiopaques or radiocontrast agents used in roentgenographic examination. The high atomic number of the iodine atom confers radiopacity on these molecules. These agents may be classified according to their application as angiographic, cholegraphic, urographic, and myelographic agents, or according to their route of administration as oral, intravascular, or intrathecal agents. They are used in high concentrations for better image resolution. An estimate in 1993 put the use of these agents as diagnostic aids on a worldwide basis at a market value of over $2 billion per annum (170). In the 1970s, the annual consumption of this group of agents in the United States surpassed 2000 metric tons and, even with the advent of computed tomography, the aggregate consumption of these media was still about 1400 tons as late as 1990 (171).

Grainger (172) in 1982 traced the early history of development of intravenous contrast media and the initial use of pyridine compounds containing iodine as radiocontrast media. Binz (173) found this group of iodinated pyridine compounds to be excreted by the kidney and the liver in substantial concentration and therefore labeled them the "Selectan group," to indicate their selectivity. Some of them were used for retrograde cystography and pyelography. Graham et al. (174) in 1926 used tetraiodophenolphthalein as the first contrast agent for intravenous cholecystography. This compound was widely used for gall bladder visualization until 1940, when Dohrn and Diedrich (175) introduced iodoalphionic acid and produced a better roentgenogram of the gall bladder and fewer adverse reactions; this remained the agent of choice until the early 1950s. At this time a large variety of iodinated organic radiocontrast agents, based on the 2,4,6-triiodobenzoic acid moiety, were introduced, most of which gave even better visualization and fewer side effects.

Wallingford (176) conceived the idea of using the benzene ring as carrier for iodine atoms. Strain (36), Grainger (172), Almén (177, 178), and Sovak (171) reviewed the historical development of organic iodine compounds as radiopaque agents, including the past, the present, and the future trends in the development of iodinated contrast media. Archer (179) discussed the chemical aspects of some cholecystographic agents developed before 1959, including iopanoic acid. Hoey et al. (180) compiled a list of iodinated compounds synthesized up to 1971 as X-ray contrast media to correlate their structure-activity relationship. From clinical experience and theoretical considerations, Almén (178, 181, 182) suggested the use of nonionic contrast agents and postulated that the nonionic contrast agents would be less toxic than the ionic ones because of lower osmolality. This theory stimulated the search for nonionic iodinated compounds, which included the synthesis and testing of monomers (183–186), bis compounds (9, 187–190), and polymers (191,192) as improved radiopaques. The outcome of such studies brought forth the first-generation nonionic monomer metrizamide (183, 184), the second-generation nonionic monomers iohexol (193), iopamidol (194–196), and so forth; the nonionic bis compounds iotrolan (197), iodixanol (198), and so forth; and the ionic, monoacidic bis compound ioxaglate (199, 200). Hoey and Smith (201) compiled a comprehensive list of ionic and nonionic monomers and bis compounds synthesized up to 1984, and reviewed and discussed the chemistry and physicochemical properties of the contrast agents as well as the safety and toxicity of different substituent groups. Newer drug design theories have focused on searching for safer and more effective contrast agents by masking the hydrophobic regions of the triiodophenyl nucleus with small alkyl chain polyhydroxyalkyl groups to increase hydrophilicity, to minimize protein interaction, and to reduce toxicity (171,182,202–205). The more powerful imaging techniques, such as computer-assisted tomography (CT), delayed CT, dynamic CT, spiral (or helical) CT, and digital subtraction angiography (DSA), depend on safer and more cost-effective contrast media to obtain anatomic and organ function information. The progress in clinical three-dimensional imaging has been reviewed (206–208).

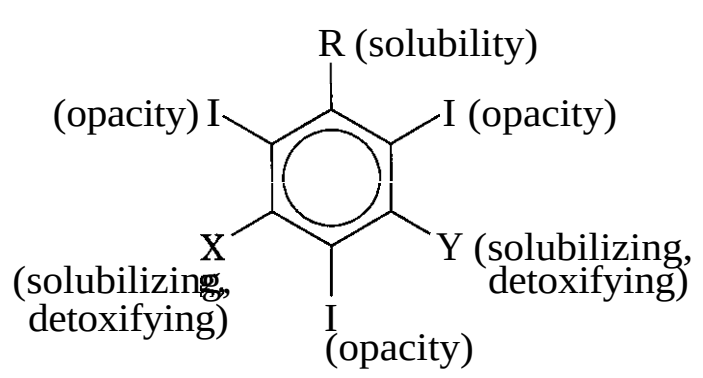
In subsequent sections, the iodinated radiopaques are discussed with respect to their classification, synthesis, structure-activity relationships, analysis, pharmacology, and uses.

The section on pharmacology includes the topics of cations, hyperosmolality, adverse reactions and toxicity, toxic effects on red blood cells, cardiovascular effects, nephrotoxicity, neurotoxicity, protein binding, histamine release, pharmacokinetics, formulation, excretion, and biotransformation.

The subject has been discussed earlier by Hoppe (209, 210), and Ackerman (211) and reviewed from the point of view of drug design by Herms and Taenzer (212). A number of monographs, from conference proceedings on various aspects of iodinated contrast media with specific information, have appeared (10, 213–218). Sovak (171) described briefly the history of the development of contrast media and his role in the search for an ideal agent.

6.1 Classification

In general, iodinated contrast agents contain either the pyridone or the benzene nucleus as the iodine carrier. With a few exceptions, newer contrast agents are derivatives of 2,4,6-triiodobenzoic acid and its congeners (1).The



(1)

adoption of the benzene ring to replace the pyridone nucleus as the iodine carrier for iodinated contrast agents originated from the observation by Wallingford et al. (219, 220) that iodobenzoic acids have very low toxicity and that the substituent groups determine the molecular and pharmacological properties. Acylation, substitution, and triiodination can further reduce the toxicity of the iodobenzoic acid neucleus (201).

Iodinated contrast agents may be classified as ionic and nonionic, monomers and bis compounds. The term dimers is often used to refer to the bis compounds. The classification is based on whether the contrast molecule contains one or two 2,4,6-triiodophenyl rings and an ionizing group. In an ionic contrast agent the R group in (1) represents a strongly hydrophilic functional group such as carboxyl, alkyl-, aralkyl- or alkoxyalkylcarboxyl group, and so forth. These groups show both hydrophilic and lipophilic properties. The X and Y groups are hydrophilic and exert a strong influence on both pharmacological properties (such as chemotoxicity, biological tolerance, and biotransformation) and physicochemical properties (such as solubility, osmolality, viscosity, and protein binding). In a nonionic contrast agent, the R, X, and Y groups are short-chain highly hydrophilic groups, in the form of 2-hydroxyethyl, 1,3-dihydroxypropyl, 2,3-dihydroxypropyl, or D-glucopyranosyl, and so forth. These substituent groups are linked to the phenyl ring by coupler groups, such as amide, reversed amide, amino, or others (201). Atoms of the coupler groups linked to the 1, 3, and 5 positions of the triiodinated phenyl ring can be expressed in designated notations as CCC, CCN, CNN, CNH, NNN, and so forth, to indicate the nature of the starting material and the ring electron density. This convention was first introduced by Gries and Mützel (221) and will be adopted here to indicate subclassification of the iodinated contrast agents by structure.

In the following, contrast agents that have been accepted for clinical use are listed by their United States Accepted Names (USAN) (222) or by their International Nonproprietary Names (INN). No proprietary names of the contrast agents will be given. For a listing of the proprietary names, chemical formulas, and uses, the International Drug Directory (223) and early compilations by Knoefel (224) and Strain and Rogoff (225) should be consulted. Fischer (226) has catalogued, up to 1986, current higher and lower osmolality intravascular contrast media (abbreviated as HOCM and LOCM, respectively) and listed their iodine content (milligrams of iodine per milliliter of fluid), osmolality, viscosity, and available dosage form sizes, by use of information obtained from manufacturers.

Classifications of ionic and nonionic monomers and dimers or bis compounds are shown with their structures in Table 10.2. Table 10.3 contains the chemical names of these radiopaque agents and available properties;

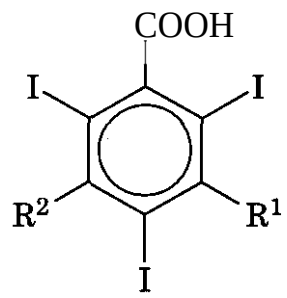
Table 10.2 Classification and List of Names and Formulae of Contrast Agents

A. Ionic monomers
1. Triiodobenzoates (2)
Acetrizoate (a)
Diatrizoate (**b**)
Diprotrizoate (**c**)
Iodamide (**d**)
Iodamide meglumine
Iotrizoic acid (**e**)
Ioxotrizoic acid (**f**)
Metrizoate (**g**)
2. Triiodoisophthalamates (3)
Ioglicic acid (a)
Ioseric acid (**b**)
Iothalamic acid (**c**)
Ioxitalamic acid (**d**)
3. Triiodophenyl alkanoates (**4**)
Iopanoic acid (**a**)
B. Nonionic monomers
1. Triiodo-1,3-benzenedicarboxamides (**7**)
Iobitridol (a)
Iohexol (**b**)
Iomeprol (**c**)
Iopamidol (**d**)
Iopentol (**e**)
Iopromide (**f**)
Iosimide (**g**)
Iotriside (**h**)
Ioversol (**i**)
C. Ionic dimers or bis compounds
1. Bis-triiodobenzoates (**9**)
Iocarmic acid (a)
Iodipamide (**b**)
Iodoxamic acid (**c**)
Iodoxamate meglumine
Ioglycamic acid (**d**)
Iosefamic acid (**e**)
D. Nonionic dimers or bis compounds
1. Bis-triiodo-1,3-benzenedicarboxamides (**10, 10′**)
Iodecol (**a**)
Iodixanol (**b**)
Iotasul (**c**)
Iotrolan (or iotrol) (**d**)
Iofratol (**e**)
E. Miscellaneous
1. Other dimer and polymer
Ioxabrolic acid (**11**)
Tris(iothalamic acid) (**12**)
2. Diiodophenyl alkanoate
Iodoalphionic acid (**13**)
3. Iodophenyl alkanoate
Iophendylate (14)
4. Diiodopyridones (**15**)
Iopydone (**a**)
Iodopyracet (**b**)
Iophenoxic acid (**b**)
Ioprocemic acid (**c**)
Ipodic acid (**d**)
Tyropanoic acid (**e**)
Iolidonic acid (**f**)
Iomorinic acid (**g**)
4. Triiodophenoxy alkanoates
Iolixanic acid (**h**)
Iopronic acid (**i**)
Iobutoic acid (**j**)
5. Triiodobenzamides (**5**)
Iobenzamic acid (a)
6. Triiodoanilides (**6**)
Iocetamic acid (a)
Iomeglamic acid (**b**)
Iosumetric acid (**c**)

Ioxilan (**j**)
3H2-Iopiperidol-A (**k**)
2. Triiodophenyl-D-gluconoamides (**8**)
Ioglucol (**a**)
Ioglucomide (**b**)
Ioglunide (**c**)
Iogulamide (**d**)
Iosarcol (**e**)
Metrizamide (**f**)
Iosulamide (**f**)
Iotetric acid (**g**)
Iotranic acid (**h**)
Iotroxic acid (**i**)
Ioxaglic acid (**j**)
Ioxaglate meglumine
Iozomic acid (**k**)

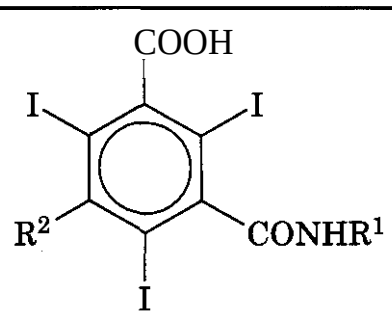
Iopydol (**c**)
Propyliodone (**d**)
Sodium iodomethamate (Iodoxyl) (**e**)
5. Iodophthaleins
Iodophthalein (**16**)
6. Others
Methiodal sodium (**17**)
Dimethiodal sodium (**18**)
Iodohippurate sodium (**19**)

Table 10.2 *(Continued)*



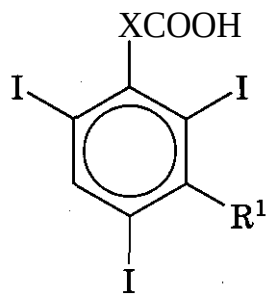
(2)

(2)	Type	R^1	R^2
a	CNH	$-NHCOCH_3$	$-H$
b	CNN	$-NHCOCH_3$	$-NHCOCH_3$
c	CNN	$-NHCOCH_2CH_3$	$-NHCOCH_2CH_3$
d	CNC	$-NHCOCH_3$	$-CH_2NHCOCH_3$
e	CNH	$-NHCOCH_2[OCH_2CH_2]_4OCH_3$	$-H$
f	CNN	$-NHCOCH_3$	$-NHCOCH_2OH$
g	CNN	$-NHCOCH_3$	$-N(CH_3)COCH_3$



(3)

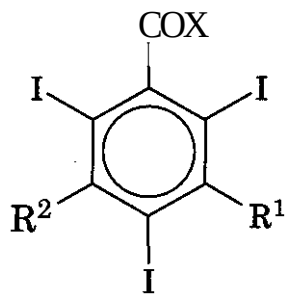
(3)	Type	R^1	R^2
a	CCN	$-CH_2CONHCH_3$	$-NHCOCH_3$
b	CCN	$-CH(CH_2OH)CONHCH_3$	$-NHCOCH_2OCH_3$
c	CCN	$-CH_3$	$-NHCOCH_3$
d	CCN	$-CH_2CH_2OH$	$-NHCOCH_3$



(4)

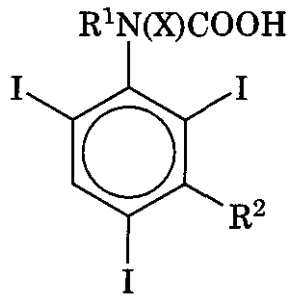
(4)	Type	X	R^1
a	CNH	$-CH_2CH(C_2H_5)-$	$-NH_2$
b	COH	$-CH_2CH(C_2H_5)-$	$-OH$
c	CNH	$-CH_2CH_2-$	$-N(C_2H_5)COCH_3$
d	CNH	$-CH_2CH_2-$	$-N{=}CHN(CH_3)_2$
e	CNH	$-CH_2CH(C_2H_5)-$	$-NHCO(CH_2)_2CH_3$
f	CNH	$-CH_2CH(C_2H_5)-$	$-NCOCH_2CH_2CH_2$
g	CNH	$-CONHCH_2CH(CH_3)-$	$-N{=}C(CH_3)N(CH_2CH_2)_2O$
h	ONH	$-O(CH_2)_2OCH(CH_3)-$	$-N(C_2H_5)COCH_3$
i	ONH	$-O(CH_2)_2OCH_2CH(C_2H_5)-$	$-NHCOCH_3$
j	OCH	$-O(CH_2)_3-$	$-N(CH_2CH_2)_2O$

Table 10.2 (Continued)



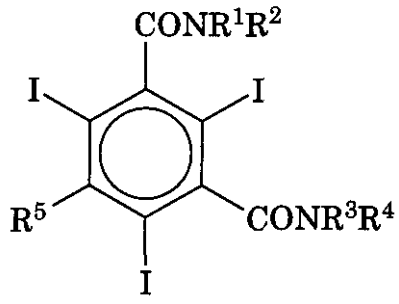
(5)

(5)	Type	X	R^1	R^2
a	CNH	$-N(C_6H_5)CH_2CH_2COOH$	$-NH_2$	-H



(6)

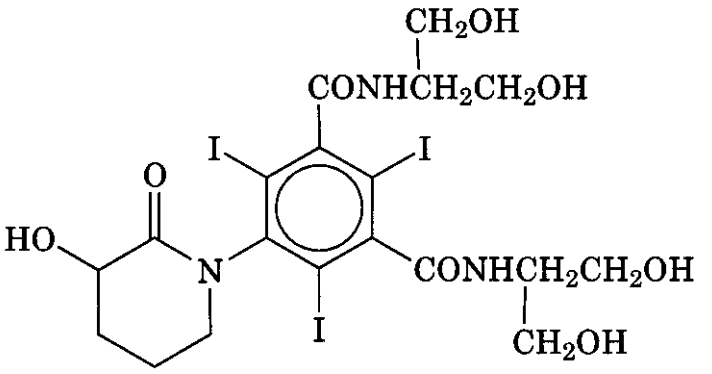
(6)	Type	X	R^1	R^2
a	NNH	$-CH_2CH(CH_3)-$	$-COCH_3$	$-NH_2$
b	NNH	$-CO(CH_2)_3-$	$-CH_3$	$-NH_2$
c	NNH	$-CO(CH_2)_2-$	$-CH_2CH_3$	$-NHCH_3$



(7)

(7)	Type	R^1	R^2	R^3	R^4	R^5
a	CCN	$-CH_2CHOHCH_2OH$	$-CH_3$	$-CH_2CHOHCH_2OH$	$-CH_3$	$-NHCOCH(CH_2OH)_2$
b	CCN	$-CH_2CHOHCH_2OH$	-H	$-CH_2CHOHCH_2OH$	-H	$-N(COCH_3)CH_2CHOHCH_2OH$
c	CCN	$-CH_2CHOHCH_2OH$	-H	$-CH_2CHOHCH_2OH$	-H	$-N(CH_3)COCH_2OH$
d	CCN	$-CH(CH_2OH)_2$	-H	$-CH(CH_2OH)_2$	-H	$-NHCOCH(OH)CH_3$[a]
e	CCN	$-CH_2CHOHCH_2OH$	-H	$-CH_2CHOHCH_2OH$	-H	$-N(COCH_3)CH_2CHOHCH_2OCH_3$
f	CCN	$-CH_2CHOHCH_2OH$	$-CH_3$	$-CH_2CHOHCH_2OH$	-H	$-NHCOCH_2OCH_3$
g	CCC[b]	$-CH_2CH_2OH$	$-(R^1)$	$-CH_2CH_2OH$	$-(R^1)$	$-CON(CH_2CH_2OH)_2$
h	CCC[b]	$-CH_2CHOHCH_2OH$	$-CH_3$	$-CH_2CHOHCH_2OH$	-H	$-CONH_2$
i	CCN	$-CH_2CHOHCH_2OH$	-H	$-CH_2CHOHCH_2OH$	-H	$-N(COCH_2OH)CH_2CH_2OH$
j	CCN	$-CH_2CHOHCH_2OH$	-H	$-CH_2CH_2OH$	-H	$-N(COCH_3)CH_2CHOHCH_2OH$
k	CCN	$-CH_2CHOHCH_2OH$	-H	$-CH_2CHOHCH_2OH$	-H	$-NCOCHOHCH_2CH_2CH_2$[c]

Table 10.2 *(Continued)*



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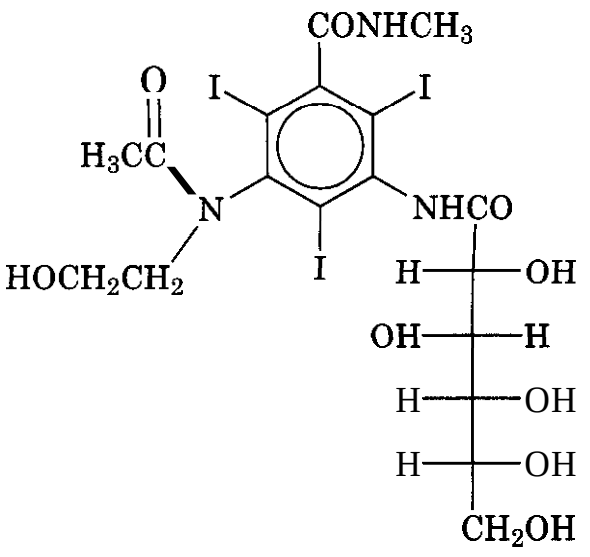

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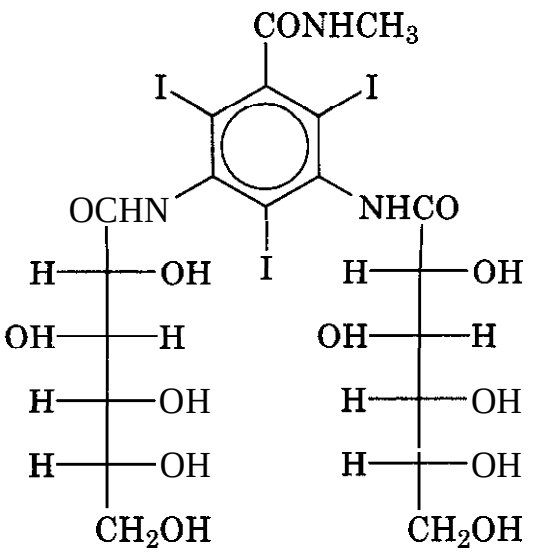

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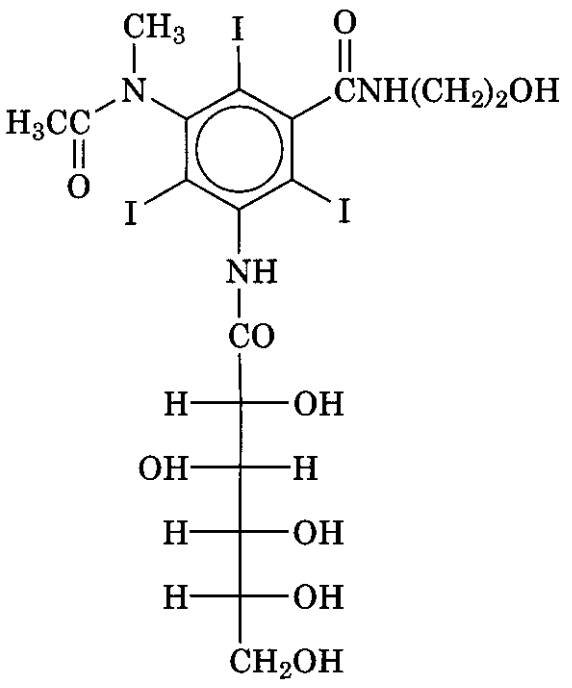

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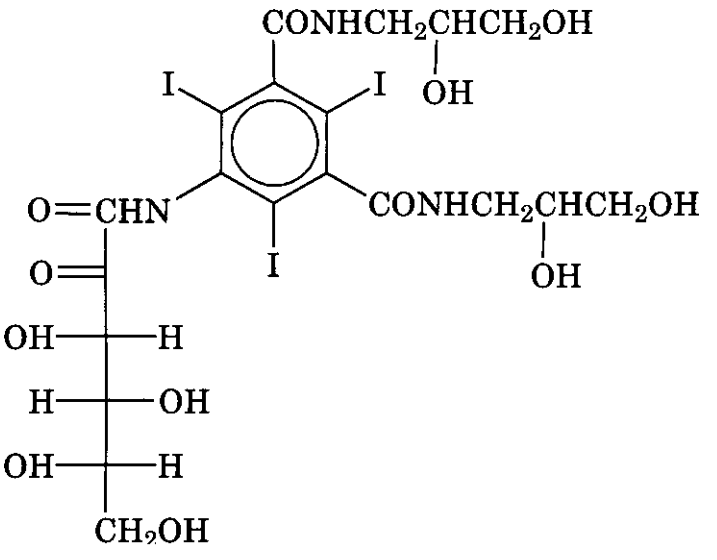

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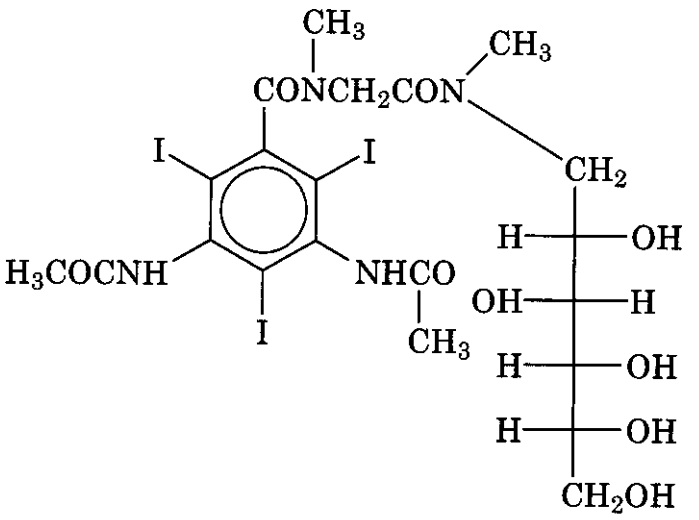

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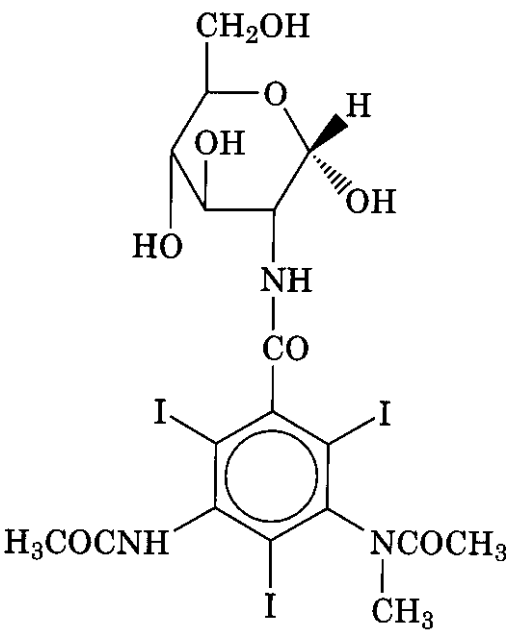

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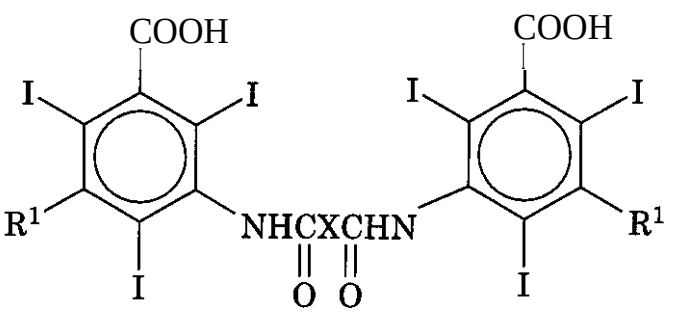
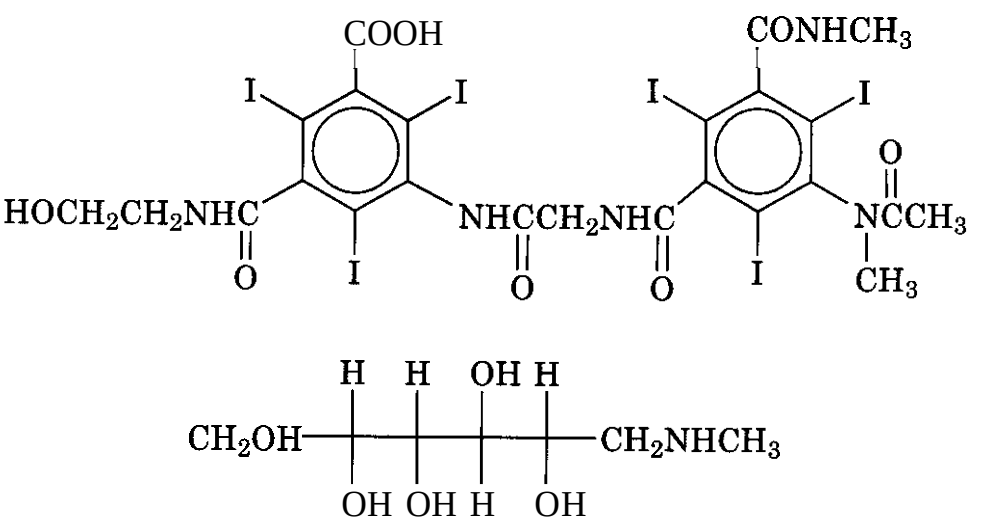


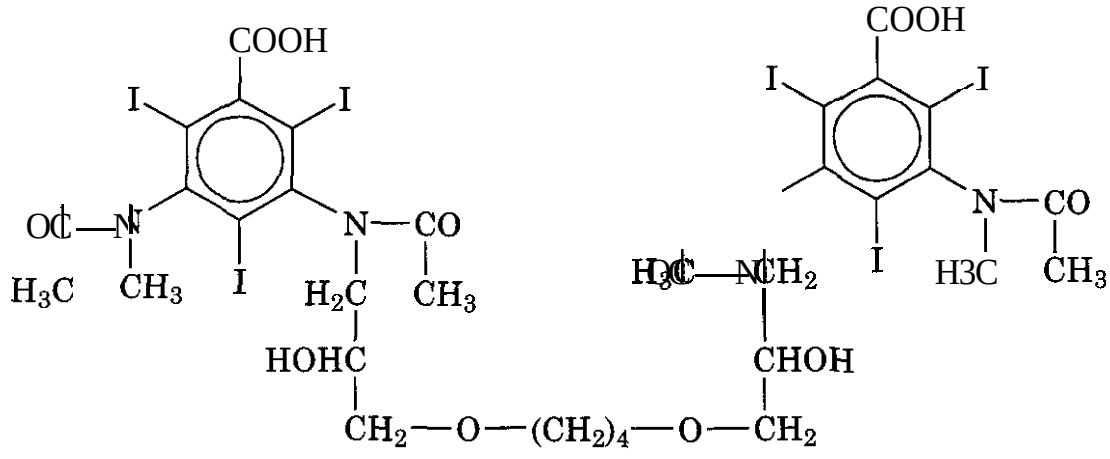
(9)

(9)	Type	X	R^1
a	CNC	$-(CH_2)_4-$	$-CONHCH_3$
b	CNH	$-(CH_2)_4-$	$-H$
c	CNH	$-CH_2(CH_2OCH_2)_4CH_2-$	$-H$
d	CNH	$-CH_2OCH_2-$	$-H$
e	CNH	$-(CH_2)_8-$	$-CONHCH_3$
f	CNN	$-CH_2CH_2SO_2CH_2CH_2-$	$-N(C_2H_5)COCH_3$
g	CNH	$-(CH_2OCH_2)_4-$	$-H$
h	CNH	$-CH_2(CH_2OCH_2)_3CH_2-$	$-H$
i	CNH	$-(CH_2OCH_2)_3-$	$-H$

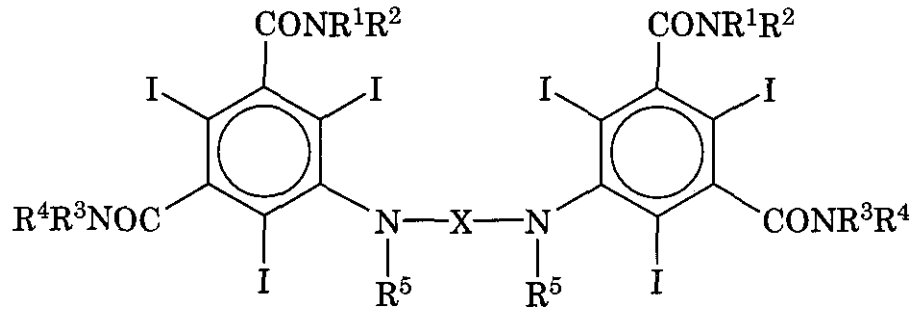


(9j) (Ioxaglate meglumine)

Table 10.2 (Continued)

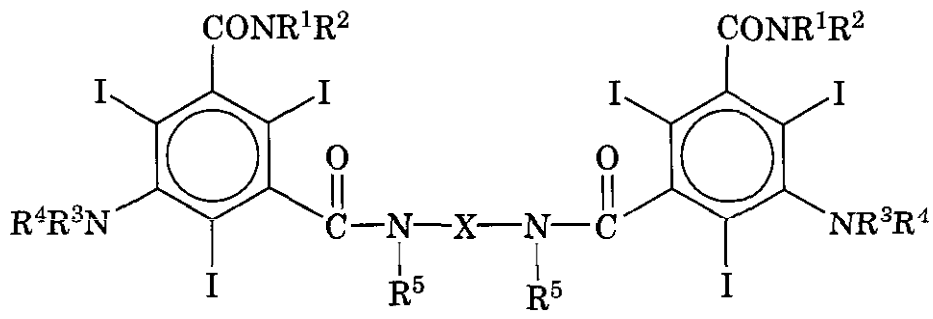


(9k)



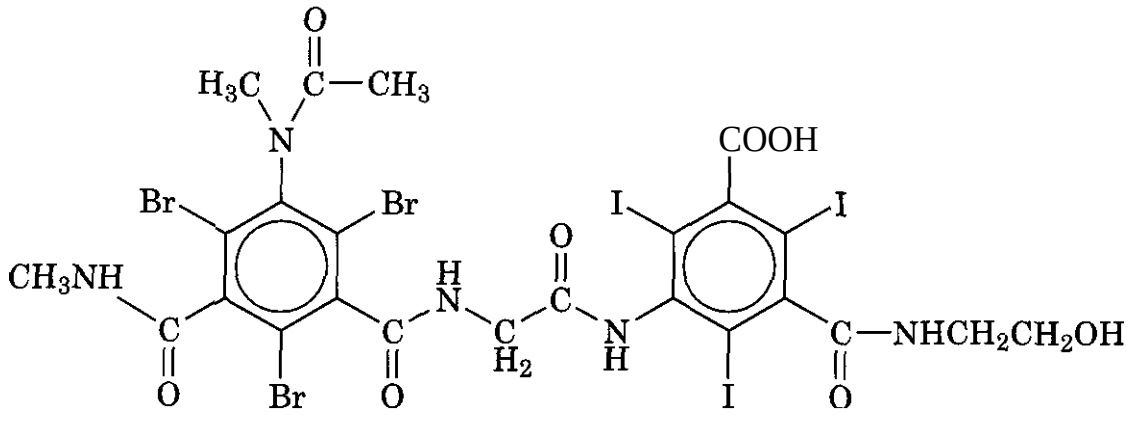
(10)

(10)	Type	X	R^5	R^1	R^2	R^3	R^4
a	CCN	$-COCH_2CO-$	$-CH_2CH_2OH$	$-CH(CH_2OH)_2$	$-H$	$-CH(CH_2OH)_2$	$-H$
b	CCN	$-CH_2CHOHCH_2-$	$-COCH_3$	$-CH_2CHOHCH_2OH$	$-H$	$-CH_2CHOHCH_2OH$	$-H$
c	CCN	$-CO(CH_2)_2S(CH_2)_2CO-$	$-H$	$-CH_2CHOHCH_2OH$	$-CH_3$	$-CH_2CHOHCH_2OH$	$-CH_3$
d	CCN	$-COCH_2CO-$	$-CH_3$	$-CH(CHOH)CH_2OH$ CH_2OH	$-H$	$-CH(CHOH)CH_2OH$ CH_2OH	$-H$



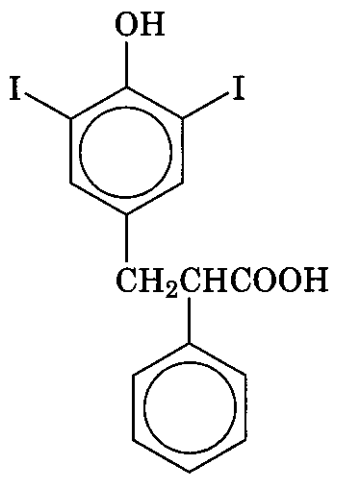
(10′)

(10′)	Type	X	R^5	R^1	R^2	R^3	R^4
e	CNC	$-CH_2CHOHCH_2-$	$-H$	$-CH(CH_2OH)_2$	$-H$	$-COCH(OH)CH_3$	$-H$



(11)

Table 10.2 *(Continued)*



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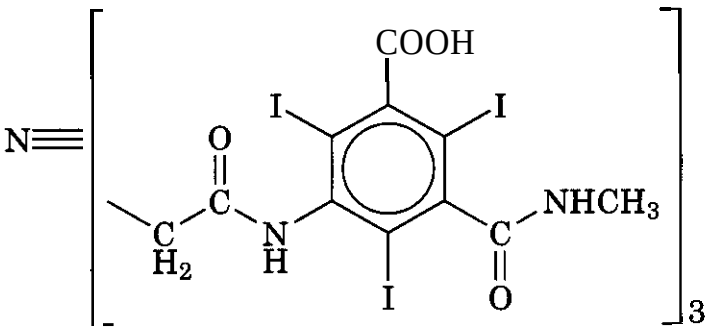

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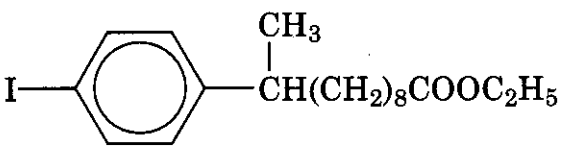

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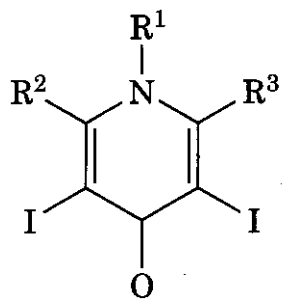
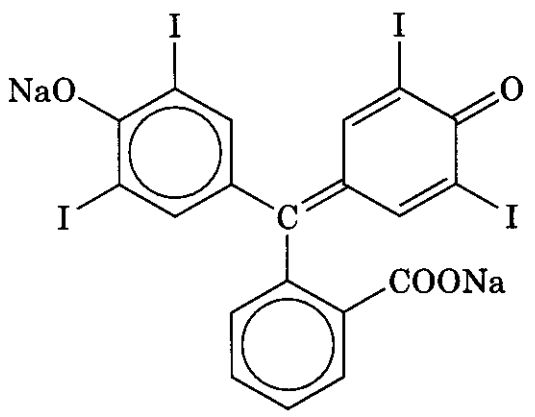


(15)

(15)	R^1	R^2	R^3
a	–H	–H	–H
b	$-CH_2COOH$	–H	–H
c	$-CH_2CHOHCH_2OH$	–H	–H
d	$-CH_2COOC_3H_7$	–H	–H
e	$-CH_3$	–COONa	–COONa



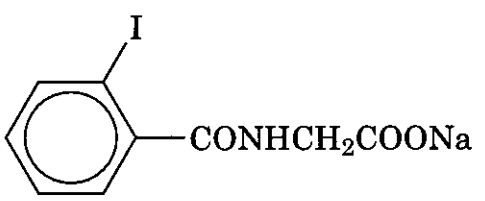
(16)

ICH_2SO_3Na

(17)

I_2CHSO_3Na

(18)



(19)

[a] L-Lactoyl residue;
[b] a 1,3,5-benzenetricarboxamide or trimesic acid amide;
[c] 2-oxo-3-hydroxy-1-piperidinyl;

Table 10.3 Characteristics of Ionic and **Nonionic** Iodine-Containing Contrast Agents

USAN or INN Name	Chemical Name	Mol. wt.	Iodine Content (%)	Melting Point (°C)	LD_{50}, g/kg or I (=g I/kg)	Solubility, g/100 mL H_2O at 20°C	$pK_{a\ H_2O}^{25}$	Ref.
A. Ionic monomers								
Acetrizoate (**2a**) [85–36-9][a]	3-Acetylamido-2,4,6-triiodobenzoic acid			278–283 (*d*)	9.56	94.20	2.08	
	Sodium salt	578.85	65.77		5.51[b]	Freely soluble		
	Meglumine salt		50.62					176
Diatrizoate (**2b**) [737–31-5]	3,5-Bis(acetamino)-2,4,6-triiodobenzoic acid			300	11.0[c]		2.05	
	Sodium salt	635.9	59.87	261–262	11.4[c]; 8.41[b]	60	2.70	233
	Meglumine salt		47.05	189–193 (*d*)	13.81'	89		272,273
Diprotrizoate (**2c**) [129–57-7]	3,5-Dipropionamido-2,4,6-triiodobenzoic acid			>300	0.0118[b]			
	Sodium salt	663.95	57.34			- 50		233
Iodamide (**2d**) [440–58-4]	3-(Acetylamino)-5-[(acetylamino)methyl]-2,4,6-triiodobenzoic acid	627.95	60.63	255–257	108[b]	0.3 (22°C)	1.88	274,275
	Meglumine salt		46.25			>75		
Iotrizoic acid (**2e**) [16024–67-2]	2,4,6-Triiodo-3-[(1-oxo-3,6,9,12,15-pentaoxa-hexadec-1-yl)amino]benzoic acid	673.11	49.89	148–150				276
Ioxotrizoic acid (**2f**) [19863–06-0]	3-(Acetylamino)-5-[(hydroxyacetyl)amino]-2,4,6-triiodobenzoic acid	629.92	60.44					277
Metrizoate (**2g**) [7225–61-8]	3-(Acetylamino)-5-(acetylmethylamino)-2,4,6-triiodobenzoic acid			281–282				278
	Sodium salt	649.93	60.63			86		
Ioglicic acid (**3a**) [49755–67-1]	3-(Acetylamino)-2,4,6-triiodo-5-[[[2-(methylamino)-2-oxoethyllaminol carbonyl]benzoic acid	670.97	65.78	284–285				279

Ioseric acid (**3b**) [51876–99-4]	3-[[[1-(Hydroxymethyl)-2-(methylamino)-2-oxoethyl] amino]-carbonyl]-2,4,6-triiodo-5-[(methoxyacetyl)amino]benzoic acid	731.02	52.12	267–268 (*d*)		(±)-form		279
Iothalamic acid (**3c**) [2276–90-6]	3-(Acetamino)-2,4,6-triiodo-5-[(methylamino) carbonyl]-benzoic acid	613.92	62.01	285 (*d*)		1.74		
	Sodium salt	635.9	59.87		85			280
	Meglumine salt	809.13	47.05					
Ioxitalamic acid (**3d**) [28179–44-4]	3-(Acetylamino)-5[[(2-(hydroxyethyl)amino] carbonyl]-2,4,6-triiodobenzoic acid	643.94	59.12	349				281
Iopanoic acid (**4a**) [96–83-3]	3-Amino-alpha-ethyl-2,4,6-triiodobenzene-propanoic acid	570.94	66.69	155.2–157 (*d,l* form)		Insol.	5.06	260, 282
Iophenoxic acid (**4b**) [96–84-4]	Alpha-Ethyl-3-hydroxy-2,4,6-triiodobenzene-propionic acid	571.92	66.57	143–144			4.66 (pK_1) 6.11 (pK_2)	283, 284
Ioprocemic acid (**4c**) [1456–52-6]	3-(Acetylethylamino)-2,4,6-triiodobenzene-propionicacid	612.97	62.11	166–170				285
Ipodic acid (**4d**) [5587–89-3]	3-[[(Dimethylamino)-methylenel amino]-2,4,6-triiodobenzene-propanoic acid	597.96	63.67	168–169		Insol.	5–5.5	261, 286
	Sodium salt	619.94	61.41	303–304 (*d*)		Freely sol.		
	Calcium salt	1233.99	61.70	298–302		0.10		
Tyropanoate (**4e**) [7246–21-1]	Alpha-Ethyl-2,4,6-triiodo-3-[(1-oxobutyl)-amino]benzene-propanoic acid			172–185.5		Sol.		283
	Sodium salt	663.01	57.42					
Iolidonic acid (**4f**) [21766–53-0]	Alpha-Ethyl-2,4,6-triiodo-3-(2-oxo-1-pyrrolidinyl)benzenepropionic acid	639.01	59.58	179–181			4.89	287
Iomorinic acid (**4g**) [51934–76-0]	2-Methyl-3-[2,4,6-triiodo-3-[1-(4-morpholinyl)-ethylidene)amino]benzoyl]aminopropionic acid	711.08	53.54					288
Iolixanic acid (**4h**) [22730–86-5]	2-[2-[3-(*N*-Ethylacetamido)-2,4,6-triiodo-phenoxy]ethoxy]propionic acid	673.02	56.65	150–151				289

Table 10.3 (*Continued*)

USAN or INN Name	Chemical Name	Mol. wt.	Iodine Content (%)	Melting Point (°C)	LD_{50}, g/kg or I (=g I/kg)	Solubility, g/100 mL H_2O at 20°C	$pK_{a\ H_2O}{}^{25}$	Ref.
Iopronic acid (**4I**) [37723–78-7]	(±)-2-[[2-[3-(Acetylamino)-2,4,6-triiodo-phenoxy]ethoxy]methyl]butanoic acid	673.03	56.57	130	.09[b] 1.95[d]		4.89	290, 291
Iobutoic acid (**4J**) [13445–12-0]	4-[2,4,6-Triiodo-3-(morpholino-carbonyl)-phenoxy]-butyric acid	671.01	56.37	180				292
Iobenzamic acid (**5a**) [3115–05-7]	*N*-(3-Amino-2,4,6-triiodoben-zoyl)-*N*-phenyl-beta-alanine	662.01	57.51	133–134.5	2.87[d]	Insol.	4.38	293, 294
Iocetamic acid (**6a**) [16034–77-8]	3-[Acetyl (3-amino-2,4,6-triiodo-phenyl)amino]-2-methyl-pro-panoic acid	613.96	62.01	224–225; 191–212 (Korver)	0.7[c] 2.21[e]	Insol.	4.89	240, 295
Iomeglamic acid (**6b**) [25827–76-3]	5-[(3-Amino-2,4,6-triiodophenyl)-methylamino]-5-oxo-pentanoic acid	613.96	62.01	169	6.35[d]	Insol.		229, 296–298
Iosumetic acid (**6c**) [37863–70-0]	4-[Ethyl [2,4,6-triiodo-3-(meth-ylamino)phenyl]amino]-4-oxobutanoic acid	627.99	60.62					299, 300
B. Nonionic monomers								
Iobitridol (**7a**) [136949–58-1]	N,N′-Bis-(2,3-dihydroxypropyl)-5-[[3-hydroxy-2-(hydroxy-methyl)-1-oxopropyl]amino]-2,4,6-triiodo-*N,N′*-dimethyl-1,3-benzene-dicarboxamide	835.16	45.58					301
Iohexol (**7b**) [66108–95-0]	5-[Acetyl(2,3-dihydroxypro-pyl)amino]-*N,N′*-bis-(2,3-dihy-droxypropyl)-2,4,6-triiodo-1,3-benzenedicarboxamide	821.15	46.96	174–180	12.1 g I/kg (rats) 24 I[b]	Sol.		193, 302
Iomeprol (**7c**) [78649–41-9]	*N,N′*-Bis(2,3-dihydroxypropyl)-5-[(hydroxy-acetyl)methyl-amino]-2,4,6-triiodo-1,3-ben-zenedicarboxamide	777.09	48.99	283–284				303
Iopamidol (**7d**) [60166–93-0]	(*S*)-*N,N′*-Bis[2-hydroxy-1-(hy-droxymethyl)-ethyl]-5-[(2-hy-droxy-1-oxopropyl)amino]-2,4,6-triiodo-1,3-benzenedicar-boxamide	777.09	48.99	>300 (dec)	11.3 g I/kg (rats) 21 I[b]	Very sol. (*S*)-form	10.79	196, 302, 304, 305

Iopentol (**7e**) [89797–00-2]	5-[Acetyl(2-hydroxy-3-methoxypropyl)amino)-*N,N′*-bis(2,3-dihydroxypropyl)-2,4,6-triiodo-1,3-benzenedicarboxamide	835.17	45.58		10.3g I/kg (rats)	302, 306
Iopromide (**7f**) [73334–07-3]	*N,N′*-Bis-(2,3-dihydroxypropyl)-2,4,6-triiodo-5-(methoxyacetyl)amino]-*N*-methyl-1,3-benzenedicarboxamide	791.12	48.12		10.3 g I/kg (rats)	302, 307
Iosimide (**7g**) [79211–10-2]	*N,N,N′,N′,N″N″*-Hexakis(2-hydroxyethyl)-2,4,6,-triiodo-1,3,5-benzenetricarboxamide	849.2	44.83	132–135		308
Iotriside (7h) [79211–34-0]	(±)-*N,N′*-Bis(2,3-dihydroxy-propyl)-2,4,6-triiodo-*N*-methyl-1,3,5-benzenetricarboxamide	747.07	50.96	202		309
Ioversol (**7i**) [87771–40-2]	*N,N′*-Bis(2,3-dihydroxypropyl)-5-[(hydroxy-acetyl)(2-hydroxy-ethyl)amino-2,4,6-triiodo-1,3-benzene-dicarboxamide	807.12	47.17	186–198	18.3 g I/kg (m. mice) 18.1 g I/kg (f. mice)	310, 311
Ioxilan (**7j**) [107793–72-6]	5-[Acetyl(2,3-dihydroxypropy)-[amino]-*N*-(2,3-dihydroxypropyl)-*N′*-(2-hydroxyethyl)-2,4,6-triiodo-1.3-benzenedicarboxamide	791.12	48.12		22.4 g I/kg (m. rats) 20.1g I/kg (f. rats)	312
3H2-Iopiperidol-A (**7k**)	5-[[Dihydro-5-(hydroxymethyl)-2(3H)-furanylidene]-amino]-*N,N′*-bis[(2-hydroxy)-1-(hydroxymethyl)-ethyl]-2,4,6-triiodo-1,3-benzenedicarboxamide			208–210(softens) 258–262(froths) 285–289(dec)		313, 314
Ioglucol (**8a**) [63941–73-1]	*N*-[3-[Acetyl(2-hydroxyethyl)-amino]-2,4,6-triiodo-5-[(methylamino)carbonyl]phenyl]-D-gluconamide	807.12	42.34	165–174		315
Ioglucomide (8b) [63941–74-2]	*N,N′*-[2,4,6-Triiodo-5-[(methyl-amino)carbonyl]] 1,3-phenylene]bis-D-gluconamide	899.17	42.34	144–148		315

Table 10.3 (Continued)

USAN or INN Name	Chemical Name	Mol. wt.	Iodine Content (%)	Melting Point (°C)	LD_{50}, g/kg or I (=g I/kg)	Solubility, g/100 mL H_2O at 20°C	$pK_{a\ H_2O}^{25}$	Ref.
Ioglunide (**8c**) [56562–79-9]	*N*-[3-(Acetylmethylamino)-5-[[(2-hydroxy-ethyl)amino]carbonyl]-2′,4′,6′-triiodophenyl-D-gluconamide	807.12	47.29					316
Iogulamide (**8d**) [75751–89-2]	*N*,*N*′-Bis(2,3-dihydroxypropyl)-5-[L-xylo-2-hexulosonoyl)&hyphen amino]-2,3,4-tri-iodo-1,3-benzenedicarboxamide	881.16	43.21					317
Iosarcol (**8e**) [97702–82-4]	1-[[[[3,5-Bis(acetylamino)-2,4,6-triiodobenzoyl]-methylamino]-acetyl]-methylamino]1-deoxy-D-glucitol	862.19	44.16					318
Metrizamide (**8f**) [31112–62-6]	2-[[3-(Acetylamino)-5-(acetyl-methylamino)-2,4,6-triiodo-benzoyl]-amino]-2-deoxy-D-glucose	789.10	48.20	230-240 (*d*)	14 I[b]	17.5[l]	>80	183, 184
C. Ionic dimers (bis compounds)								
Iocarmic acid (**9a**) [10397–75-8]	3,3′-[(1,6-dioxo-1,6-hexanediyl)-diimino]bis-[2,4,6-triiodo-5-[(methylamino)carbonyl]-benzoic acid[l]	1253.87	60.72	302 (*d*)				281, 319
	Meglumine salt	1644.31	46.31		14–17.5[b]	65 (25°C)		
Iodipamide (**9b**) [606–17-7]	3,3′-[(1,6-Dioxo-1,6-hexanediyl)-diimino]bis-[2,4,6-triiodobenzoic acid[l]	1139.77	66.80	306–308 (*d*)		Insol.	1.74 (pK_1)	256, 262
	Meglumine salt	1530.2	49.76		34[c]	Sol.	2.76 (pK_2)	320,321
Iodoxamic acid (**9c**) [31127–82-9]	3,3′-[(1,16-Dioxo-4,7,10,13-tetraoxahexadecane-1,16-diyl)-diimino]bis[2,4,6-triiodobenzoic acid[l]	1287.93	59.12	125	52[b]	0.0364(20°C)	1.8(pK_1)	189,263
	Meglumine salt	1678.36	45.37			14 (20°C, pH 4.70)	2.8 (pK_2)	

Ioglycamic acid (**9d**) [2618–25-9]	3,3′-[Oxybis[(1-oxo-2,1-ethanediyl)imino]] bis[2,4,6-triiodobenzoic acid]	1127.72	67.55	222			1.67 (pK_1)	256, 320
	Meglumine salt			281 (*d*) (three crystalline modifications)		Sol.	2.68 (pK_2)	
Iosefamic acid (**9e**) [5591–33-3]	3,3′-[(1,10-Dioxo-1,10-decanediyl)diimino]bis-[2,4,6-triiodo-5-[(methylamino)carbonyll-benzoic acidl	1309.98	58.12	279	131[b]			319, 322
Iosulamide (**9f**) [63534–64-5]	3,3′-[Sulfonylbis[(1-oxo-3,1-propanediyl)-imino]]bis[(5-acetyl-ethylamino)-2,4,6-triiodo-benzoic acidl	1374.04	55.41	249–254				
	Meglumine salt	1569.26	48.52		11.5[c]			323
Iotetric acid (**9g**) [60019–19-4]	3,3′-[(1,14-Dioxo-3,6,9,12-tetraoxatetra-decane-1,14-diyl)-diimino]bis[2,4,6-triiodo-benzoic acidl	1259.88	60.44					
Iotranic acid (**9h**) [26887–04-7]	3,3′-[Oxy-bis(ethyleneoxy-ethylenecarbonylimino)]bis-[2,4,6-triiodobenzoic acid]	1234.88	61.21	155				188
Iotroxic acid (**9i**) [51022–74-3]	3,3′-[Oxybis[2,1-ethanediyloxy-(1-oxo-2,1-ethanediyl)imino]]-bis[2,4,6-triiodobenzoic acidl	1215.82	62.63					324
Ioxaglic acid (**9j**) [59017–64-0]	3-[[[[3-(Acetylmethylamino)-2,4,6-triiodo-5-[(methylamino)-carbonyl]benzoyl]amino] acetyl]amino]-5-[[(2-hydroethyl)amino] carbonyl]-2,4,6-triiodobenzoic acid	1268.89	60.01					
	Meglumine salt	1464.11	52.01		121[b]			199
	Sodium-meglumine salt							
Iozomic acid (**9k**) [31598–07-9]	3,3′-[1,4-Butanediylbis[oxy(2-hydroxy-3,1-propanediyl)(acetylamino)]]bis[5-(acetyl-methyl-amino)-2,4,6-triiodobenzoic acid	1458.14	52.22					325,326

Table 10.3 (Continued)

USAN or INN Name	Chemical Name	Mol. wt.	Iodine Content (%)	Melting Point (°C)	$LD_{,,}$ g/kg or I (=g I/kg)	Solubility, g/100 mL H_2O at 20°C	$pK_{a\ H_2O}^{25}$	Ref.
D. Nonionic bis compounds								
Iodecol (**10a**) [81045–33-2]	5,5′-[1,3-Dioxo-1,3-propanediyl)-bis[2-hydroxy-ethyl)imino]]-bis[*N,N′*-bis[2-hydroxy-1-(hydroxymethyl)ethyl]-2,4,6-triiodo-1,3-benzenedicarboxamide]	1566.20	48.62					259
Iodixanol (**10b**) [92339–11-2]	5,5′-[(2-Hydroxy-1,3-propanediyl)bis(acetyl-imino)]-bis[*N,N′*-bis(2,3-dihydroxypropyl)-2,4,6-triiodo-1,3-benzenedicarboxamide]	1550.2	49.12	240-250				198
Iotasul (**10c**) [71767–13-0]	5,5′-[Thiobis[(1-oxo-3,1-propanediyl)imino]]bis[*N,N′*-bis(2,3-dihydroxypropyl)-2,4,6-triiodo-*N,N′*-dimethyl-1,3-benzene-dicarboxamide]	1608.35	47.34	240-260				327
Iotrolan (**10d**) [79770–24-4]	5,5′-[(1,3-Dioxo-1,3-propanediyl)-bis(methyl-imino)]bis[*N,N′*-bis[2,3-dihydroxy-1-(hydroxyethyl)propyl]-2,4,6-triiodo-1,3-benzene-dicarboxamide]	1626.25	46.82		26 g/kg (mice) 12.7 g/kg (rats)			197, 328
Iofratol (**10′e**) [141660–63-1]	*N,N′*-(2-Hydroxy-1,3-propanediyl)bis[*N′*-[2-hydroxy-1-(hydromethyl)ethyl]-5-[(2-hydroxy-1-oxopropyl)amino]-2,4,6-triiodo-1,3-benzenedicarboxamide], [*S*-(*R**,*R**)]							329
E. Miscellaneous								
Ioxabrolic acid (**11**) [96191–65-0]	*N*-(2-Hydroxyethyl)-2,4,6,-triiodo-5-[2-[2,4,6-tribromo-3-(*N*-methylacetamido)-5-(methyl-carbamoyl)benziamido]acetylamino-1,3-benzenecarboxamide]	1127.88	Br: 21.25 I: 33.75					330

Tris(iothalamic acid) (**12**)	5,5′,5″-(Nitrilotriacetyltriimino)-tris (2,4,6-triiodo-*N*-methyl-isophthalamic acid)		61.67					331
Iodoalphionic acid (13)[577–91-3]	3-(4-Hydroxy-3,5-diiodophenyl)-2-phenyl-propionic acid	494.07	51.38	157–162(d)		~ 1.5		332
	Disodium salt			>230				
Iophendylate (**14**) [99–79-6]	Ethyl 10-(*p*-iodophenyl) undecylate	416.34	30.48	Bp: 196–198		Slightly sol.		333–335
Iopydone (**15a**) [5579–93-1]	3,5-Diiodo-4(1*H*)pyridinone	346.88	73.17	321 (*d*)		Insol.		336
Iodopyracet (**15b**) [300–37-8]	3,5-Diiodo-4-oxo-(4*H*)-pyridine acetic acid	388.92	62.68	245–249	6.3[b]; 3.2 I[b]		2.15	213, 337
	2,2′-Iminodiethanol(1:1) salt		49.76	155–157	2 (i.v. in dogs)	60		
Iopydol (**15c**) [5579–92-0]	1-(2,3-Dihydroxypropyl)-3,5-diiodo-4(1*H*)-pyridinone	420.96	60.29	161				338
Propylidone (**15d**) [587–61-1]	3,5-Diiodo-4-oxo-1-(4*H*)-pyridineacetic acid, propyl ester	446.99	56.78	186–187	0.3[b]	0.014 (15°)		339, 340
Sodium iodomethamate (Iodoxyl) (**15e**) [519–26-6]	1,4-Dihydro-3,5-diiodo-1-methyl-4-oxo-2,6- pyridinedicarboxylic acid, disodium salt	448.94	51.5	174 (*d*)	4.6; 2 I[b]	Freely sol.		213, 336
Iopax	5-Iodo-2-oxo-1(2*H*)-pyridine acetic acid, sodium salt		42.16		8[c]	Very sol.		341, 342

[a]CAS Registry Number;
[b]i.v. in mice;
[c]i.v. in rats;
[d]oral in mice;
"oral in rats.

their Chemical Abstracts Service (CAS) Registry Numbers are listed in square brackets beneath each name.

6.2 Synthesis

The synthesis of iodinated ionic and nonionic contrast agents follows a general pattern, which includes (1) the selection and synthesis of an intermediate containing the ring nucleus, (2) introduction of an activating group, (3) iodination, and (4) acylation.

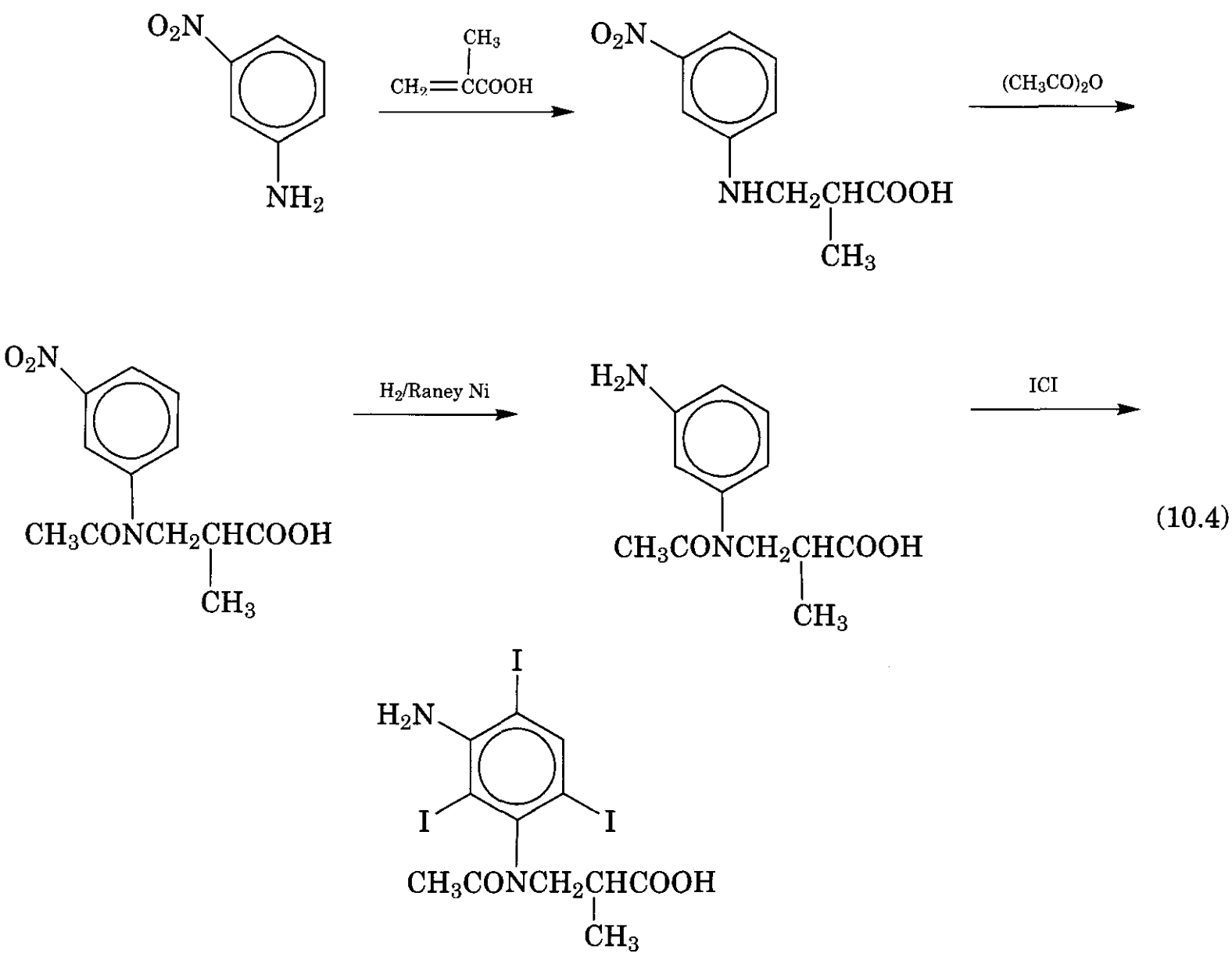
The compound usually selected as the starting material is a substituted benzoic, isophthalic, isophthalamic acid, or one of their derivatives (227). The activating group is introduced as a nitro group followed by reduction to an amino group before iodination. Reduction of the nitro group is effected by catalytic reduction using 5–10% palladium on charcoal or Raney nickel as catalyst (228,229). The latter gives a higher yield and a cleaner product than those of other reduction methods. Ammonium or sodium sulfide may also be used as a reductant (186, 230–232). The amino group can also be diazotized to form a phenolic hydroxyl group.

A vinyl C-I bond is more stable than an alkyl C-I bond. The phenyl ring, in the presence of an activating group, can accept three iodine atoms upon iodination with iodine monochloride (233). Iodination is usually carried out with iodine monochloride in dilute hydrochloric acid or dilute acetic acid or in potassium chloride solution (180, 233, 234). Acetylation of the amino group changes the electron density of the ring and locks the carbon-iodine bond in place (201, 227). The substitution also increases the solubility and reduces the toxicity of the iodinated product. Acetylation may be effected by using ketene in the presence of sulfuric acid (232). A general method of acylation is the dissolution of the amine in dimethylformamide or dimethylacetamide (235), followed by addition of the appropriate acyl chloride.

More synthetic steps and more different intermediates are required to complete the synthesis of nonionic contrast agents than that of ionic contrast agents. The commonly used intermediates for introducing (poly)hydroxyalkyl groups to the triiodophenyl ring in a nonionic contrast molecule are amino-alcohols, epihalohydrins, 1,3-dihydroxy-2-aminopropane (serinal) (236), 3-(N-2-hydroxy-ethyl)amino-1,2-propanediol (237), and so forth. Other reagents, such as 1,1,2-trichloroethane (238) and acetoxyacetyl chloride (238), may also be needed for the synthesis. Acetoxyacetyl chloride is a protective reagent for the hydroxyl groups. Any of the intermediates required for the synthesis of nonionic contrast agents would have to be prepared, purified, and recovered for reuse if necessary. Felder (196) remarked on the complexity of the preparation of iopamidol that "Methods of analysis were developed with specifications for 35 raw materials, five intermediate products, and the final product, for a total of 289 single analytical tests."

In the following sections, selected examples of synthesis for ionic and nonionic, and monomeric and dimeric contrast agents are given to illustrate the general synthetic approach. The synthesis of an ionic monomer iocetamic acid (6a), a triiodoanilide of the NNH type, used for oral cholecystography (240), is shown in Equation 10.4.

The synthesis of an ionic contrast agent is almost complete at this stage. The product is crystalline and can be readily isolated. The crude product can be purified by precipitation from a salt solution with acid or by recrystallization from solvent (201). In the synthesis of nonionic contrast agents, additional synthetic steps are required to attach the (poly)hydroxyalkyl substituent groups to the ring. The presence of these groups in the molecule increases reactivity and water solubility and also makes the product isolation from an aqueous solution difficult. If the product is amorphous and cannot be crystallized, its isolation may require flash evaporation or vacuum distillation. To concentrate and purify iopamidol from aqueous solution, permeable filtration membranes are used in special filtration procedures (241). Also, electrolytes introduced in the intermediate synthesis should be removed by desalination or ion exchange. Deionization and purification of the radiopaque can be achieved by column chromatography by use of silanized silica gel for adsorption, followed by washing with water until free of electrolyte, and desorption with dilute organic solvent (242). For example, crude ioversol can be ei-



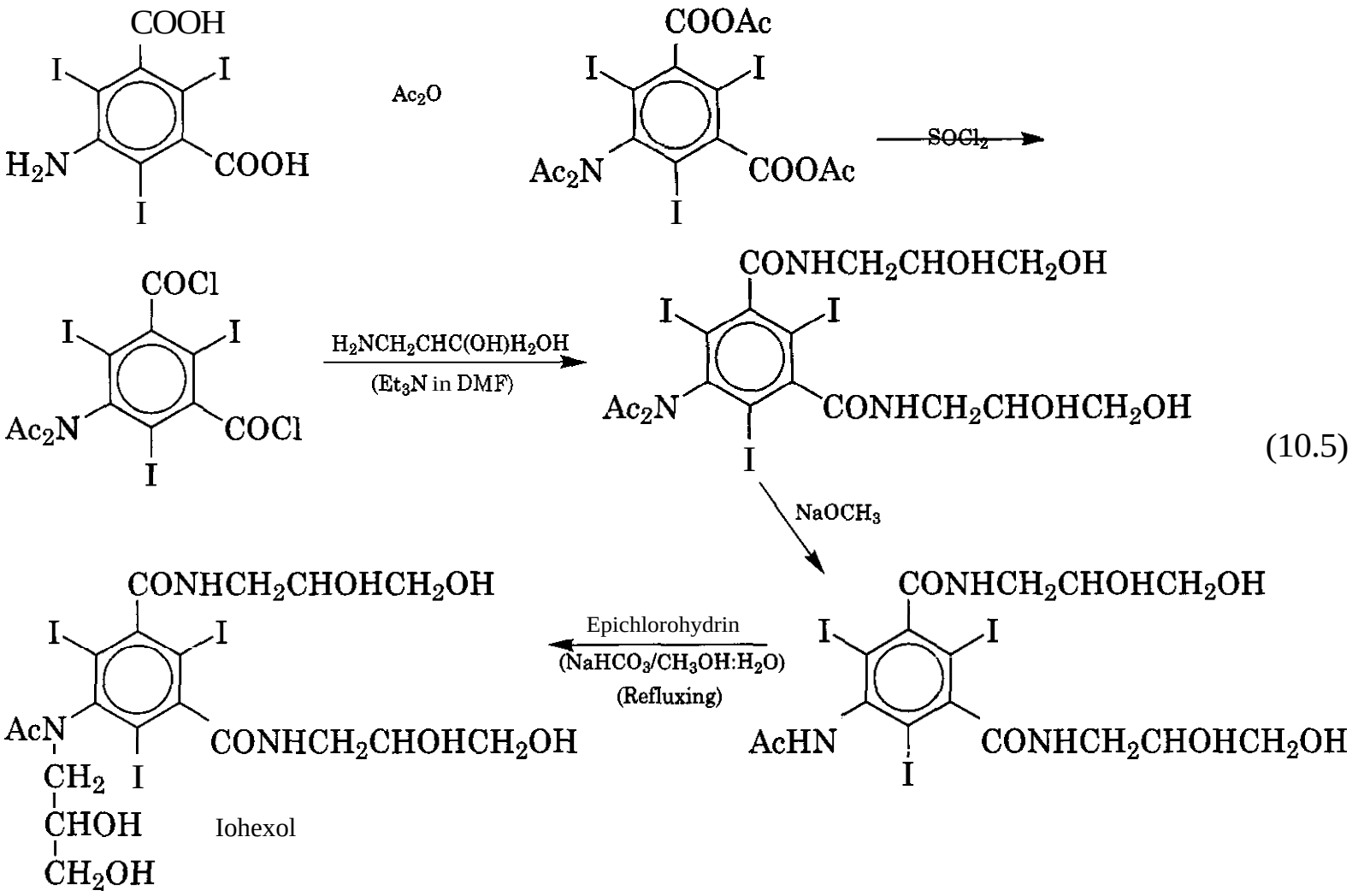
(10.4)

Iocetamic acid

ther purified by reversed-phase liquid chromatography (243) or purified continuously with a bed of mixed ion-exchange resin contained by selectively permeable membranes with an electric potential imposed across the membrane (244). Recrystallization from a butanol mixture is another way of purifying iopamidol to pharmacopoeia standard (245).

One of the methods for synthesis of iohexol (**7b**), a nonionic contrast agent, is shown in equation (10.5) (**246**). Nonionic contrast media are more costly to manufacture than are ionic constrast media. The cost of nonionic contrast media is determined by the number of synthetic steps, overall yield, cost of the intermediates, difficulties in isolation and purification, and other associated manipulations. Many alternate paths of synthesis and purification of nonionic contrast agents have been reported. For example, the nonionic contrast agent ioxilan (**7j**) can alternately be prepared from the ionic contrast agent ioxitalamic acid (**3d**) by reaction with epihalohydrin, to selectively produce the N-hydroxyalkylated product (202, 247). Iohexol **can** be prepared from the triacetyl derivative of **5-amino-2,4,6-tri**iodoisophthaldiamide by N-allylation with **al**lyl chloride or allylamine, followed by oxidation and hydrolysis (**i.e.,** deprotection), to give the product (**248**). The cost of manufacture can be reduced by using a minimal excess of an expensive reagent (acetoxyacetyl chloride) or by shortening the purification process (239). Selective hydrolysis of the protected product in ioversol synthesis can simplify the purification procedures (249). Methods for iodine recovery from manufacturing wastes of **iodin**ated contrast media may also contribute to the reduction of the manufacturing cost (250,251).

Novel reactions can also be used to prepare nonionic contrast media (252). For example, iomeprol in alkaline solution undergoes the



Iohexol (10.5)

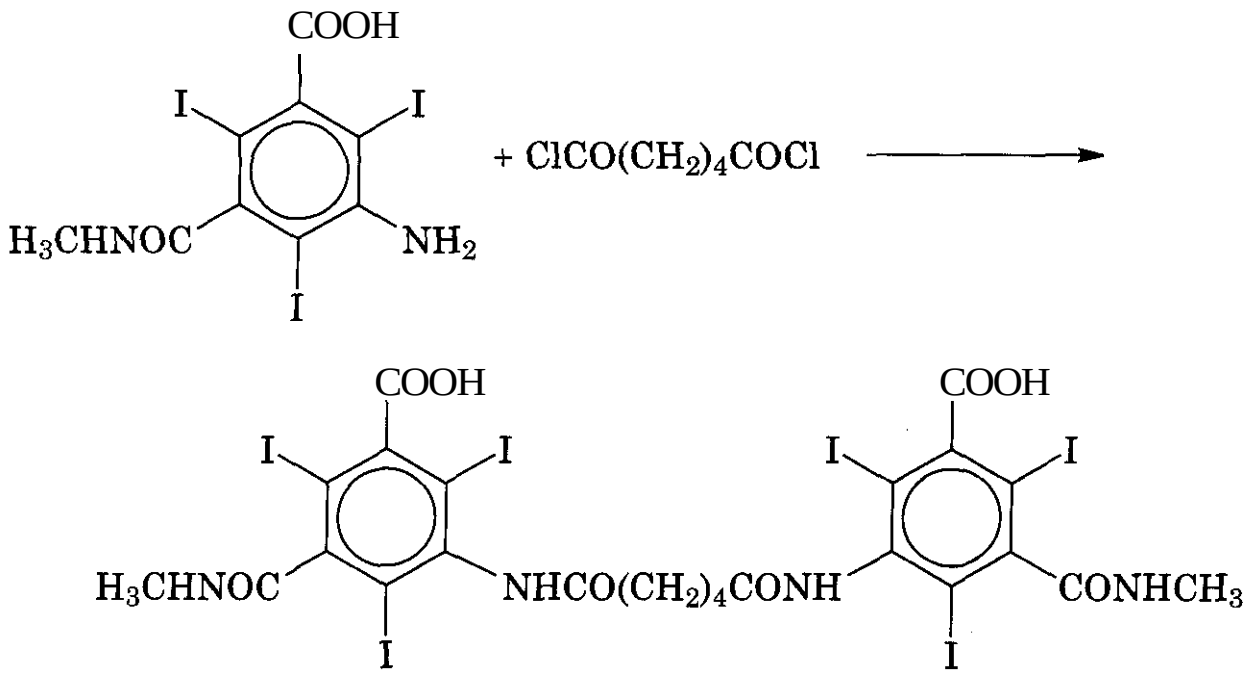
Smiles rearrangement, an *intramolcular nu*cleophilic aromatic substitution, in which the substituent 5-[(hydroxyacetyl)methylamino] group is rearranged to form the 5-[methylaminocarbonylmethoxy] group. This reaction uncouples and replaces the N—C bond with the O—C bond between the substituent and the ring intramolecularly (253). The rearrangement is reversible, with sodium hydroxide or sodium methoxide catalyzing the forward reaction. Ioversol, iopamidol, and a dimer also undergo the Smiles rearrangement. The reaction is useful for preparing nonionic iodinated radiopaques that cannot be obtained by conventional synthetic methods, such as by converting from a CCO type to a CCN type molecule (252).

Many side reactions can occur during the alkylation of an intermediate containing many nucleophilic sites. Bjoersvich et al. (254) studied the influence of various cations on the N/O regioselectivity in the N-alkylation of acetamidotriiodoisophthalamide derivatives with 3-chloro-1-methoxy-2-propanol and found that the Ca^{2+} ion gives the best selectivity and the highest yield of the desired nonionic product. A recent patent showed that the presence of $Ca(OH)_2$ can selectively influence the hydrolysis of the protected groups (255).

Hexaiodinated bis compounds are formed using an alkylene bridge or a substituted alkylene bridge through coupler groups, such as amide (—CO—NH—) or reversed amide (—NH—CO—) or mixed amide and reversed amide, to link two triiodinated monomers. The bis compounds with amide-linked bridges are synthesized by condensing two molecules of a triiodinated monomer containing a free amino group with one molecule of a dicarboxylic acid dichloride (189, 235, 256). The bis compounds with reversed amide groups are prepared by condensing two molecules of substituted triiodinated isophthalic acid monochloride with an (un)substituted alkylene diamine (201). Bis compounds (e.g., ioxaglate) containing mixed linkages with one amide and one reversed amide group in the molecule are asymmetric (199, 257), whose synthesis is more involved than that of compounds with symmetric anchoring groups. The synthesis of an amide-linked ionic bis compound iocarmic acid (9a) is shown in Equation 10.6.

The synthesis of nonionic bis compounds is analogous to that of ionic compounds, except

2



(10.6)

Iocarmic acid

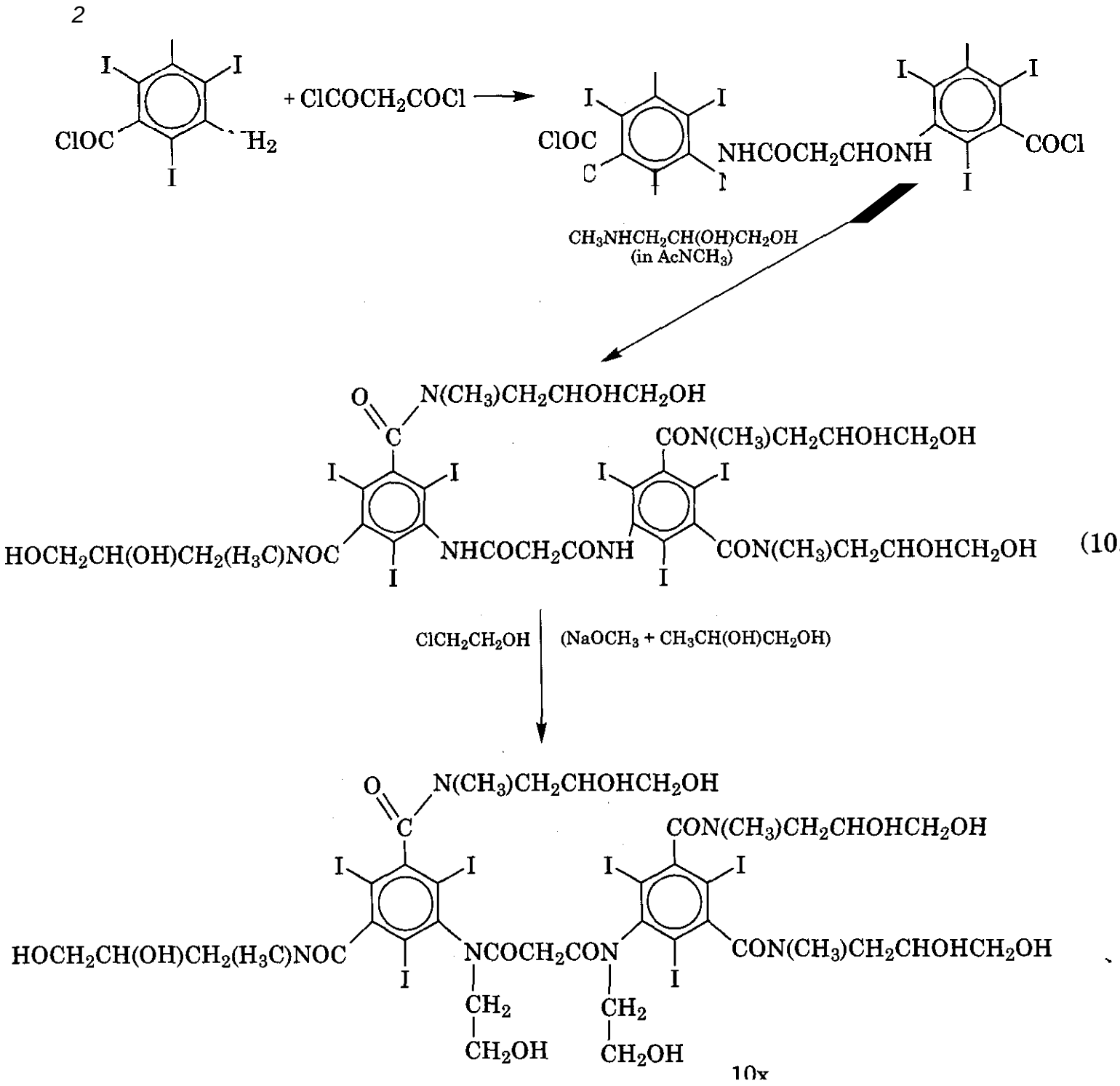
that provisions should be made for the subsequent introduction of the polyhydroxyalkyl groups (258). An example taken from the patent literature (259) is shown in Equation 10.7.

6.2.1 **Isomerism.** Iodinated radiopaque compounds that contain a chiral center or chiral centers form optical isomers or diastereoisomers. The presence of an asymmetric carbon atom in iopanoic acid (220), iophenoxic acid, or iodoalphionic acid (225), for example, leads to the formation of d and *l* forms. In iocetamic acid, free rotation around the axis connecting the iodinated phenyl nucleus with the tertiary nitrogen atom in an alkylamido substituent is impeded by the bulky ortho-substituted iodine atoms, creating d and *l* forms (192). The molecule also contains an asymmetric carbon atom that gives rise to d' and I' forms. The racemates contain *d,d'* and *l,l'* as well as *d,l'* and *d',l* forms, which are mutually diastereoisomeric and have different melting points and solubilities. Mixtures of diastereoisomers exhibit a wide melting range over many degrees.

Besides diastereoisomerism, iodinated radiopaques may show polymorphism; crystals formed under different conditions show identical infrared absorption bands but with different intensities (262,263).

Nonionic contrast media have a multiplicity of optical and rotational isomers and diastereoisomers (201, 261, 264–267). Optical isomerism is derived from the chiral centers in polyhydroxyalkyl groups, and geometric isomers from the restricted rotation of the pendant substituents attributed to steric hindrance about (1) the amide (CO—N) bond, (2) the aryl-nitrogen bond in N-substituted acetanilides, and (3) the aryl-carbon bond in benzamides (266). Nonionic contrast agents, such as iopamidol, iohexol, iopentol, ioversol, ioxilan, and iotrolan, exist in aqueous solution as mixtures of isomers. Iohexol exists in two isomers in a ratio of 1:3 (*endo/exo*) separable by HPLC, and the exo-iohexol has a longer retention time than that of the *endo*-iohexol (267). Iopentol exists in several diasteromers that may not be readily resolvable and identifiable by available analytical techniques (266). Ioversol has four apparent rotamers that can be resolved by reversed-phase HPLC chromatography and confirmed as acetonide derivatives by high resolution NMR spectroscopy (264). HPLC and NMR spectroscopy show that the rotational isomers of iohexol and of ioxilan are interconvertible after equilibration (265).

The rotational isomers exist in cis and trans, anti and syn, or *endo* and exo forms. The cis and trans forms arise from slow rotation of the side-chain carbonyl groups, hindered by adjacent ortho-substituted iodine atoms in the ring, resulting in the two carbonyl groups aligned either parallel or antiparallel to each other (268,269). The anti and syn forms refer to the position of N-methyl or N-alkyl group relative to that of adjacent carbonyl oxygen (266). The *endo* and exo forms refer to the po-



(10.7)

sition of the amide group or the N-acetyl's methyl group with reference to the plane of triiodophenyl ring. The various geometric isomers will coalesce into one form at higher temperature **(265).** The dimer or bis compound iodixanol has three geometric isomers: *exo*-exo, *exo-endo*, and endo-endo forms that can be adjusted to elute as a single peak by HPLC for the purpose of quantitation **(270).A** multiplicity of isomeric forms in nonionic contrast agents is necessary for high water solubility because it increases the energy barrier of desolvation and slows down the process of crystallization and precipitation. From the viewpoint of drug design, a higher water solubility minimizes the binding to biomacromolecules, decreasing the toxicity and increasing the biological tolerance. It is an attribute that should be custom designed into any improved nonionic contrast molecules.

6.2.2 Potential Radiopaques. A large number of organic iodine compounds synthesized in search of improved contrast agents are described in the patent literature; limitation of space does not allow **a** full tabulation of these compounds. Because information concerning them may prove to be of value in the design and synthesis of new potential contrast agents, this chapter lists only the potential nonionic radiopaques, where the main current research effort is centered. Summaries of research on ionic contrast media may be found in early references **(211,271).**

Table 10.4 lists potential nonionic contrast agents containing one triiodinated 1,3-benzenedicarboxamide nucleus and variations. Table 10.5 lists hexaiodinated nonionic compounds containing two such rings per molecule. Tables 10.6–10.9 contain examples of polymers and other derivatives that have a different iodine-carrying moiety. Physicochemical information, toxicity, and proposed use are given when available. Contrast media are used in large quantities in medical diagnostic radiology. The nonionic agents generally surpass ionic ones in offering higher acceptance and safety to the patients.

6.3 Structure-Activity Relationship

With few exceptions, organic iodine compounds for X-ray diagnostic use contain an iodinated benzene ring or an iodinated pyridone nucleus. For reasons of low toxicity and high biotolerance, modern contrast agents are variations of structures based on the triiodophenyl moiety as the iodine carrier. According to Hoey and Smith (180, 201), the desirable properties of intravascular contrast agents should include the following:

1. Opacity to X-rays.
2. High water solubility at high iodine content.
3. Chemical stability.
4. Biological safety.

Intravascular contrast agents should have low viscosity and low osmolality as close as possible to those of body fluids, to improve tolerance and minimize adverse reactions. Contrast agents intended for oral cholecystography should possess hydrophilic and lipophilic properties, so that the compound can be orally absorbed, should be excreted from the liver and bile ducts in sufficient amount to provide radiopacity, and should have no adverse side effects. Because each of these properties is associated with certain molecular features and not all of them are structurally compatible, the best radiopaques represent a compromise of a maximum of these desirable qualities.

In the following sections, relations between structure-dependent properties and characteristics of radiopaques are given.

6.3.1 Substituent Structures. The basic structure in an iodinated contrast agent, the triiodinated phenyl ring, is lipophilic. The lipophilicity can be partially or completely modified by the substituent groups, shown as R, X, and Y in **(1)**.The carboxylic acid moiety in the R group is responsible for the water solubility and ionic property of ionic contrast media. The X and Y substituent groups, containing acylamino, substituted acetamido, substituted carbamoyl, oxo, carbonyl, hydroxy, methoxy, or ether linkages, determine or modify the physicochemical properties (such as water solubility and hydrophilicity) and the pharmacological properties (such as biotolerance, toxicity, distribution, excretion of the molecule). For example, N-alkylation increases the distribution of the contrast agent in the liver as compared to the corresponding nonalkylated compound (263,400). Asymmetry in substitution, such as replacement of an acetamido group with an N-methylcarbamoyl group, can increase the water solubility of iothalamate compared to that of diatrizoate (9). Many ionic contrast molecules show distinct hydrophilic and lipophilic regions, and only a few have hydroxyl groups. The nonionic contrast molecules have a large number of hydroxyl groups, masking the lipophilic regions of the molecule to achieve high water solubility.

The ionic charge of the carboxyl group leads to the high osmolality of ionic contrast media and is the cause of neurotoxicity and vascular pain in arteriography (181,182). Metrizamide, the first-generation nonionic contrast agent, induced no adverse reactions when first introduced in X-ray diagnosis. This confirmed Almén's theory (181)that nonionic radiopaques are safer and better tolerated than ionic ones and led to the rapid development of nonionic radiopaques in the 1980s, which has continued to the present. The use of nonionic contrast media has been beneficial to high risk patients. The compounds listed in Table 10.3 are second-generation monomeric and dimeric contrast media, consisting mainly of derivatives of 5-amino-1,3-benzenedicarboxamide or 5-amino-isophthalamide. These compounds have no ionizable carboxyl groups and rely on the presence of 4–6 hydroxyl groups per monomer molecule or 8–12 hydroxyl groups per dimer or bis compound mol-

Table 10.4 Potential Nonionic Monomeric Radiopaques

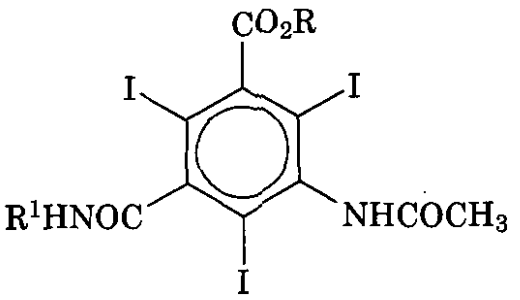
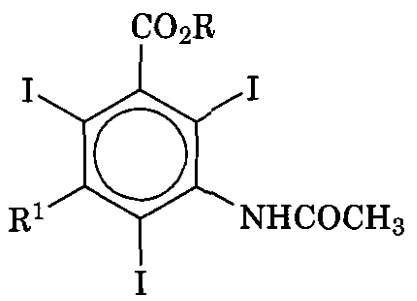
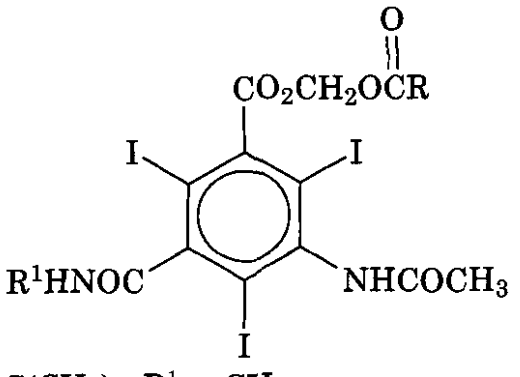
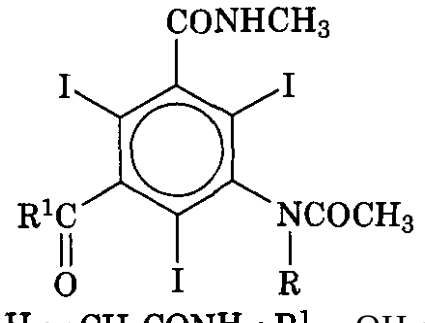
Type (Subclass)	Stem Structure	Comments	Ref.
1. CCN type			
			
R = alkyl, CH_2Ph, CH_2OCOCH_3, $CH_2OCOC(CH_3)_3$, or CH_2CCl_3; $R^1 = COCH_3$.		Synthesis	343
R = C_{1-12} alkyl, $R^1 = CH_2CH_2OH$		Synthesis	230, 231
			
R = phthalidyl, C_{5-8} alkyl, CH_2Ph, $CH(CH_3)OAc$, $CH(CH_3)O_2CC(CH_3)_3$, $CH_2O_2CC(CH_3)_3$, or $CH_2CH_2N(CH_3)_2$; R = $N(CH_3)COCH_3$, $CONHCH_3$		Synthesis (13 esters prepared)	344
			
R = CH_3, $C(CH_3)_3$; $R^1 = CH_3$		Synthesis; bronchography, hepatography.	345
			
R = CH_2CO_2H or CH_2CONH_2; R^1 = OH or NH CH_3		Synthesis, toxicity studies . Urography, arteriography, myelography	346

Table 10.4 (Continued)

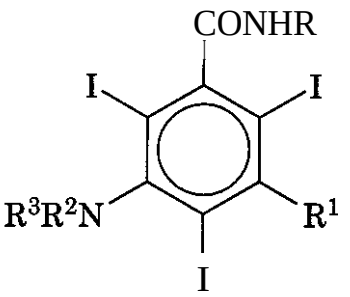
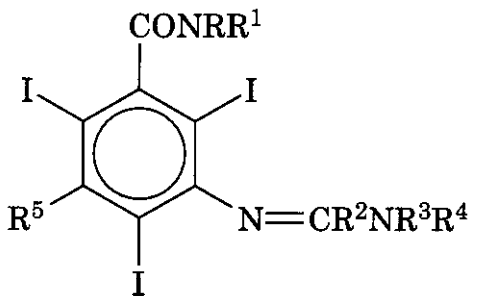
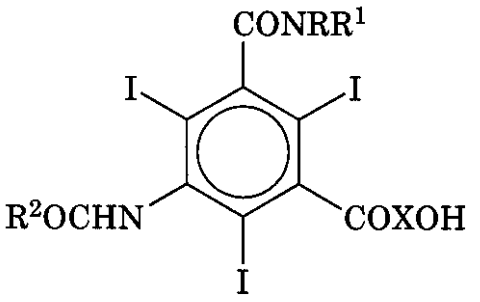
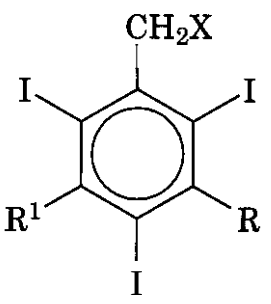
Type (Subclass)	Stem Structure	Comments	Ref.
			
R = CH_3, CH_2CH_2OH; R^1 = CONHMe or NMeAc; R^2 = gluconoyl; R^3 = H or Me.		Synthesis	347
			
R = H, Me, Et, Pr, $CHMe_2$, CH_2, $CH{=}CH_2$, $(CH_2)_3OMe$, Ph, CH_2Ph; R^1 = Me, Et, $CH_2CH{=}CH_2$, or QCO_2H, Q = CH_2CHMe; R^2 = H, Me, Et, CH_2CO_2H, or $(CH_2)_2CO_2H$; R^3 = H, Me, or Et; R^4 = Me, Et, or Ph; R^5 = H, CO_2H, or CONHMe		Synthesis (60 compounds prepared)	348, 349
			
R = H; R^1 = H, Me, or CH_2CH_2OH; NRR^1 = morpholino; X = amino acid residue; R^2 = Me, CH_2OMe, or CH_2OH		Synthesis	350
			
X = OH or Cl; R = NH_2, NHAc, NAc_2 or I; R^1 = NH,, NHAc, NAc_2, NHCOPr, OMe, or I.		Synthesis (11 compounds prepared)	351

Table 10.4 (Continued)

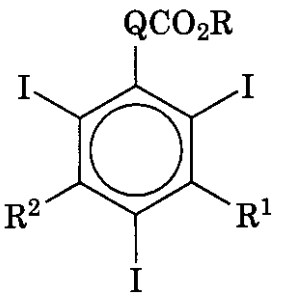
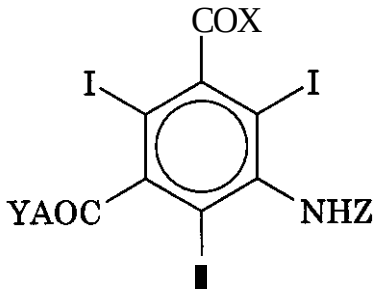
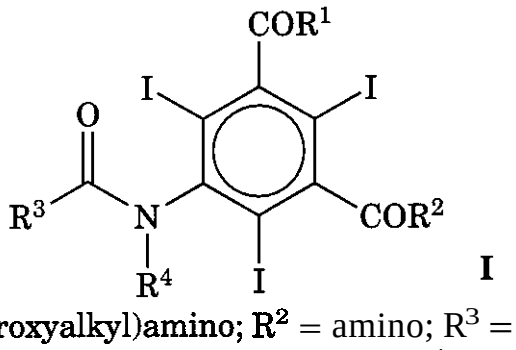
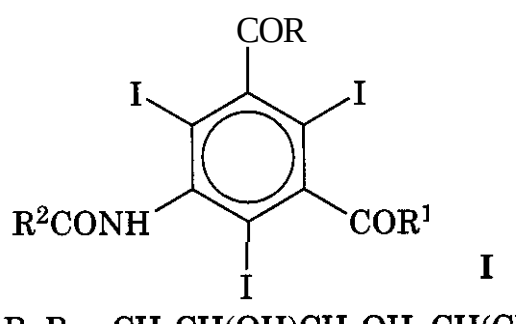
Type (Subclass)	Stem Structure	Comments	Ref.
Q = chemical bond, $(CH_2)_2$, or OCHEt; R = OH or Cl; R^1 = NH_2, NHAc, NAc_2, or I; R^2 = NH_2, NHAc, NAc_2, NHCOPr, OMe, or I.		Synthesis (11 compounds prepared)	351
Q = chemical bond, $(CH_2)_2$, or OCHEt; R = CH_2, CCl_3, Bu, CH_2CO_2Me, or CH_2CMe_2COMe; R^1 = H, NH_2, or NHAc; R^2 = H or CONHMe.		Synthesis	352
X, Y = OH or NRR^1 with R = H, Me, or CH_2CHOH; R^1 = H or Me; NRR^1 = morpholino; (A = residue of an amino acid, e.g., glycine, DL-serine, DL-alanine, sarcosine, proline, or glycyl-L-leucine; Z = H or COR, with R = Ac, $COCH_2OMe$, COBu, or $COCH_2OH$		Synthesis	353
R^1 = (hydroxyalkyl)amino; R^2 = amino; R^3 = alkyl, hydroxymethyl, ethoxymethyl, etc.; R^4 = H, Me, alkoxymethyl, etc.; R^3R^4 = alkylene	 I	Dimers prepared from I	354
R_1 = NMeR; R = $CH_2CH(OH)CH_2OH$, $CH(CH_2OH)_2$; R_2 = $CH(CH_2OH)_2$	 I		355

Table 10.4 (Continued)

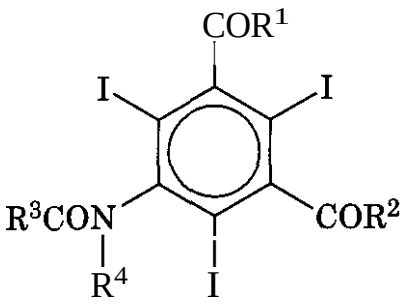
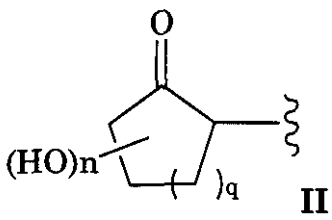
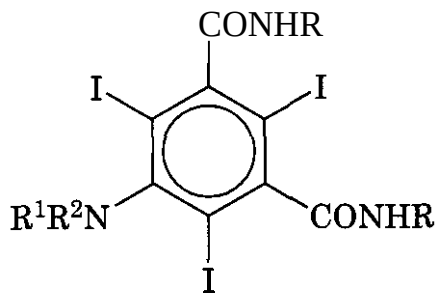
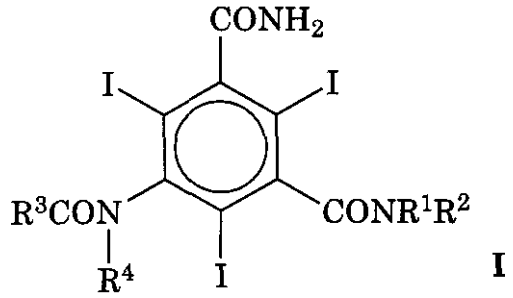
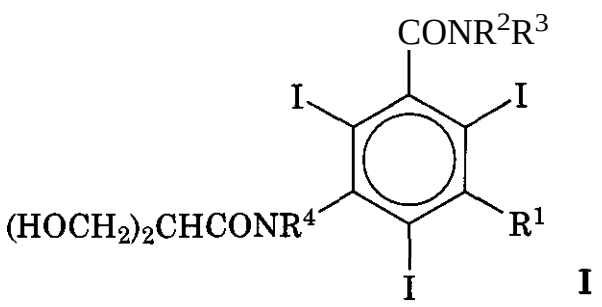
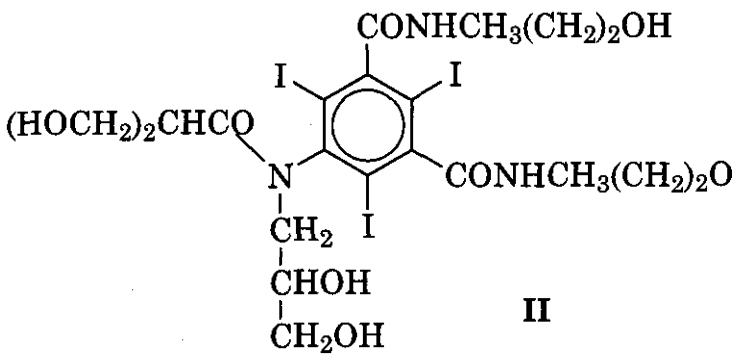
Type (Subclass)	Stem Structure	Comments	Ref.
	 I	 II	
I: R^1 = $N(CH_2CH_2OH)CH_2CHOHCH_2OH$; R^2 = e.g., R^1, NHMe, $NHCH_2CH_2OH$; R^3 = e.g., Me, CH_2OH, CHMeOH; R^4 = e.g., H, Me, CH_2CH_2OH; or R^3 and R^4 together from group **II** (n = 0, 1, 2, 3; q = 0, 1, 2, 3.		At least one amide group is derived from amino-alc., 3-(N-2-hydroxy-ethyl)-amino-1,2-propanediol. Methods of preparation given.	237
	 I		
R = $CH(CH_2OH)_2$, $CH_2CHOHCH_2OH$; R^1 = Ac, R^2 = $CH_2CHOHCH_2OMe$.			356
	 I		
R^1 = H, alkyl, hydroxyalkyl; R^2 = (poly)hydroxyalkyl; R^3 = alkyl, hydroxyalkyl, alkoxyalkyl; R^4 = H, alkyl, (poly)hydroxyalkyl.			357
	 I	 II	
I: R^1 = NR^6COR^5, $CONR^7R^8$; R^2, R^7 = C_{1-4} (poly)-hydroxyalkyl; R^3, R^8 = H, C_{1-4}alkyl; R^4, R^6 = H, C_{1-4} (hydroxy)alkyl, C_{1-4} (poly)hydroxy-alkyl; R^5 = C_{1-4} (hydroxy)alkyl, C_{1-4} (poly)hydroxyalkyl.		Preparation of **II** is given.	358

Table 10.4 (Continued)

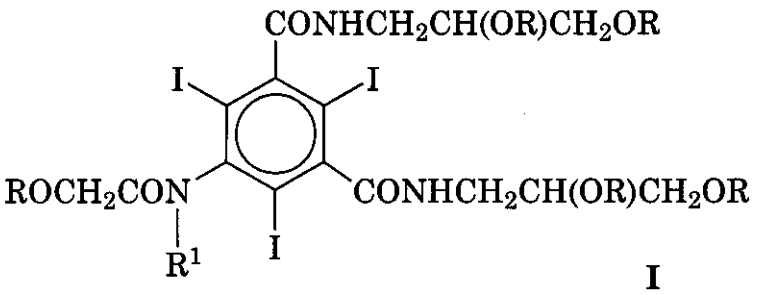
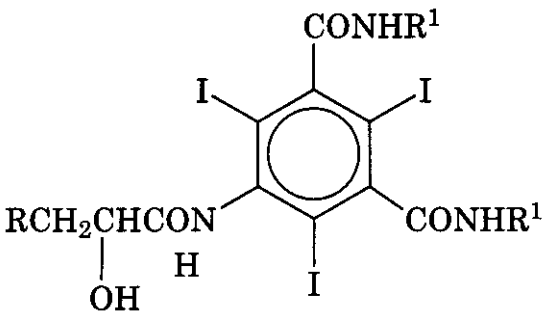
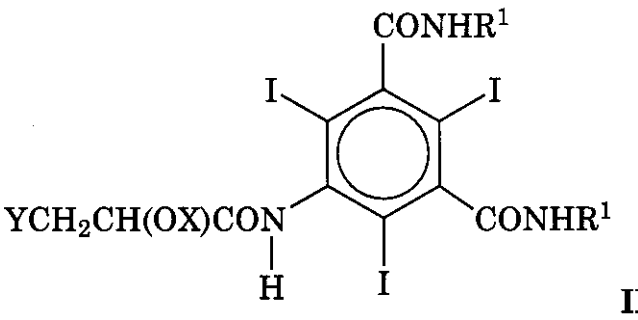
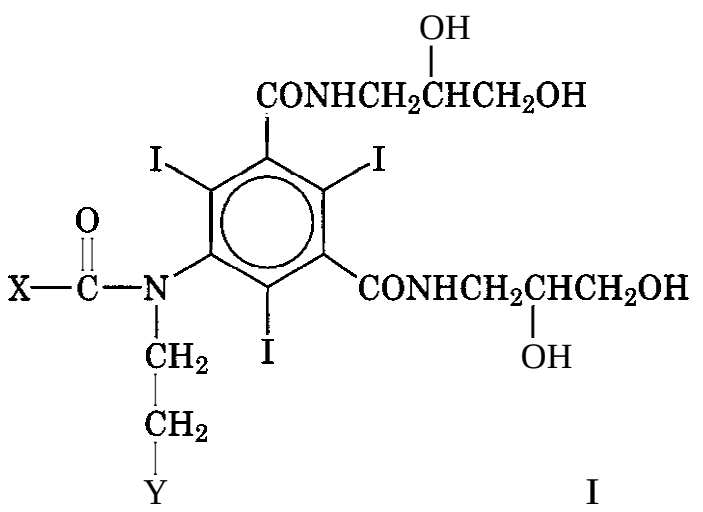
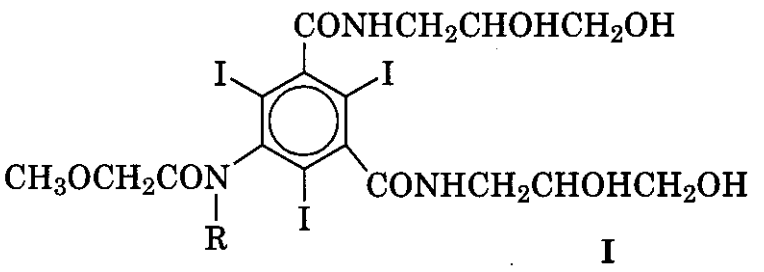
Type (Subclass)	Stem Structure	Comments	Ref.
	 I		
R = Ac, R^1 = H		A variation of process for preparation of ioversol.	359
	 I	 II	
I: R = H, OH; **R^1 = 1,3-dihyroxypropyl, 2,3-**dihydroxypropyl. **11:** X = **acyl,** Y = H, **acyloxy; R^1 = 1,3-dihydroxypropyl, 2,3-dihydroxypropyl.**		**$Ca(OH)_2$** used to promote yield of main product.	255
	 I		
I: X = **CH_2OH, CH_2OCH_3;** Y = OH, **OMe.**		Two examples given with **LD_{50}** values of 17.3 and 16.4 *g* **I/kg in** mice.	360
	 I		
R = H (I), **CH_2CH_2OH (II), $CH_2CHOHCH_2OMe$ (III)**		**II** at 350 mg **I/mL** has **osmolality** 735 **mOs/kg** and viscosity 9.4 **cP** at **37°C, LD_{50}** 1.460 mg **I/kg** in rat. Better than iohexol.	361

Table 10.4 (Continued)

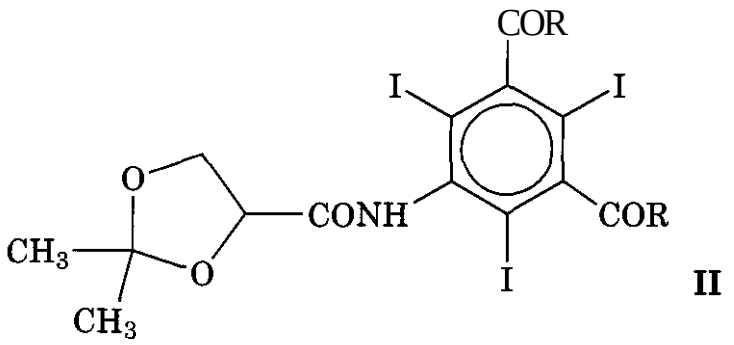
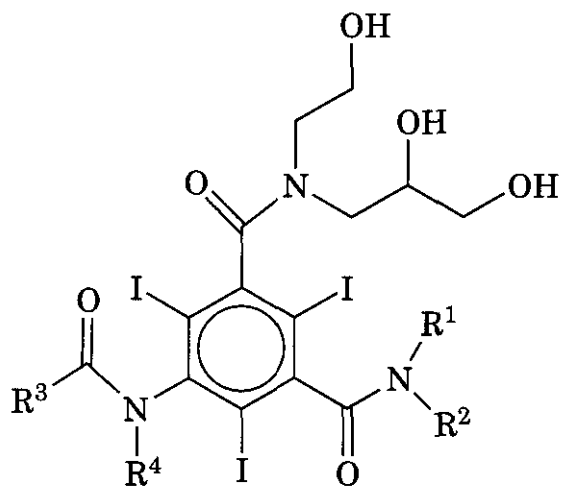
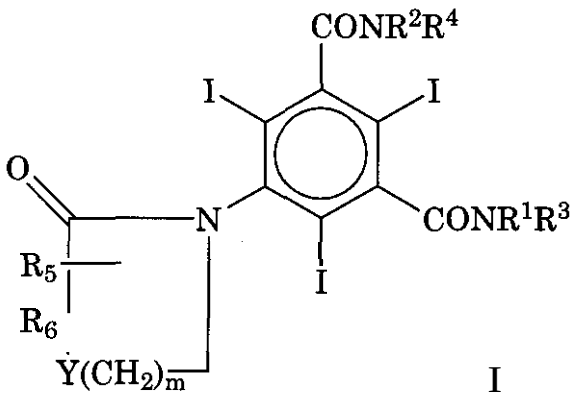
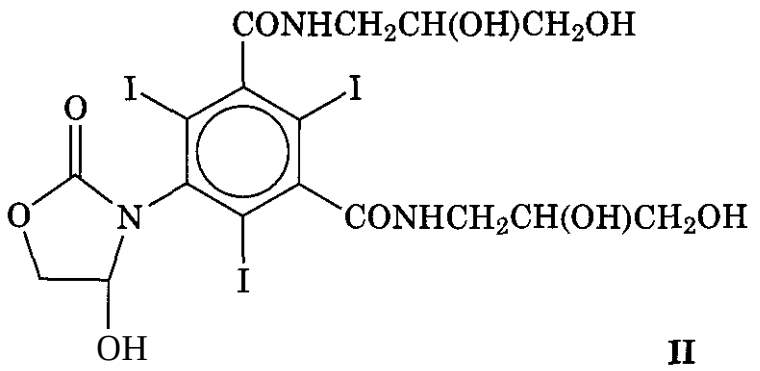
Type (Subclass)	Stem Structure	Comments	Ref.
11: R = −Cl, −$NHCH_2CH_2OH$	 II	Intermediates for synthesis of nonionic contrast agents	**362**
I: R^1, R^2 = H; R^3 = CH_3, CH_2OH, $CH(CH_3)OH$; R^4 = CH_2CH_2OH, $CH_2CH(OH)CH_2OH$; R^3R^4 = $-CH(OH)CH_2CH_2CH_2-$.		Synthesis of ioversol derivatives and testing of the safety of subsituent group	**363**
11: R^1 = H; R^2 = CH_3; R^3 = H, CH_3; R^4 = H, CH_2CH_2OH.		Synthesis	**363**
III: R^1 = H; R^2 = CH_2CH_2OH; R^3 = CH_3, CH_2OH, $CH(CH_3)OH$, $CH(OH)CH_2OH$, CH_2OCH_3; R^4 = H; R^3R^4 = $-CH(OH)CH_2CH_2CH_2-$.		Synthesis	**363**
IV: R^1 = H; R^2 = $CH_2CH(OH)CH_2OH$; R^3 = CH_3, CH_2OH, $CH(CH_3)OH$, R^4 = H.		Synthesis	**363**
V: R^1 = CH_2CH_2OH; R^2 = $CH_2CH(OH)CH_2OH$; R^3 = CH_3, CH_2OH, $CH(CH_3)OH$; R^4 = H.		Synthesis	**363**
I: R^1, R^2 = $CH_2CH(OH)CH_2OH$, $CH(CH_2OH)_2$, CH_2CH_2OH, etc.; R^3, R^4 = H, Me, CH_2CH_2OH; R_5, R_6 = H, alkyl, CH_2CH_2OH, CH_2OH, OH; Y = bond, CH_2CH_2, CH_2O, OCH_2, NCH_2, CH_2NCH_2, O, N; m = 0,1.	 I	Synthesis	**364**
II:	 II	Method of preparation given	**364**

Table 10.4 (Continued)

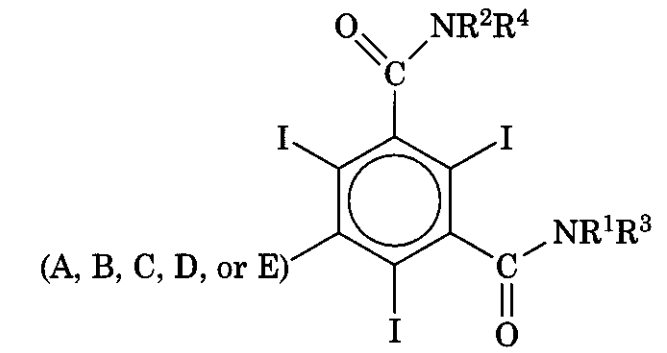
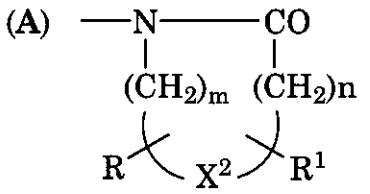
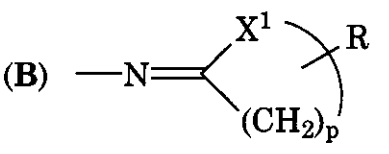
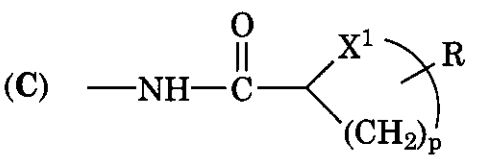
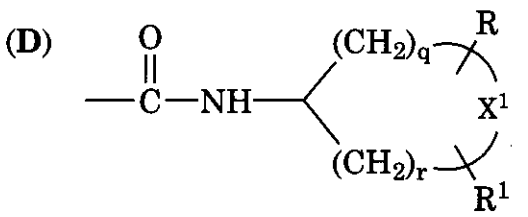
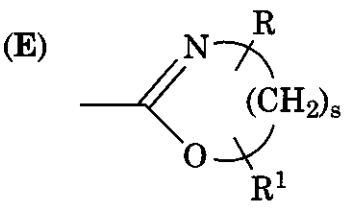
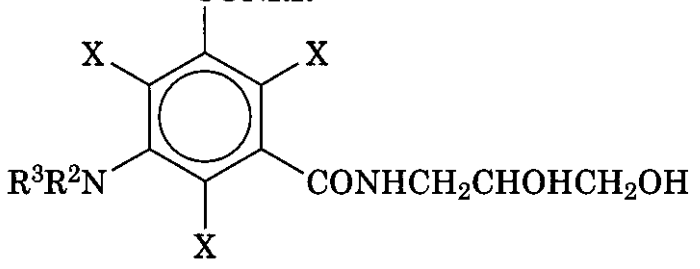
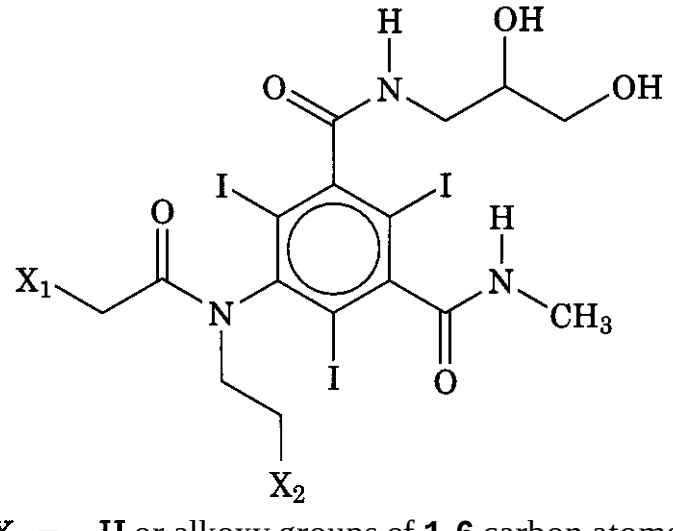
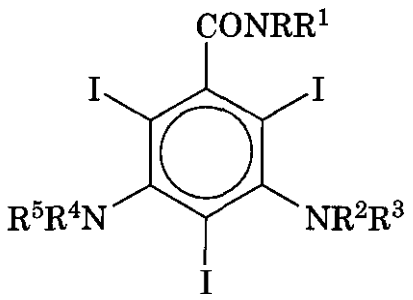
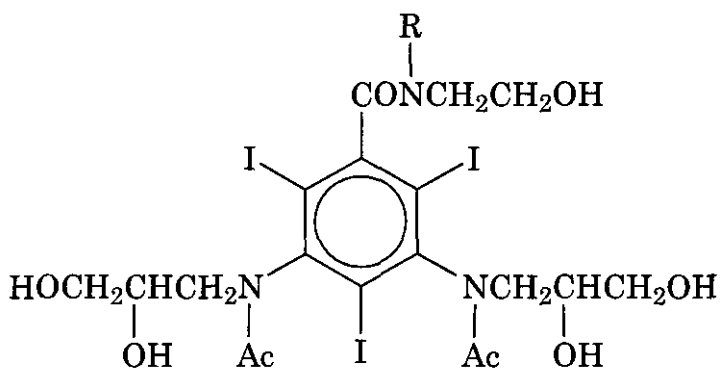
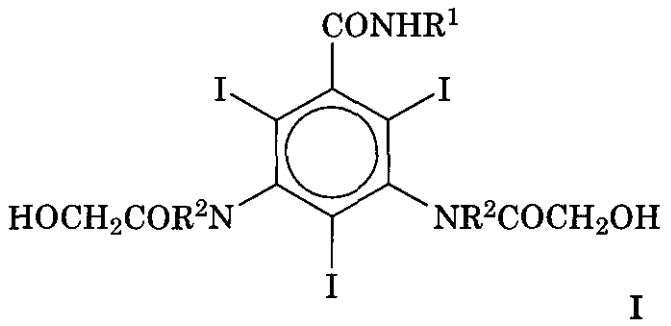
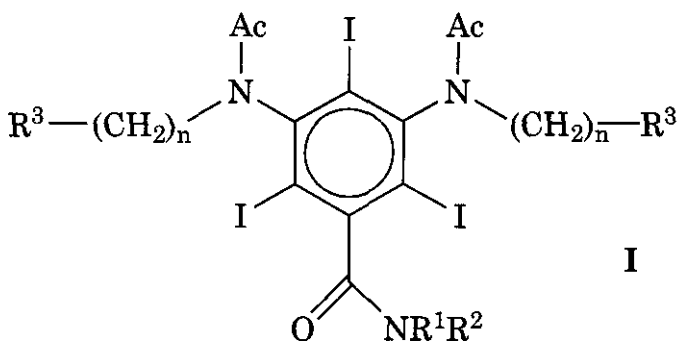
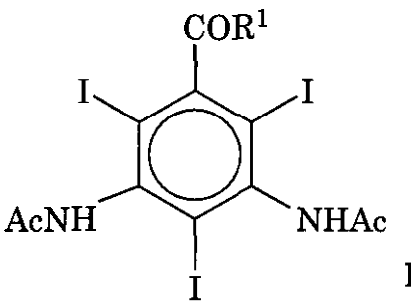
Type (Subclass)	Stem Structure	Comments	Ref.
	     		
(A), (B), (C), (D), (E): R, R^1 = −H, **−R,** −OH, −OR, **-hydroxyalkyl, -alkoxyalkyl;** X^1 = **=N(CO)H, =N(CO)-alkyl,** −S-, or − 0-; X^2 = X^1 or **$-CH_2-$;** m = **1-4,** or if X^2 **is $-CH_2-$,** m = 0 4 ; n = **0–3;** p = 2–5; **q, r** = **1** or **2** independently; and s = 2–5.		Synthesis (This group of compounds with a heterocyclic ring has an unexpected degree of anticoagulant behavior.)	**313, 314**
R = **$CH_2CHOHCH_2OH$, $CH(CH_2OH)_2$;** R^1 = H; R_2 = H, Ac, R_3 = H, **$CH_2CHOHCH_2OH$, $CH_2CHOHCH_2OMe$, $CH_2CH(OMe)CH_2OH$.**		Synthesis and testing	**365**
X_1, X_2 = −**H** or alkoxy groups of **1-6** carbon atoms		Synthesis; intravascular and CNS uses	**366**

Table 10.4 (Continued)

Type (Subclass)	Stem Structure	Comments	Ref.
2. The CNN type			
			
NRR^1 = glucosimino or N-methylglucosimino, R^2 = H, Ac, R^3 = Ac, R^4 = H, Me, R^5= Ac.		Synthesis, angiography, myelography	367
 **I**			
R = H, Me; R^1 = $NHCH_2CH_2OH$		I (R = H) has a solubility of >80% (w/v) in H_2O at 20°C and a toxicity of >16 g/kg i.v. in rats.	368
 **I**			
R^1, R^2 = CH_2CH_2OH, $CH_2CHOHCH_2OH$		Preparation and formulation	369
 **I**			
I, II: Ac = acetyl, propionyl, 2-hydroxyacetyl, 2-methoxyacetyl or 2-hydroxy propionyl; R^1, R^2 = methyl, ethyl, propyl, butyl, 2-hydroqethyl, 2,3-dihydroxypropyl, 1,3-dihydroxyisopropyl; R^3 = hydroxymethyl, 1,2-dihydroxyethyl, 1,2,3-trihydroxypropyl, 2-hydroxyethoxy, 2,3-dihydroxy-propoxy; n = 2, 3, 4.		Analogs with n = 4 have lower osmolality and higher viscosity than analogs with n = 2.	370


II

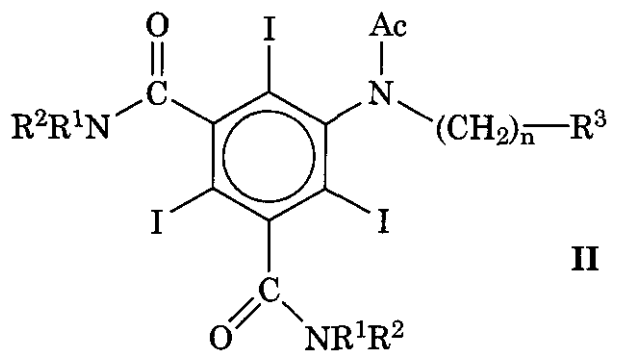

II

Table 10.4 (Continued)

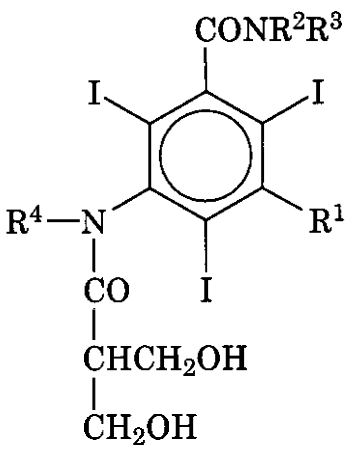
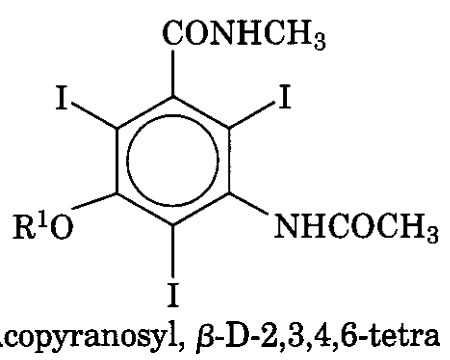
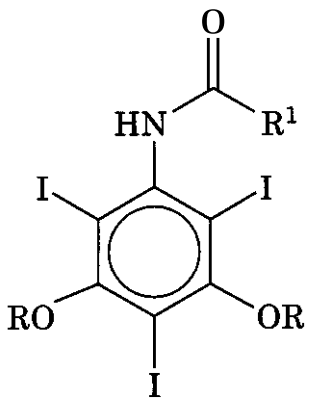
Type (Subclass)	Stem Structure	Comments	Ref.
	 **I**		
I: R^2 = H, C_{1-4} (poly)hydorxyalkyl; R^3 = H, C_{1-4} alkyl; R^4 = H, alkyl or (poly)hydroxyalkyl; R^1 = $NR^4COCH(CH_2OH)_2$, NR^6COR^5; R^5 = C^{1-4} alkyl or (poly)hydroxyalkyl; R^6 = groups cited for R^4, $CONR^7R^8$; R^7 = C_{1-4} (poly)-hydroxyalkyl; R^8 = H, C_{1-4} alkyl.		**An** example prepared in 3 steps and purified by HPLC is given.	371
3. The CNO type			
			
R = β-D-glucopyranosyl, β-D-2,3,4,6-tetra acetylglucopyranosyl		Synthesis	186
4. The NOO type			
			
R_1 = CH_3, CH_2OH, CH(MEOH(1)-, CH_2OCH_3, arabinos-2-yl; R = glucos-3-yl, allos-3-yl, fructos-3-yl		Synthesis and testing	372

Table 10.4 (Continued)

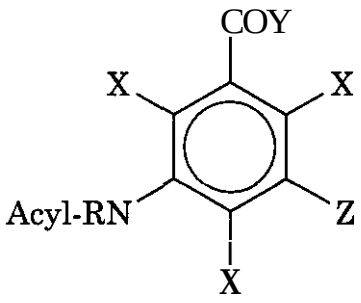
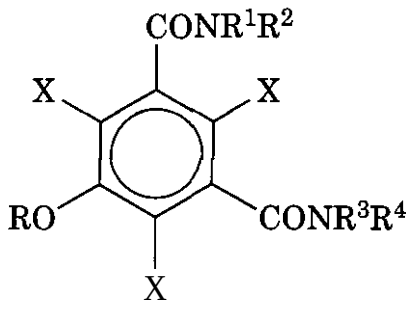
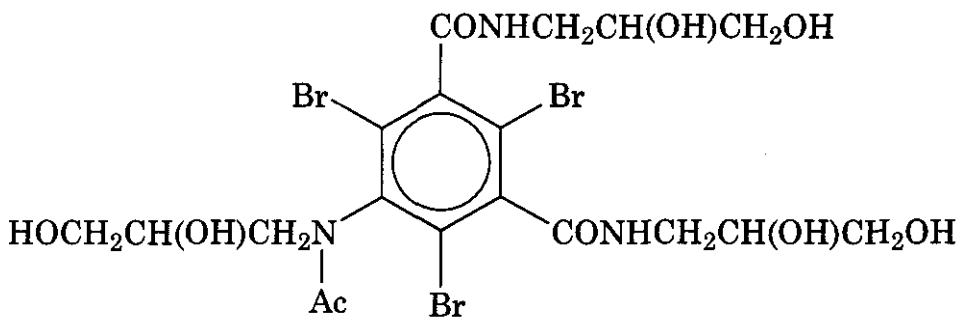
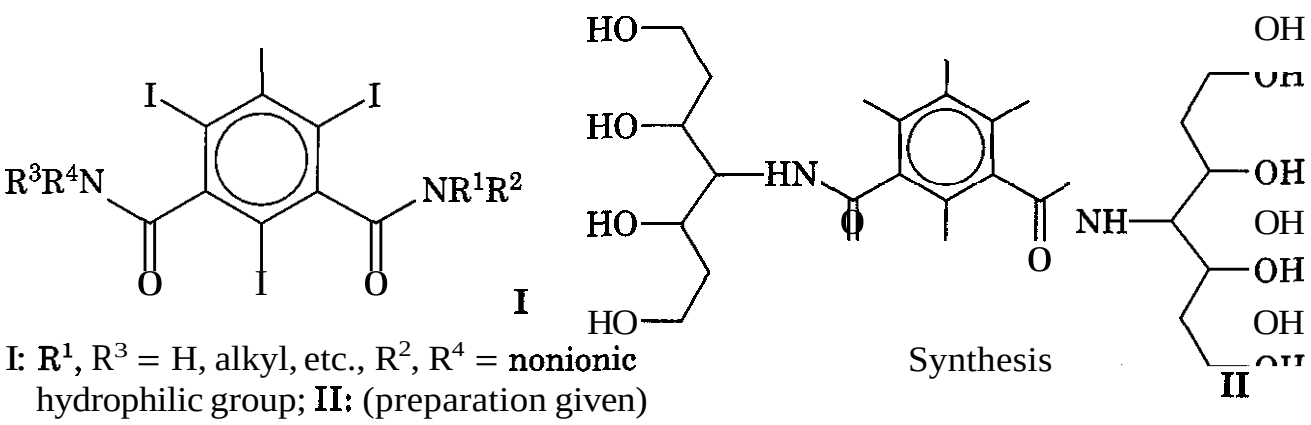
Type (Subclass)	Stem Structure	Comments	Ref.
5. Mixed brominated and iodinated CCN and CNN type			
 I			
X = Br, iodo; Acyl = C_{2-6} hydroxyalkanoyl, alkoxyalkanoyl, alkoxyhydroxyalkanoyl, (un)substituted C alkanoyl; R = H, C_{1-6} alkyl, hydroxyalkyl, alkoxyalkyl, alkoxyhydroxyalkyl, $H(OCH_2CH_2)_{2-5}$. $Me(OCH_2CH_2)_{2-4}$, $Et(OCH_2CH_2)_{2-4}$, alkylene analog of I; Y = HO, alkoxy, hydroxyalkoxy, alkylamino, etc.; Z = COY, hydroxyalkylaminocarbonyl, C_{2-5} acylamino, hydroxyacylamino, N-alkylacylamino, *N*-hydroxyalkylacylamino, acylaminomethyl.		Synthesis	373
 I			
R = hydroxyalkyl, (poly)alkoxyalkyl, polyhydroalkyl, aminocarbonylalkyl, etc.; R^1, R^3 = H, alkyl, hydroxyalkyl; R^2, R^4 = hydroxyalkyl; X = iodo, Br.		Synthesis	374
 I			
(Only one compound prepared)		Synthesis	375
6. Tetraiodinated CCI type			
 I II			
I: R^1, R^3 = H, alkyl, etc., R^2, R^4 = nonionic hydrophilic group; II: (preparation given)		Synthesis	376

Table 10.5 Potential Dimeric Radiopaques: Bis(2,4,6-triiodophenyl) Derivatives

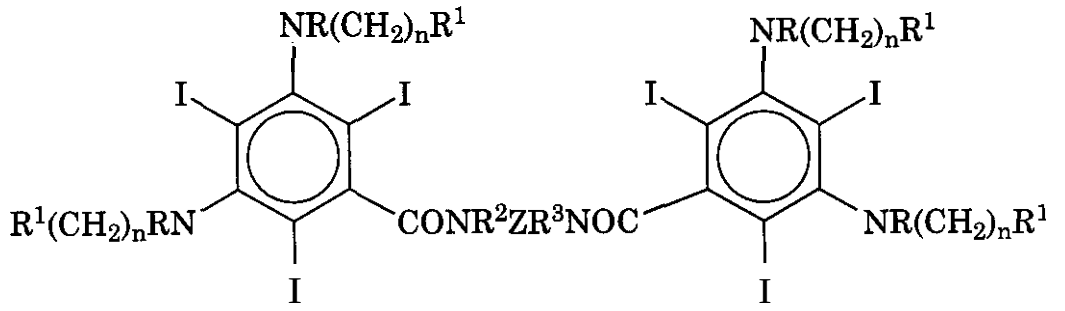
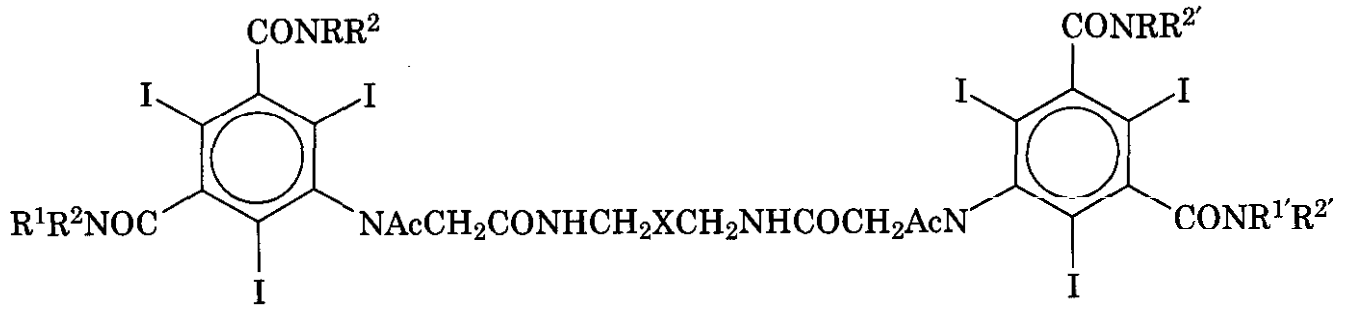
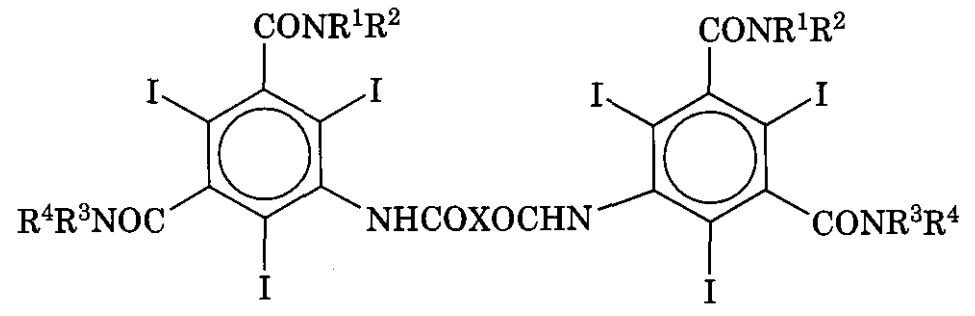
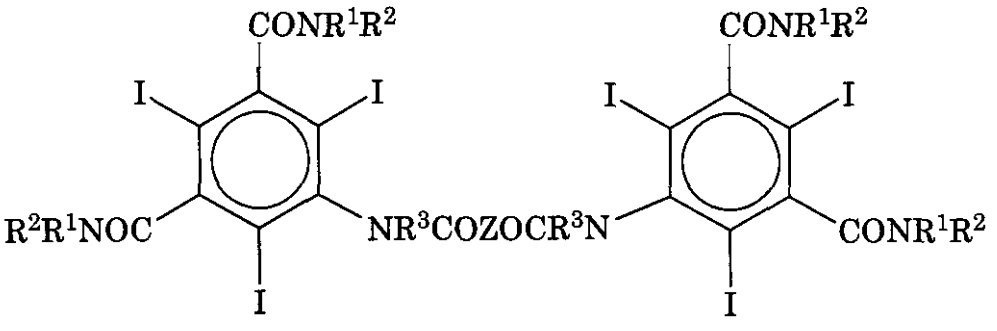
Type (Subclass)	Stem Structure	Comments	Ref.
1. CNN type (iodobenzamides)			
R = (un)substituted acyl; R^1 = hydroxylalkyl, hydroxyalkoxy, etc.; R^2, R^3 = H, alkyl, hydroxyalkyl, etc.; Z = bond, alkylene, etc.; n = 2 4 .		High water solubility, low osmolality, low viscosity	370
2. CCN type (iodoanilides)			
R, R', R^1, $R^{1\prime}$ = same or different = H, C_{1-4} alkyl, linear or branched C_{1-4} mono- or poyhydroxyalkyl; R^2, $R^{2\prime}$, R^3, $R^{3\prime}$ = same or different = CH_2CH_2OH, $CH_2CHOHCH_2OH$, $CH(CH_2OH)CHOHCH_2OH$, $CH_2(CHOH)_4CH_2OH$, $CH(CH_2OH)_2$; X = CH(OH), $CH(CH_2OH)$, $C(OH)(CH_2OH)$, $C(CH_2OH)_2$, enantiomers, diastereoisomers, and/or rotamers.		Methods of preparation given	377
3. CCN type (iodoanilides)			
X = $(CH_2)_4$, $CH_2OCH_2CH_2OCH_2$, $CH_2CH_2SCH_2CH_2$, $CH_2(OCH_2CH_2)_2OCH_2$ R^1 = CH(CONHMe) CH_2OMe, CHMeCONHMe, $CH_2CONHMe$, Me, H; R^2 = H, Me; R^3 = H, Me; R^4 = H, $CHMeCO_2H$, CH_2CO_2H		19 compounds prepared	235
			
R^1 = mono- or polyhydroxyalkyl; R^2 = H, alkyl, R^1; R^3 = mono- or dihydroxyalkyl; Z = bond, alkylene optionally with O interrupters or OH or alkoxy substituents.		Methods of preparation given	259

Table 10.5 (Continued)

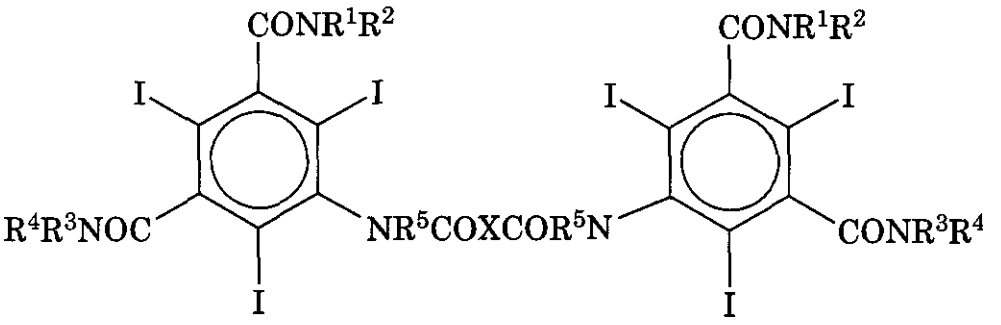
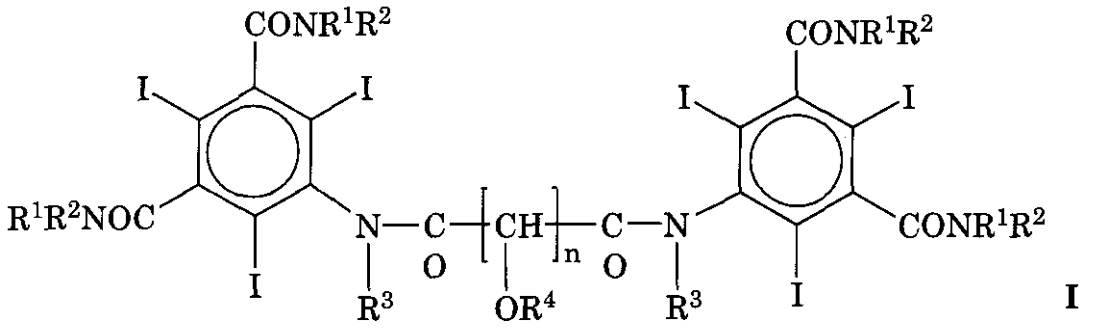
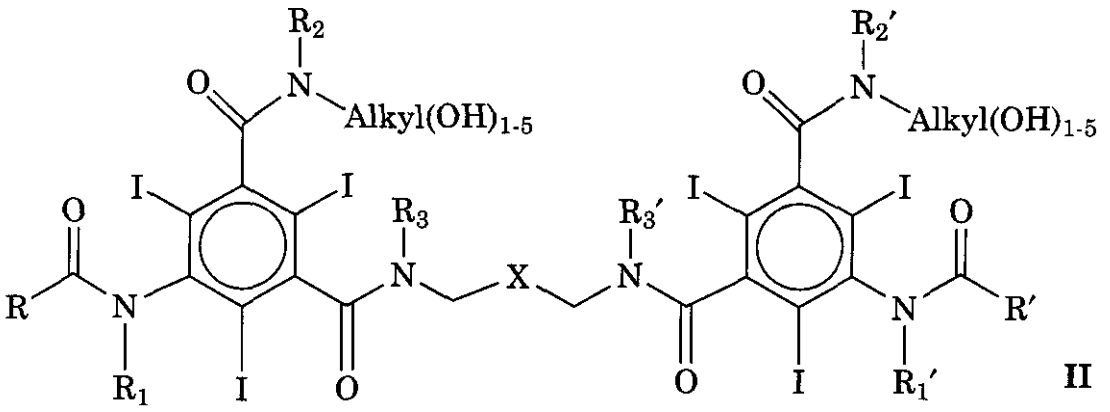
Type (Subclass)	Stem Structure	Comments	Ref.
4. CCN type (iodoanilides)			
R^1 = H, alkyl, R^2; R^4 = (poly)hydroxyalkyl; R^3 = alkyl, R^4; R^5 = H, alkyl, poly(hydroxyalkyl); X = (HO- or alkoxy-substituted)(O-interrupted) C_{1-6} alkylene.		Methods of preparation given	378
5. CCN type (iodoanilides)			
I: R^1 = H, lower alkyl, R^2; R^2 = straight or branched mono- or polyhydroxyalkyl; R^3, R^4 = H, lower alkyl; n = 1, **2.**		Methods of preparation given	379
II: R, R^1 = same or different, a straight or branched mono- or poly-hydroqalkyl C_1–C_3 residue containing 1 to 2 OH groups; R,, R_2, R_3 = H or CH_3; R_1', R_2', R_3' = H, or CH,; Alkyl $(OH)_{1-5}$ = $-CH(CH_2OH)CH(OH)CH_2OH$, $-CH(CH_2OH)_2$, $-CH_2CH-(OH)CH_2OH$, $-CH_2(CHOH)_4CH_2OH$, or $-CH_2CH_2OH$; X = $-CH(OH)-$, $-CH((CH_2OH)-$ $-CH(OH)(CH_2OH)-$ or $-C(CH_2OH)_2-$.		**28** compounds and methods of preparation given	380

Table 10.5 (Continued)

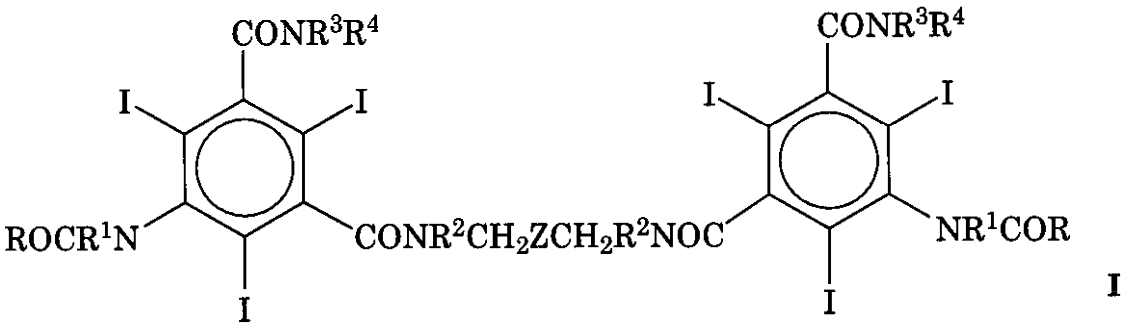
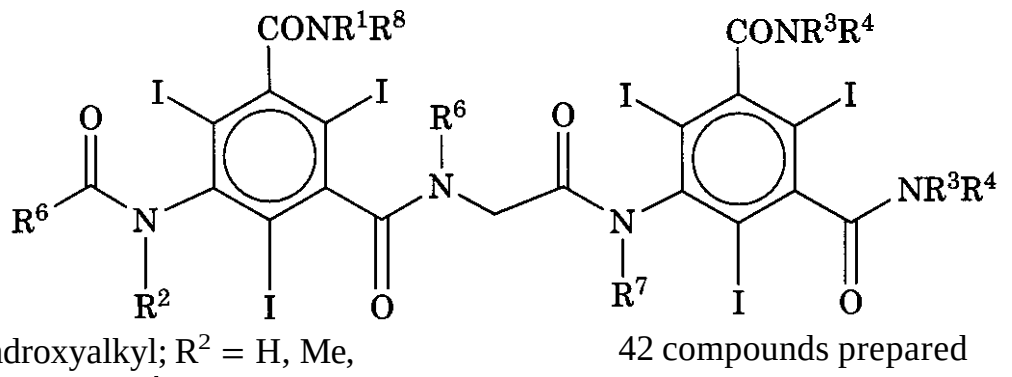
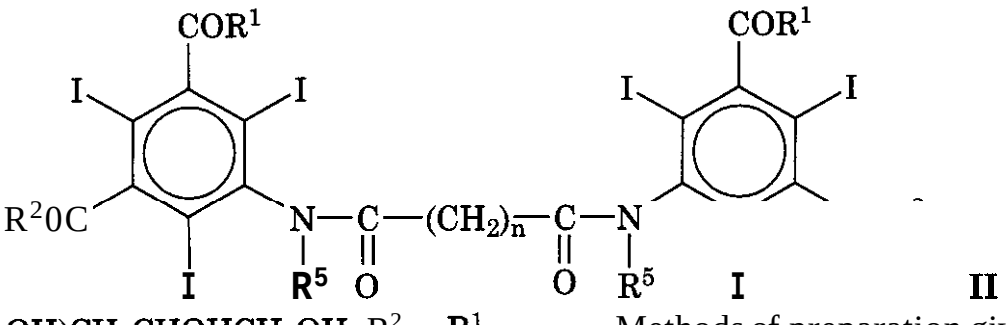
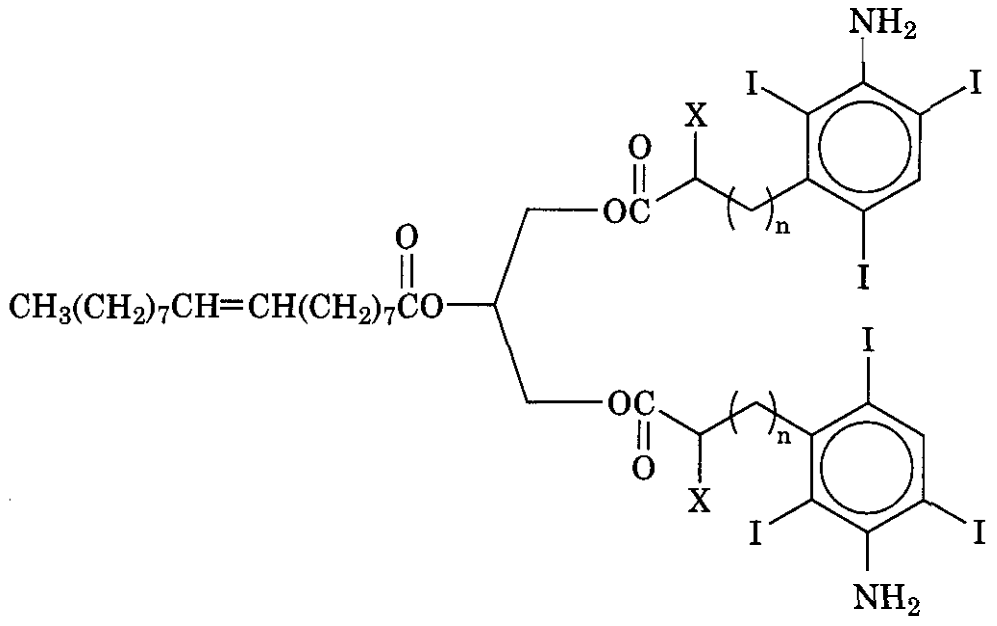
Type (Subclass)	Stem Structure	Comments	Ref.
6. CNC type (**iodobenzamides**)			
R = straight or branched mono- or **polyhydroxyalkyl** C_1–C_3 residue containing **1** to **2** OH groups; R^1, R^2, R^3 = H, Me; R^4 = $HOCH_2CHCH(OH)CH_2OH$, $CH(CH_2$, $CH_2CH(OH)CH_2OH$, $CH_2(CHOH)_4CH_2OH$ or CH_2CH_2OH; Z = CH(OH), $CH(CH_2OH)$, $CH(OH)CH_2OH$, or $C(CH_2OH)_2$).		Methods of preparation given	**380**
7. CCN type (iodobenzenedicarboxamides)			
R^1, R^4 = C_{2-4} polyhydroxyalkyl; R^2 = H, Me, polyhydroxyalkyl, CH_2CH_2OMe, $CH_2CH(OH)CH_2OMe$; R^3, R^8 = H, Me, CH_2CH_2OH; R^5 = Me, CH_2OH, CH_2OMe; R^6, R^7 = H, Me, CH_2CH_2OH, CH_2CH_2OMe, $CH_2CHOHCH_2OH$, $CH_2CHOHCH_2OMe$; or R^5R^2 = $CH_2CH_2CH_2$ bearing **0–3** OH groups.		42 compounds prepared	**381, 382**
11: R^1 = R^4 = $CH_2CHOHCH_2OH$, R_2 = R_3 = R_8 = H, R_5 = HOCH,, R_6 = R_7 = Me.		LD_{50}: **25–28 g/kg** (rats); **152 mOsm/kg** at **300** mg I/mL; **7.0 mPa·, 52.1%**I; comparable to iotralan.	**381,382**
8. CCN type (iodobenzenedicarboxamides)			
11: R^1 = $N(CH_2CH_2OH)CH_2CHOHCH_2OH$; R^2 = R^1, NHMe, $NHCH_2CHOHCH_2OH$; R^5 = H, Me, CH_2CH2OH, $CH_2CHOHCH_2OH$, CH_2OMe, m = **0 4 .**		Methods of preparation given	237

Table 10.5 (Continued)

Type (Subclass)	Stem Structure	Comments	Ref.
9. CNH type (bis-iodophenylalkanoates)			
n = 0 to 5 carbon atoms alkylene chain, X = H, C_2H_5.		Synthesis and testing	383

ecule for their high water solubility. To minimize solution viscosity, all nonionic contrast agents are designed to be small and globular molecules. Substituent groups most frequently found in nonionic radiopaque molecules are, for example, hydroxyethyl, 2,3-dihydroxypropyl, and 1,3-dihydroxypropyl. These susbstituents are attached to the triiodophenyl ring by the coupler groups, such as amide (—CO—NH—), reversed amide (—NH—CO—), amino (—NH—), and so forth. Depending on the nature of substituent groups, hydroxyl groups from different substituent groups can have different hydrophilicities. For example, the hydroxyl groups in metrizamide from a sugar molecule are highly hydrophilic but unstable toward heat at 120°C. To avoid decomposition, metrizamide is sterilized at low temperature and marketed as lyophilized powder to be reconstituted with sterile water just before use (401). On the other hand, hydroxyl groups from hydroxyalkyl substituents are less hydrophilic and heat stable at 120°C. Nonionic monomers and bis compounds, such as iopamidol, iohexol, and iotrolan, containing alkyl hydroxyl groups, are heat stable and can be sterilized at 120°C for 2 h (401).

In designing improved nonionic contrast agents, high water solubility is achieved by masking and shielding the lipophilic regions of the molecule (i.e., around the bulky iodine atoms and about both faces of the triiodophenyl ring) with polar groups to achieve lateral and facial hydrophilicity (204). High water solubility is necessary to minimize protein binding and to achieve high biological tolerance. It is difficult to predict the water solubility of a molecule from the number of hydroxyl groups it contains because hydrogen bonding and the prevalence of multiple isomeric forms, for example, can exert a strong influence on the molecular interaction with water. Nonionic contrast agents in mixed isomeric forms are more water soluble than those present in only one pure isomeric form, the only exception being the pure L-lactoyl form of iopamidol, which is more soluble than the mixed isomeric forms (194).

Nonionic contrast agents are less anticoagulant than ionic contrast agents (402–405). Ranganathan et al. (313, 314) showed that placing a heterocyclic ring substituent in the 5-position of the 2,4,6-triiodo-1,3-benzenedicarboxamide moiety can confer an unexpected anticoagulant behavior to the nonionic contrast molecule.

6.3.2 Radiopacity. Radiopacity is dependent on the number of iodine atoms in the molecule, with more iodine atoms per molecule yielding better images, provided that other properties are equal (181). An ionic con-

Table 10.6 Potential Radiopaques: Polymeric Substituted 2,4,6-Triiodobenzoic Acids

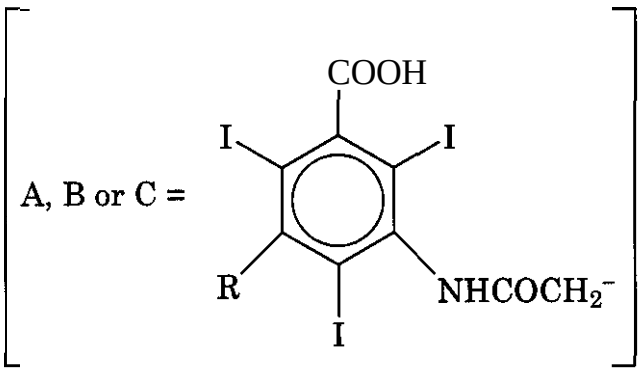
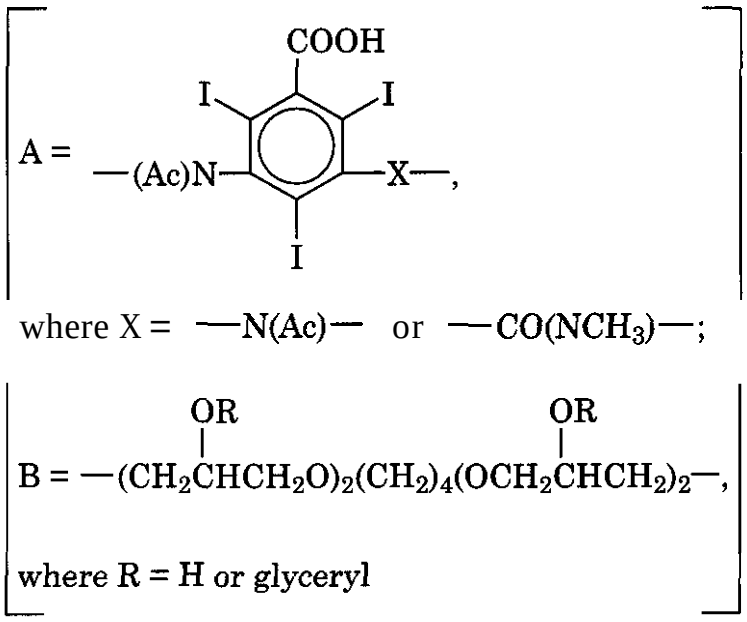
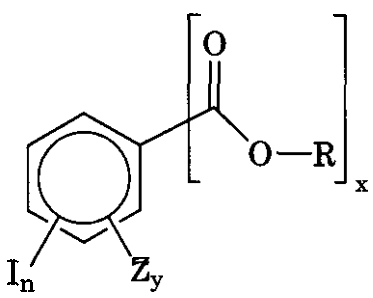
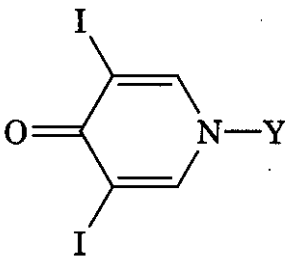
Compound	Uses	Comment	Ref.
1. N ≡ (A, B, C)  	Angiography	Synthesis	331
2. - - - -A-B-A-B-A-B- - - -  	Roentgenography of gastrointestinal tract		191, 192
3. Mixtures of iodobenzoic acid derivatives and cellulose derivatives  Z = H, halo, C_1–C_{20} alkyl, cycloalkyl, lower alkoxy, cyano; R = C_1–C_{25} alkyl, cycloalkyl or halo-lower alkyl, fluoro-lower alkyl, aryl, lower alkoxy, hydroxy, carboxy, lower-alkyl carbonyl or lower alkoxy-carbonyloxy; or $(CR_1R_2)_p$-$(CR_3{=}CR_4)_m$ Q or $(CR_1R_2)_p$-C≡C-Q; R_1, R_2, R_3, R_4 = lower alkyl or halo-alkyl; X = 1 3 ; y = 1–4; n = 1–5; m = 1–15; p = 1–15; p = 1–10; and Q = H, lower alkyl, alkenyl, alkynyl, lower alkylene, aryl or aryl-lower alkyl.	A mixture of the derivatives—used for oral or retrograde examination of GI tract		384

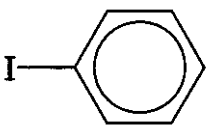
Table 10.7 Potential Radiopaques: Substituted 3,5-Dilodo-4(1*H*)-pyridones



Y	Uses	Comment	Ref.
$-CH_2CO_2R$ [R = Bu, CH_2Ph, C_4–C_{10} alkyl, CH_2OAc, $CH_2OCOCMe_3$, CH_2CCl_3]		Synthesis	343,352,385
$-(CH_2)_nCO_2R$ [R = H, Me, Et, Bu, Am, octyl, $CH_2CH_2NMe_2$, CH_2CH_2OH, NH_2]	Urography, lymphography	Synthesis (18 compounds prepared)	386
$-CH_2CONH(CH_2)_nNR^1R^2$ [n = 2,3; R^1 = R^2 = Me, Et, R^1R^2 = $CH_2CH_2OCH_2CH_2$]		Synthesis (high toxicity)	387
$-CH_2CH_2R$ R = $N(CH_3)_2$, NEt_2, —N⟨⟩O, and their $(CH_3)_2$ salts, $N^+(Et)_2CH_2CH_2OH \cdot Cl^-$		Syn thesis (high toxicity)	387

trast molecule can dissociate in solution into an iodine-containing anion and an iodine-free cation, thus reducing the ratio of three iodine atoms per molecule by a factor of 2 to a ratio of 1.5 iodine atoms on average per ion (177,180). All the ionic monomers are therefore known as iodine ratio 1.5 contrast agents. The monoacidic bis compound ioxaglate with one carboxylic acid group and six iodine atoms in the molecule is a ratio 3.0 contrast agent. The nonionic monomers, which contain no ionizable groups and do not undergo dissociation in solution, are ratio 3.0 contrast agents, and their dimers or bis compounds ratio 6.0 agents. Higher ratio contrast agents with higher iodine content per particle will give bet-

Table 10.8 Potential Radiopaques: Monoiodophenyl Derivatives



(1)

(1)	Uses	Comment	Ref.
$-CO_2XO_2CR$ [X = $(CH_2)_2$, $(CH_2)_3$, CH_2CHMe, or $(CH_2)_4$; R = Me, Et, Pr, i-Pr, Bu, $(CH_2)_4Me$, $(CH_2)_5Me$, or CH_2OMe]	Myelography	Synthesis	388
$-CO_2(CH_2)_nO_2CR$ [n = 2 4 , R = Me, Et, Pr, i-Pr, $(CH_2)_3Me$, $(CH_2)_4Me$, $(CH_2)_3Me$, CH_2CHMe, or CH_2OMe]	Myelography	Synthesis	389
$-X\text{-}OCO_2R$ [X = $(CH_2)_2$, $(CH_2)_3$, $CHMeCH_2CH_2$, $CH_2CHEtCH_2$, or $CH_2CHBuCH_2$; R = Et, i-Pr, Bu, i-Bu, pentyl, hexyl, octyl, decyl, $CHMeC_6H_{13}$, $CH_2CH(Et)Bu$, $CHMeCH_2CH(CH_3)_2$, $CH(Et)Pr$, or CH_2CH_2OMe]	Myelography, lymphography, bronchography, salpingography	Synthesis	390

Table 10.9 Miscellaneous Potential Radiopaques

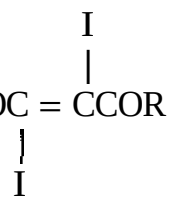
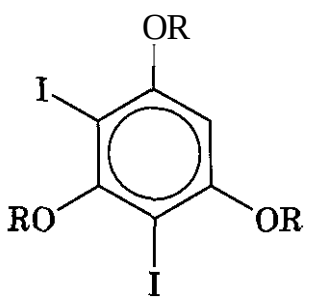
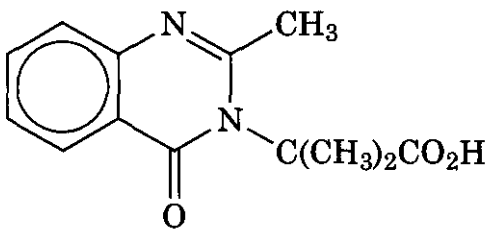
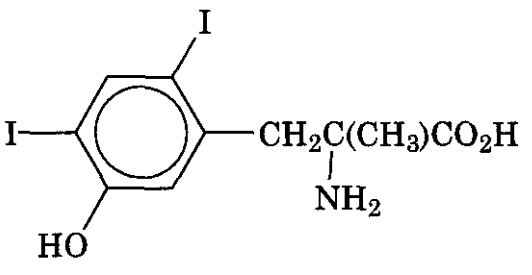
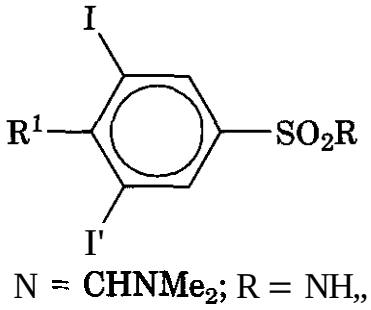
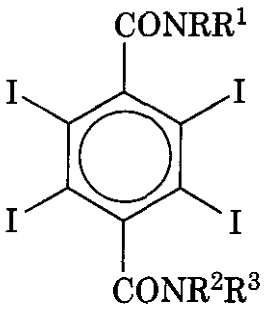
Compound	Uses	Comments	Ref.
$ICH_2SO_2NRR^1$ [R = H, Me; R^1 = $CH_2CH(OH)CH_2OH$, $CH(CH_2OH)_2$, CH_2CH_2OH, $CH_2CH_2OCH_2CH_2OH$]	Myelography	Synthesis	391,392
		Synthesis	393
[R = OH, $O(CH_2)_2OMe$, $O(CH_2)_2OEt$, $(OCH_2CH_2)OMe$, $(OCH_2CH_2)2OEt$, $O(CH_2)_3Me$, $O(CH_2)_2CHMe_2$, $NH(CH_2)_2OH$, $NHCH_2CH(OH)Me$, $NHC(CH_2OH)_2Me$, $N(CH_2CH_2OH)_2$, $NHCHCO_2H$, or $NMeCH_2CO_2H$]			
BuNHCONHR (R = 2,3,5,6-tetraiodo-*p*-tolylsulfonyl)	Radiography of pancreas and prostate gland	Synthesis	394
			
(R = glycosyl)	Urography		185
		Synthesis, toxicity studies	395
	Cholescystography	Synthesis	396
	Cholecystography	Synthesis	397
[R^1 = NH_2, N = $CHNMe_2$; R = NH_2, OH, NMe_2, NEt_2, NBu_2, morpholino, piperidino, $NHCH_2CH_2OH$, $N(CH_2CH_2OH)_2$, or $NMeCH_2(CHOH)_4CH_2OH$]			

Table 10.9 (Continued)

Compound	Uses	Comments	Ref.
 [NRR^1, NR^{2}R^3 = NHMe, $NHCH_2CO_2H$, $NHCH(Pr)CO_2H$, morpholino, 2-carboxy-1-pyrrolidinyl, $NHCHEtCH_2CO_2H$, $NMeCH_2CO_2H$, $NEtCH_2CO_2H$]		Synthesis	398
$Me_2N(CH_2)_nNMe_2 \cdot 2X^-$ (R, R^1) [n = 2, 4, 6, or 10; R, R^1 = H, ethylacetyl, 3-iodobenzyl, 2,4,5-triiodobenzyl, 5-amino-2,4-didiodobenzyl, 3-amino-2,4,6-triiodobenzyl; X = Cl, I]	Binding to cartilage	Synthesis	399

ter X-ray image and resolution. Radiopacity is a physical property, affected not only by the atomic number but also by the localization and concentration of contrast medium in the organ.

Gadolinium chelates provide contrast enhancement in CT and also in MRI. Gadolinium has a higher atomic number than that of iodine and can attenuate X-rays more efficiently than iodine at equivalent mass concentration, although iodinated contrast media yield better resolution at higher concentrations (91).

6.3.3 Acidity. The inductive effect of the iodine atoms in the molecule makes the substituted 2,4,6-triiodobenzoic acids stronger acids than the substituted 2,4,6-triiodophenyl or triiodophenoxy alkanoic acids. The pK_a values of many ionic contrast agents were measured by Felder et al. (263). The nonionic contrast agent ioparnidol has a pK_a value of 10.7 for the nitrogen proton next to the triiodiophenyl ring, but the molecule is practically undissociated at the physiologic pH 7.0–7.5 (194).

6.3.4 Electron Density. Substituent groups attached to the triiodinated phenyl ring can donate electrons to the ring or withdraw electrons from it. Substituent groups attached to the ring through oxygen and nitrogen atoms will donate electrons to the ring and those attached through the carbonyl carbon atoms will withdraw a-electrons from the ring. Sovak (203) related the decrease in toxicity of a series of model compounds to the decrease in a-electrons in the ring. The following groups are listed in the order according to their increasing ability to withdraw a-electrons from the ring: methyleneoxy (ϕ-CH_2-O-) < methyleneamino (ϕ-CH_2-N-) < reversed amido (ϕ-NH-CO-) < alkyloxy (ϕ-O-CH_2-) < carbamoyl (ϕ-CO-NH-) group. Among the nonionic contrast media, listed in Table 10.3, iosimide (7g) and iotriside (7h) are derivatives of trimesic acid, which belong to the CCC subclass and have the lowest a-electron density in the ring.

Gries and Mützel (205) gave the number of a-electrons in the benzene ring, in addition to the six benzene electrons, for a series of model compounds ranging from subclass NNN to subclass CCC. These a-electron values are given for each subclass in parentheses as follows:

"NNN" (a: 0.1088) > "CNN" (a: 0.1077) > "CCN" (a:0.0947) > "CCC" (a: 0.0815).

This means that the model compound derived from triiodotrimesic acid (subclass CCC) contains fewer π-electrons than the model compound derived from isophthalic acid (subclass CCN) and thus has a greater biosafety and a higher intravenous median lethal dose (LD_{50}) than that of the latter. Average values of neural tolerance, expressed in mg iodine per kg body weight, for these model compounds are given in parentheses below:

"CNN" (4.2 mg I/kg)

> "CCN" (11.4 mg I/kg)

> "CCC" (22.7 mg I/kg).

These values support the hypothesis that associates the least toxicity of an iodinated contrast molecule with the smallest π-electron density in the benzene ring.

6.3.5 Hydrophilicity and Solubility. Contrast agents for angiography are by necessity administered intravascularly in large doses and, for this, high water solubility is required. Agents for oral cholecystography need an optimum oil-and-water solubility so that upon ingestion, the molecule can be absorbed and transported across the intestinal cell membrane, from blood to the liver, and concentrated in the gall bladder. Agents for myelography may be oil-soluble or water-soluble compounds. The molecular requirements for different contrast agents differ and do not necessarily focus on the same substituent groups.

Solubility of contrast agents is determined mainly by the presence of hydrophilic groups (180, 209). In an ionic contrast molecule the carboxyl group in the R group in (1) confers a high water solubility to the molecule; and an acylamino, alkylcarbamoyl, or hydroxylated alkylamino group in the X or Y group in (1) modifies such properties as its hydrophilicity, toxicity, distribution, and excretion. Thus, substituted triiodobenzoates and triiodoisophthalamates are highly water soluble compounds. Solutions with concentrations as high as 90% can be achieved with iothalamates and metrizoates. These highly soluble contrast agents are also strong acids with pK_a values less than 3. In nonionic contrast molecules the hydroxyl groups, hydrogen bonding, and hydrophobic associations determine the water solubility (171, 182, 202), which may be further enhanced by introducing more hydrophilic groups (199).

Asymmetry in the substitution of contrast molecules also influences the solubility (9). The sodium salt of diatrizoic acid with a symmetrical 3,5-diacetamido substitution has relatively low water solubility and concentrations greater than 50% cannot be obtained. The solubility is enhanced when one of the acetamido groups is replaced with a *N*-methylcarbamoyl, N-methylacetamido, or acetamidomethyl group, as in iothalamate, metrizoate, or iodamide. N-Methyl substitution can substantially increase the water solubility of nonionic contrast agents (199), but the hydroxyl groups have to be evenly distributed to mask the lateral and facial lipophilic regions in the molecule, distinguished conceptually as lateral hydrophilicity and facial hydrophilicity (204).

Oral cholescystographic agents must possess an optimum oil-and-water solubility for duodenal absorption. Substituents, such as carboxyl, alkyl, or aralkyl groups, can impart both hydrophilicity and lipophilicity to the molecule. Iopanoate, ipodate, and tyropanoate, for example, are substituted triiodophenyl alkanoic acids and will meet this requirement. The chain length of the substituent can affect the quality of the image. Epstein et al. (406) observed that in a series of iodinated *p*-hydroxyphenylalkanoic acids, optimal visualization of dog bladder was achieved with five to eight carbon atoms in the alkanoic acid chain. Felder et al. (291) reported that the insertion of a methyl group between the oxygen and the a-carbon in the series of substituted triiodophenoxyalkoxyalkanoic acids can improve oral absorption, biliary excretion, and gall bladder visualization.

6.3.6 Chemotoxicity. The development of modern contrast agents began with the observation by Wallingford et al. (219) that iodobenzoic acids have very low toxicity. Iodination, substitution, and acetylation can modify the acute toxicity of substituted benzoic acids (209,210). For example, amination of sodium benzoate decreases toxicity, and the intrave-

nous median lethal dose (LD_{50}) values of 3-aminobenzoate and 3,5-diaminobenzoate (i.e., 3270 and 2600 mg/kg, respectively) in mice are higher than that of benzoate (1440 mg/kg). Acetylation decreases toxicity, as is shown by the even higher LD_{50} values of 3-acetamidobenzoate and 3,5-diacetamidobenzoate (3400 and 5580 mg/ kg) than of the corresponding amines given above. In the series of 3-acylamino-2,4,6-triiodobenzoates, the detoxifying effect of substitution by acylation reaches a maximum of two carbon atoms in the acetyl group and further lengthening of the acyl chain causes an increase in toxicity (219).

Iodination may either decrease or increase the toxicity, depending on whether the parent compound is acetylated or unacetylated. Both 3-acetamido-2,4,6-triiodobenzoate and 3,5-diacetamido-2,4,6-triiodobenzoate (LD_{50}: 8300 and 14,000 mg/kg) have lower toxicities than their corresponding noniodinated parent compounds. A fully substituted benzene ring further decreases the toxicity. 3,5-Diacetamido-2,4,6-triiodobenzoate is the least toxic of the series and is available commercially as diatrizoate sodium and meglumine salts for clinical use in angiography, pyelography, urography, and other related roentgenographic procedures.

Although contrast media are remarkably safe, when injected intravascularly in high concentrations as a bolus, the blood is replaced for a very brief period with the contrast medium. Such high concentrations can produce a myriad of dose-dependent pharmacological effects, often manifested as undesirable side effects. Rosati and de Haën (407) classified these toxic effects as (1) chemotoxicity in distinction to molecular toxicity, arising from unique structural features that show affinity to binding with biomacromolecules; and (2) osmotic toxicity, attributed to many side effects caused by the considerably higher osmolality of ionic contrast media relative to that of body fluids. These authors correlated the LD_{50} values of ionic and nonionic iodinated contrast media for uroangiography directly with their osmolality. Nonionic contrast agents, because of their lower osmolality, high water solubility, and a fully substituted benzene ring, do not combine with proteins or macromolecules and are considerably less toxic than ionic contrast media. Contrast agents, regardless of their ionic and osmolality differences, in the presence of X-ray irradation, may cause chromosome aberrations (408–411). Iodinated contrast agents in combination with X-ray radiation can develop synergistic cytotoxicity, possibly mediated by energetic photoelectrons, and this cytotoxicity increases with iodine concentration (410). Norman et al. (413) showed that in iodine dose-enhancement therapy for brain tumors, the iodine contrast media help localize the tumor and increase the absorbed radiation dose.

The contrast bis compounds, in general, have lower toxicity than that of the corresponding monomers (258). The toxicity of hexaiodinated ionic bis compounds increases with increasing length of the alkylene bridge and also with increasing length of the substituent group at position 5 of the triiodophenyl ring (224,414). Bis compounds with open positions in the triiodophenyl rings linked by a polyoxymethylene bridge have a higher intravenous toxicity in mice than those with the fully substituted benzene rings. Replacement of the substituent n-butyramido group with a butyrolactamyl group decreases the toxicity of the bis compounds linked by a short alkylene bridge but increases the toxicity of those linked by a long alkylene bridge. Introduction of one or more oxygen atoms into the alkylene bridge greatly reduces the toxicity. For this series of bis compounds, optimum tolerability was achieved in the compound formed by joining two molecules of 3-amino-5-acetylaminomethyl-2,4,6-triiodo-benzoic acid with tetraoxahexadecane-dicarboxylic acid dichloride (224). Ioxaglate is an asymmetric bis compound, consisting of a dipeptide bridge linked to two dissimilar monomers, one ionic and the other nonionic (189). Ioxaglate is a ratio 3.0 contrast agent and has lower osmolality and lower toxicity than that of the ratio 1.5 symmetric ionic bis compounds.

Hexaiodinated nonionic bis compounds are ratio 6.0 contrast agents and owe their low osmolality, high hydrophilicity, and high biological tolerance to 8–12 hydroxyl groups in the molecule. Iotrolan containing 12 hydroxyl groups has an osmolality isotonic to blood. In concentrated solutions nonionic contrast me-

dia may undergo molecular association (i.e., hydrophobic interaction), to form relatively small aggregates (quasi-dimers or quasi-oligomers), thus giving a lower osmolality than that in dilute solutions (202,415).

6.3.7 Protein Binding and Excretion. The capacity of contrast agents to bind serum proteins is related to certain structural features (414, 416, 417). Contrast agents with a fully substituted benzene ring show little or no protein binding and those having an open position 5 in the ring bind readily to serum protein. Protein binding favors bilitropism (i.e., being hepatotrophic, excreted through the liver) rather than urotropism (i.e., being nephrotrophic, excreted through the kidney). The relative magnitude of biliary and urinary excretion of the contrast agent, also known as the B/U ratio, is determined by the structural features of the molecule, the dose, and the patient's condition. Fumagalli et al. (418, 419) observed that N-alkylation promotes bilitropism.

According to Hansch (420, 421), protein binding is nonspecific and occurs with many compounds with sufficient lipophilicity. The structural requirements for bilitropic agents are sufficient hydrophobicity and an open position 5 in the triiodophenyl ring. Knoefel and Huang (422) observed that protein binding correlates with increasing toxicity in a series of substituted iodinated benzoic acids. In hexaiodinated ionic bis compounds, an increase both in the length of substituents and in the length of the alkylene bridge enhances hydrophobicity and increases the protein binding and the B/U ratio (423). Higher B/U excretion ratios are observed in bis compounds with open position 5 in the phenyl ring than in those that are fully substituted. Introduction of one or more oxygen atoms into the alkylene bridge increases water solubility and reduces toxicity (189).

Nonionic contrast media, by virture of the large number of hydroxyl groups, 4 to 6 in monomers and 8 to 12 in bis compounds, are highly water soluble. These hydroxyl groups are evenly distributed throughout the contrast molecule and leave no lipophilic regions in the molecule free to bind with proteins. The hexaiodinated nonionic bis compound iodixanol, similar to others of this class, is not metabolized and is excreted in the urine unchanged by the kidney (270,258).

6.3.8 Osmolality and Viscosity. Ionic contrast media exert osmotic pressure in solution, and high concentrations of ions cause adverse reactions in the patient (181, 424). The osmotic pressure of a solute is measured in terms of osmolality, in relation to the pressure exerted by a gram-molecular weight of an ideal un-ionized substance dissolved in 1 kg of water (425), measured as mOsm or Osm/kg H_2O. Ionic and nonionic contrast media are referred to as high osmolality contrast media (HOCM) and low osmolality contrast media (LOCM), with values of 1600 and 600 mOsm/kg, respectively; for reference, the osmolality of the body fluids is about 300 mOsm/kg H_2O. Ioxaglate, the monoacidic asymmetric dimer, belongs to the LOCM category. The hexaiodinated nonionic dimers, such as iodecol, iotasul, and iotrolan, may have an even lower osmolality than that of the nonionic monomers (203, 258, 415 mOsm/kg). Krause et al. (415) measured the osmolality of a number of commercial products of X-ray contrast media and ranked their osmolalities at 300 mg I/mL in increasing order as follows: iotrolan $\ll$ ioxaglate $<$ iopromide $<$ iopamidol $<$ ioversol $=$ iohexol $<$ iopentol $\ll$ diatrizoate. The physicochemical parameters of the commercial products of iotrolan (iotrolan-280, iotrolan-300, iotrolan-320) are, respectively: iodine concentrations 278,296,321 (mg I/mL); iotrolan concentrations 0.595, 0.632, 0.687 (g/mL); osmolalities 272,291,317 (mOsm/kg); product densities at 20°C, 1.333, 1.353, 1.379 (g/mL); and product viscosities at 20°C, 13.4, 17.4, 25.3 (mPa·s). At 37°C the density is decreased only slightly but the viscosity is reduced by more than 50%. At 37°C iotrolan-300 has a viscosity of 8.1 mPa·s (426). In comparison, the blood has a viscosity of 4 mPa·s. To include ionicity with osmolality, Hardeman (427) suggested a more exact nomenclature of ILO (ionic low-osmolar), NILO (nonionic low-osomolar, and IHO (ionic high-osmolar) in referring to contrast media.

Osmolality and viscosity depend on the ionic character and structure of the contrast molecule (10, 11, 181, 428). Almén (181) con-

cluded from physicochemical principles that both osmolality and viscosity of a contrast medium can be reduced if nonionic and spherical polymeric molecules are involved. Polymers can have more iodine atoms per molecule than can monomers and a lower osmolality. A prominent example of more iodine atoms and low osmolality in a molecule is the monoacidic asymmetric dimer ioxaglate (203). Spherical molecules show less resistance to flow than do linear molecules and tend to have lower viscosity. Nonionic molecules make no contribution to electrolyte concentration and show no osmotic toxicity. The LD_{50} of ionic diatrizoate when injected into the subarachnoid space is about 50 mg I/kg in mice; in comparison, the LD_{50} values for nonionic contrast agents, metrizamide and iohexol, are in excess of 1500 mg I/kg (182). The remarkable safety of the early nonionic contrast agents provides a stimulus for the search for improved contrast agents, especially as dimers, trimers, polymers, and nonionic compounds. Nonionic iodinated organic compounds, such as triglycosyldiiodobenzene, 2,4,6-triiodo-3-acetamido-5-*N*-methylcarboxamido-phenyl-D-glycopyranoside (186) and others, have been synthesized for clinical tests as potential contrast agents.

Iodinated nonionic dimers iodixanol, iodecol, iofratol, and iotrol (or iotrolan) have respectively lower osmolalities (200, 320, 140, and 320 mOsm/kg H_2O) and higher viscosities (8.7, 7.2, 8.5, and 8.1 mPa·s at 37°C) than those of iodinated nonionic monomers. Felder and de Haën (429) found unexpectedly that solution mixtures of iodinated nonionic monomers and dimers, such as iomeprol and iofratol, have a lower toxicity and a lower viscosity than those of either of the components and can supply a favorable iodine delivery rate through less invasive catheters during the injection, but have an intermediate osmolality compared to that of the pure solutions of the monomer and dimer components.

6.4 Analysis

A contrast medium may be analyzed by conventional means for its iodine content and functionality or by capillary electrophoresis (CE) and high performance liquid chromatography (HPLC) for its components. Nonionic contrast media, such as iosimide, iopentol, ioversol, iopamidol, and iohexol, can be analyzed in a borate buffer by capillary electrophoresis (430, 431). Borate ions combine with the 1,2-diol and 1,3-diol groups in the molecule, to form negatively charged complexes that can migrate in an electric field. Iohexol in serum can be measured and quantitated by CE against an internal standard after deproteinization with acetonitrile (431, 432) or by HPLC after deproteinization with tetrahydrofuran (433). A serum sample of iohexol may also be injected directly onto an HPLC column for analysis after dilution (434). HPLC is a versatile analytical technique and can qualitatively and quantitatively assay contrast agents as intact molecules, geometric isomers, and metabolites. For example, HPLC can separate isomeric iohexol into endo and exo forms (1:3) (267). An automated HPLC system, with online dialysis and column-switching to enrich dialysate, can analyze samples of iopentol in human serum or whole blood (433). An automated analytical HPLC, using a workstation originally designed for the analysis of iopamidol, can be adapted for the analysis of other contrast agents (435). The early HPLC methods relying on elaborate sample purification and higher column temperature for analysis and separation of ioversol, iomeprol, and iopamidol in plasma and urine samples are inconvenient for routine assay (436, 437). An improved method using reversed-phase HPLC and a new mobile-phase system can routinely analyze samples of diatrizoate, iopamidol, and iohexol in the serum and testes of the mouse to study the uptake and clearance kinetics (432). Alonoso-Silva et al. (438) used reversed-phase HPLC to measure and predict the lipophilicities or partition coefficients of nonionic contrast agents. Liquid-liquid partition coefficients measured by HPLC correlate well with the values calculated by the Hansch method (439).

Contrast agents in plasma may also be determined by spectrophotometric procedures (440, 443) or by radiotracer techniques using ^{125}I- or ^{131}I-labeled materials (442–446). Felder et al. (447) measured the concentration of water-soluble radiopaques by colorimetric determination of free aromatic amine function using the Bratten-Marshall reaction. Hart-

mann and Roepke (448) reported methods for determining the purity and stability of iodine-containing contrast agents of the aminobenzoic acid series. In *vitro* analysis of iodine content of contrast media can be carried out by the Sandell-Kolthoff reaction (189) or by inductively coupled plasma atomic emission spectrometry. The iodine concentration in tissue samples can be determined by the fluorescent excitation method by the K_α and K_β characteristic X-rays generated from irradiation with a ^{241}Am source (449, 450). This method can also be applied to in *vivo* measurement of hepatic iodine concentration after administration of a radiopaque (451). The range of iodine determination is 0.05 to 40 mg/mL, with an accuracy of approximately ±10%. Measurements based on function groups or iodine atoms represent the concentration of these groups directly and not necessarily the concentration of the intact contrast agent.

6.5 Pharmacology

Contrast media by necessity are relatively nontoxic. Adverse reactions accompanying their use vary and usually decrease in intensity and complexity in the order intracerebral > intravascular > oral route of administration > topical application (442–457).Contrast molecules that bind proteins, biomacromolecules, and enzymes are more toxic than those that do not. The toxicity decreases with increasing hydrophilicity for different classes of contrast media: oral cholecystographic media > intravenous cholangiographic media > ionic triiodinated uroangiographic media > monoionic hexaiodinated molecules > nonionic triiodinated contrast media > nonionic hexaiodinated contrast media. Median lethal dose (LD_{50}) is an indication of systemic toxicity, even though it lacks predictive value (458). The intravenous LD_{50} values of the last four classes of contrast media are in the range of tens of grams per kilogram body weight when tested in rats or mice. The opacifying atom iodine has an intravenous LD_{50} in animals of 0.8–1.0 g/kg; the intravenous LD_{50} values (expressed in g Iikg in mice) of uroangiographic contrast agents increase, for example, from 7 for ionic iothalamate to 13.8 for monoacidic dimer ioxaglate and then to even greater values for nonionic contrast agents, such as 18.1 for metrizamide, 22.1 for iopamidol, and 24.3 for iohexol (178). The subarachnoid LD_{50} values of ionic diatrizoate and nonionic metrizamide and iohexol differ by a factor of 30, whereas their osmolalities differ by a factor of only 2 (459). The oral cholecystographic agents have considerably higher toxicity but the side reactions are generally restricted to gastrointestinal symptoms.

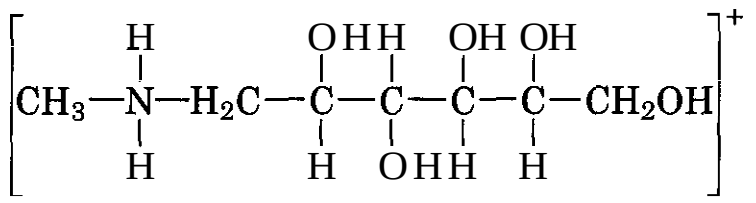
Procedures in angiocardiography, intravenous digital subtraction angiography, and rapid-scan CT require rapid and multiple intravascular injections of low osmolality contrast media, causing significant hemodynamic changes (460,461). A bolus injection can be as short as 2 s (460, 461), and a volume of up to 4 mL I/kg body weight may be injected (462). According to Golman and Almén (463), the toxicity of contrast media is dependent on injection rate and dose, and increases with either an increase in the dose or an increase in the rate of injection, or both. The dose-response curve is typically an S-shaped curve with dose as abscissa and percentage mortality as ordinate, and this curve shifts in its entirety along the dose axis according to the rate of injection. The curve begins at a lower dose level with rapid injection, indicating greater toxicity, and shifts to a higher dose level with slow injection, indicating greater safety. Although delivery by slow injection is safer than by rapid injection, rapid injection of contrast medium gives a better image quality. de Haën et al. (458) suggested that the mortality ratio, which incorporates both volume factor and injection time factor in its definition, can serve as a better predictor than the median lethal dose (LD_{50}) for acute intravenous toxicity of contrast agents. The mortality ratio is a function of three parameters: the osmotic load (referring to excess osmolality associated with isotonic volume), the volume of contrast medium, and the duration of injection. The endpoint is death, measured by the time between administration of the dose and death of the mouse, and is closely dependent on the rate of injection of the contrast medium. This suggests that the mechanism of contrast medium toxicity may involve early damage and late consequences (458).

The pharmacological aspects and toxic reactions of contrast agents have been compre-

hensively reviewed by Hoppe (464), Almén (457), Ansell (465), Berk and Loeb (466), and Speck et al. (258).

6.5.1 **The Cations.** Iodinated ionic contrast agents (ratio 1.5) used in angiography, urography, and intravenous cholangiography are substituted 2,4,6-triiodobenzoic acids. These agents are formulated for intravenous administration as solutions of salts, ionized at physiological pH. Their sodium salts have high water solubility, and solutions of 50–90% concentration, corresponding to an iodine content of about 300 to more than 400 mg I/mL, can be prepared for angiographic use (10, 11). Ionic contrast media with sodium concentrations of 118–370 mEq/100 mL rarely produce ventricular fibrillation in perfused rabbit heart (467) but sodium ions in excess amount are cardiotoxic (468). The sodium ion concentration of ionic contrast media is many times higher than that of plasma. Their toxicity in high concentrations is attributable not only to high osmolality but also to the effects of their cation and anion components (9, 10, 34, 36–43). The cations in regular use in contrast media are N-methylglucamine, sodium, calcium, and magnesium.

N-Methylglucamine (meglumine) ion has the following structure:



Meglumine salts of contrast media such as diatrizoic, iothalamic, and metrizoic acid have higher viscosities than those of the corresponding sodium salts but are better tolerated and less toxic. The high viscosity of the meglumine salts can be reduced by mixing with the sodium salt of the corresponding acid (469). Mixed salts of diatrizoate are marketed under various proprietary names (223). During coronary angiography, sodium ions in contrast media reduce the risk of ventricular fibrillation and reverse the reduction of contractile force of the myocardium. A recent study showed that meglumine diatriazoate without Na^+ caused a higher frequency of ventricular fibrillation in isolated rabbit heart than did diatrizoate with 77 or 154 mmol of Na+ added (470). Also, the meglumine salts of ionic contrast media inhibit in *vitro* leukocyte phagocytosis more than do the sodium salts (471).

Other organic bases, such as diethylamine, monoethanolamine, diethanolamine, triethanolamine, and morpholine have low toxicity and can be used in place of cations (9, 225). The monoethanolamine salt of ioxitalamic acid shows a toxicity intermediate between those of sodium and meglumine salts (9). Diethanolamine and morpholine form 1:1 salts with 3,5-diiodo-4-oxo-1(4*H*)pyridine acetic acid, named iodopyracet (Diodrast) and Joduron (225), respectively. Other cations such as basic amino acids also form salts with the acid form of contrast agents to reduce toxicity (472). For example, ionic diatrizoate, iothalamate, and metrizoate form salts with Tris, which is 2-amino-2-(hydroxymethyl)-1,3-propanediol, to yield solutions of lower viscosity than that of the corresponding meglumine salts. Tris is an aliphatic amine with a negative temperature-acidity coefficient; that is, its pK_a value decreases about 0.025 unit with each degree Centigrade increase in temperature. This property allows the nonionic contrast agent iohexol to be formulated in Tris buffer at physiologic pH (7.0–7.5) at room temperature and to be heat sterilized at 120°C, when the pH falls below 5.5 to avoid deterioration. The product after sterilization will return to its original physiologic pH at room temperature for use in angiography without causing discomfort in the patient; this would not have been the case had the solution remained at pH below 5.5 (178,473). Another approach without using an amine buffer is to adjust the pH of iohexol in a citrate-EDTA buffer to less than 5 with carbon dioxide before sterilization (474).

In normal blood, sodium, potassium, calcium, and magnesium ions are present in concentrations of 330, 20, 5–6, and 2–2.5 mg/100 mL plasma, respectively (475). The physiological functions of the cations are both antagonistic and complementary to one another. Sodium ions tend to increase the permeability of cell membrane, whereas calcium ions counteract this effect. Calcium and magnesium ions are physiological antagonists; magnesium ions can exert a deleterious effect on the nervous system if unaccompanied by the revers-

ible antagonistic effects of calcium ions. The physiological interaction between these ions suggests that the toxicity of sodium ions in contrast medium can be modified by the addition of small concentrations of calcium and magnesium, although incorporation of potassium ions affords no beneficial effect. To minimize the toxicity, an optimum concentration of calcium and magnesium ions in the contrast medium is essential and should be 2.5 times their concentration ratio in plasma (i.e., the ratio of concentration of calcium or magnesium ions to that of sodium ions). Incorporation of optimal amounts of calcium and magnesium ions in solutions of sodium metrizoate can increase the LD_{50} values by 80–100% in the rabbit. Calcium ions alone lower the toxicity of methylglucamine metrizoate in LD_{50} tests in mice but not in the rabbit, whereas magnesium ions alone cause an increase in toxicity (475). A small amount of calcium ions in methylglucamine metrizoate causes less blood-brain barrier damage than when pure methylglucamine metrizoate of identical iodine content is given alone (476). Also, addition of calcium salt inhibits metrizoate- and ioxaglate-induced serum complement activation (477). Formulations of diatrizoate that minimize calcium binding are advocated for cardiac angiography (478).

Nonionic contrast media are intrinsically ion-free but iohexol, iopentol, and iodixanol, for example, contain cations at subplasma concentration levels to avoid negative effects on myocardium contractility, electric conduction, and red blood cell aggregation during coronary arteriography. An oxygenated iohexol solution (350 mg I/mL) containing (in m*M*/L) Na^+ 30, Ca^{2+} 0.15, K^+ 0.9, and Mg^{2+} 0.1, when injected into the left coronary artery of a pig, produces fewer electrocardiographic and/or hemodynamic changes than pure iohexol or iopamidol(479–481).Compared to no sodium or excess sodium, the addition of a small amount of sodium to iohexol and iopentol lowers the risk of ventricular fibrillation and causes the least decrease in the contractile force of the isolated rabbit heart. A physiological amount of sodium ions is important in test solutions of 20% mannitol, ioxaglate, iopamidol, iotrolan, and sodium iopamidol to prevent fatal arrhythmia (482). In cardiac angiography, optimal concentrations of Ca^{2+} and Mg^{2+} additives are necessary to maintain myocardium contractile function and reduce adverse reactions to contrast media (483,484). Thus, a balanced electrolyte formulation for even low osmolality contrast media is essential to minimize ion toxicity (459,470,484).

6.5.2 **Hyperosmolality.** Ionic contrast media for angiographic use are hyperosmotic relative to plasma. The osmolalities of the substituted triiodobenzoate anions diatrizoate, iothalamate, and metrizoate are approximately the same on the basis of equal iodine content (485). Injection of hyperosmolar contrast media causes hemodynamic responses that vary according to the rate and site of injection (469). Slow injection into a peripheral vein allows visualization of the collecting systems of an organ, and rapid injection of a more concentrated solution allows visualization of the vasculature of an organ or a region of the body (469). Slow injection into the peripheral veins as in urography and intravenous cholangiography produces hypotension because of a lowering of peripheral vascular resistance (486). Acute intravenous toxicity of contrast medium is increased with the rate of injection (184, 487–489). A large volume of hypertonic contrast medium, when rapidly injected into veins, arteries, and ventricles in selective &■ giography causes an elevation of the plasma osmolality and a shift of water out of red blood cells and out from extravascular spaces to the blood, thereby increasing the circulating blood volume and peripheral blood flow and affecting various aspects of circulation (10,428,466, 468, 469, 490–496). The more concentrated the injectate, the more intense the vascular reactions, and the more precipitous the onset (469,491).

Systemic hypotension, changes in the activity of the smooth muscle cells in vessel walls, aggregation and crenation of red blood cells, and hypervolemia are examples of vascular reactions induced by the administration of ionic contrast media. Agglutination and crenation of red blood cells cause blood sludging and reduce the cells' ability to undergo deformation, thus blocking the blood flow to capillaries and leading to pulmonary hypertension and related physiological responses

(495–498).The hypervolemia resulting from a shift of water elevates the ventricular filling pressure and increases the cardiac output from increased ventricular stroke work. The potassium ions released from shrinking and crenating red blood cells contribute to the lowering of systemic vascular resistance and blood pressure (499).

That osmolality is a major determinant of hemodynamic effects is clear from the experiments conducted by Kloster et al. (500), who injected equiosmolar solutions of mannitol, sodium, and meglumine salts of diatrizoate and iothalamate, with an osmolality corresponding to 4.5 to almost 8 times the osmolality of blood serum, directly into the left ventricle of a dog and observed approximately similar hemodynamic responses. D-Mannitol is often used in osmotic effect studies on cells, organs, and organisms as a reference (458) because it is inert, distributed in extracellular space, and acts as individual molecules, not as dilution-dependent aggregates. Hilal (493) reported that the bradycardia, hypertension, and accompanying vascular reactions attributed to intracarotid injection of contrast media could be reproduced by injection of hypertonic solutions of sodium chloride of similar osmolarities. By comparing the hemodynamic effects from intracarotid injection of 50% sodium diatrizoate, 80% sodium iothalamate, and equiosmolar solutions of sodium chloride and dextrose, Cornell (501) concluded that the sodium ions may play a greater role than hypertonicity in causing these effects.

Toxic effects of sodium ions and hyperosmolality associated with monomeric ionic contrast agents are lessened when dimeric and trimeric contrast agents containing, respectively, six and nine iodine atoms per molecule are used. Both bis(iothalamic acid) (i.e., iocarmic acid) and tris(iothalamic acid) produce fewer peripheral vasodilatation and reflex responses than does the monomer iothalamic acid (9).

Ionic contrast monomers at a solution concentration of 300 mg I/mL have an osmolality in the range of 1.5–1.7 Osm/kg H_2O. At the same solution concentration, nonionic monomers, monoacidic ionic dimer (ioxaglate), and nonionic dimers have osmolalities of 0.6–0.7, 0.56, and about 0.3 Osm/kg, respectively. If made isotonic to body fluids, the isotonic ionic monomer solutions have an iodine content of 70 mg I/mL; the nonionic monomer solutions, 150; the monoacidic ionic dimer ioxaglate solution, 150; and the nonionic dimer solutions, 300. The osmolalities of nonionic dimers or bis compounds are isotonic to body fluids and are the lowest of all contrast media. Hyperosmolality causes vascular pain. Sovak et al. (502) estimated the concentration threshold for vascular pain to be about 750 to 800 mOsm. Thus if the osmolality of a contrast medium at a concentration of 350 mg I/mL does not exceed the pain threshold, the solution would not be expected to induce pain in arterial arteriography. Metrizamide at 550 mOsm/kg is essentially painless in peripheral angiography (503, 504), and all the nonionic contrast media are expected to behave similarly. Metrizamide has an extremely low cellular toxicity and can be injected at an iodine concentration of 380 mg I/mL into the common carotid artery of guinea pig without damage to the blood-brain barrier.

6.5.3 Adverse Reactions and Toxicity. The toxicity of a contrast medium is attributed to its molecular structure, solution property, formulation, and the amount used as a dose. Toxicity may be further classified as chemotoxicity, osmotoxicity, and ion toxicity (407, 459). Chemotoxicity is related to a molecular structure that allows binding to proteins, leading to interaction with biomacromolecules such as enzymes, cell membranes, and cell components. Osmotoxicity is attributed to the hyperosmolality of contrast media, and a marked difference exists between the osmolalities of ionic and nonionic contrast media. Ion toxicity refers to adverse reactions as a consequence of too high or too low ion concentrations in contrast media that interfere with cellular function. Contrast media formulations containing citrate and EDTA buffers can interfere with serum Ca^{2+} and affect myocardial contractility function. Contrast media are transported by the cardiovascular system and are excreted by the kidneys. Any reactions to contrast media are known as adverse reactions.

Contrast media are injected intravascularly into the patients in large volumes and high concentrations. The size of the injectate, together with its high osmolality and viscosity,

can produce an intolerant environment to cells, producing abnormal physiological effects. Patient safety and efficacy of iodinated contrast media are of great concern to health professionals (505, 506). Many aspects of adverse reactions to contrast media, such as etiology (459, 507, 508), adverse reactions in the patients (505, 506, 509–5181, and premedication to decrease adverse effects (519–522) have been researched and reviewed.

In humans adverse reactions to contrast media vary greatly in type and severity from slight nausea or mild "hot flush" through a broad spectrum of increasingly severe cutaneous, respiratory, neurological, and cardiovascular disturbances that may, in extreme cases, result in the sudden death of the patient (454–457,459,465,469, 507). Ansari and Baldwin (523) reported acute renal failure after urography with meglumine diatrizoate, angiography with meglumine and sodium diatrizoate, oral cholecystography with iopanoic acid, and cholangiography with iodipamide. Roylance et al. (524, 525) reported the incidence of reactions to urographic agents.

To review the incidence and severity of adverse reactions to contrast media, a committee on contrast media was formed under the auspices of the International Society of Radiology in 1969 (526). Based on the analysis of a total of 112,003 cases of adverse reactions toward contrast media from Australia, Belgium, Canada, Norway, and the United States, Shehadi (452) reported the nonfatal incidence of reactions to be 2.3% for vascular studies, 5.65% for intravenous urography, and 10.11% for intravenous cholangiography. The incidence of reactions for total intravenous and intraarterial examinations was 4.95%, and the incidence of fatal reactions was about 1110,000, which is higher than the 1160,000 reported earlier on the basis of retrospective studies. The contrast agents used in the cases studied were meglumine diatrizoate, meglumine iothalamate, sodium diatrizoate, and sodium metrizoate. These studies did not include nonionic contrast media and predated their use.

In intravenous cholangiography the frequency of toxic symptoms was 12.6% for single-dose medication and 8.16% for drip infusion (452). In intravenous urography the incidence of reactions was 5.38% for single bolus injection and 7.06% for drip infusion that required a higher dose of contrast medium (452). The incidence was higher with slow injection over a period of 3 to 10 min than with rapid injection performed in less than 2 min. This suggests a reversal of the hitherto widespread practice of administration of contrast media. Although the cause of this low incidence of reactions with rapid injection is not explained, it is not unreasonable to assume that less histamine is released (489).

In hypersensitive or allergic patients, idiosyncratic reactions may occur from a dose of contrast medium, and the overall incidence of adverse reactions in patients with allergy is about twice that in the general population (452–455,527). Pretesting the patient by intradermal, subcutaneous, or intravenous injection of small doses has no value in predicting adverse reactions (452–455, 525, 528) but testing by *in vitro* leukocyte challenge has been reported to be of some predictive value (529). Premedication with antihistamines, anticholinergics, and diazepam (468) is less effective than that with corticosteroids (454,525).

Shehadi and Toniolo (453) in 1980 reported the result of a survey on the safety of intravascular ionic contrast media in more than 300,000 cases collected from the United States, Canada, Europe, and Australia and showed that the overall incidence of reactions was slightly below 5%, with neither significant geographic or racial differences nor differences related to age, sex, and weight. Most of the patients were in their third and fourth decade of life.

Beginning in 1980s the use of low osmolality nonionic contrast media has significantly reduced the severity and frequency of adverse reactions to contrast media. The high cost of nonionic contrast media becomes an economic barrier to a complete replacement of the ionic contrast media. The price ratio of nonionic to ionic contrast media in 1989 was about 3.5–6.5 times to 1 in Europe and about 13.2–23.6 times to 1 in the United States (531). Prescribing an ionic contrast medium to a patient in the face of a better choice, on the basis of cost consideration, can have serious ethical and medical implications (532). Palmer published an interim guideline that identified high risk groups who should receive nonionic contrast

media, that is, patients with previous reactions to contrast media, patients with asthma, allergy (not to drugs), renal and cardiac impairment, diabetes mellitus, myelomatosis, and sickle-cell anemia; poorly hydrated patients; and infants and small children.

The Royal Australasian College of Radiologists (RACR) survey included 109,546 cases of mild. moderate, and severe reactions to intravenous ionic and nonionic contrast media (533, 534). The report defined mild reactions as all skin reactions requiring no therapy and excluding sensation of heat; moderate reactions as those requiring therapy but not considered at risk and no hospitalization, and severe reactions as those requiring urgent therapy and considered at risk and requiring hospitalization. The incidence of mild, moderate, and severe reactions in the high risk group were 7.20, 2.70, and 0.36%, respectively, for ionic contrast media, and 1.10, 0.10, and 0.03%, respectively, for nonionic contrast media. Incidences of moderate and severe reactions in the high risk groups receiving ionic and nonionic contrast media were 1 in 32 injections and 1 in 718 injections, respectively. This clearly demonstrates a significant advantage for the nonionic contrast media.

The Japanese Committee on the Safety of Contrast Media conducted similar studies from 1986 to 1988 (535–537). The large scale, nationwide clinical study included 337,647 cases. One-half of the group (50.1%) received ionic contrast media and the other half (49.9%) nonionic contrast media. The overall prevalence of adverse reactions was 12.66% in the ionic contrast media group, and 3.3% in the nonionic contrast media group. The prevalence of severe adverse reactions was 3.13 and 0.22% in the ionic and nonionic contrast media groups, respectively. The report detailed the prevalence of adverse reactions regarding time of onset, patient sex and age, patient history on prior reactions to contrast agents, allergy, underlying disease, premedication, injection mode, and dose. The prevalence of adverse reactions was the lowest in the subgroup of patients receiving contrast media by bolus and intravenous injection as compared to by drip infusion and by bolus plus drip infusion. The patient subgroup receiving a dose ranging from 81 to 100 mL of contrast media, ionic or nonionic, showed the lowest prevalence of adverse reactions (537). The study clearly demonstrates that nonionic contrast media can significantly reduce the prevalence of adverse reactions to contrast media. Reducing the mild adverse reactions such as nausea, vomiting, urticaria, itching, and heat sensation can make contrast media examinations more acceptable to the patient. Bettmann (537) noted that the report showed no difference between the ionic and nonionic contrast agents with regard to mortality. Fareed et al. (538) pointed out that both the Japanese and the Australian survey reports on the safety of contrast media did not include any information on thrombotic complications.

The use of premedication with steroids and H_1 and H_2 histamine receptor blockers can significantly reduce the adverse reactions to high osmolality contrast media. There are suggestions to extend their use to modify even the reactions to low osmolality contrast media. Pretesting of contrast media reactors by injecting 1 mL of the nonionic contrast medium has also been reported (539).

Greater use of nonionic contrast media for routine X-ray procedures is clearly the trend of the future. It was estimated that in 1991, approximately 69% of the procedures performed in the United States used nonionic contrast media compared to about 66% in Europe and more than 80% in Japan. In 1994 the percentage increased to about 80% in the United States, 75% in Europe, and 92% in Japan (540). In the United States, in 1994 as many as 18 million patients received intravascular contrast media, and an estimated 170 million contrast medium-enhanced radiologic studies were performed from 1978 to 1994 (541).

Spring et al. (541) analyzed the reports of adverse drug events (ADE) of iodinated contrast media, filed with the Spontaneous Reporting System and MedWatch Program of the U.S. Food and Drug Administration (FDA) from 1978 through 1994. In this 17-year period, there were 22,785 reports (86.5%) of mild or moderate ADEs, 2639 reports (10.0%) of serious nonfatal ADEs, and 920 reports (3.5%) of deaths describing 850 fatal ADEs. Reporting to the FDA Spontaneous Reporting System and MedWatch Program is voluntary. *Ad-*

verse drug event (ADE) is the term used to avoid contributing causation and to encourage reporting of adverse consequences from professional practice. When these events are specifically attributed to the drug, they are called ***adverse drug reactions*** (ADR). To identify any changes in the frequency of reporting between ionic and nonionic contrast media, the authors arbitrarily divided the 17-year period into an earlier period (1978–1986) before FDA approval and wide use of nonionic low osmolality contrast media (LOCM) and a later period (1987–1994), when the use of nonionic LOCM rose steadily over time after approval. In 1994 the use of nonionic LOCM exceeded ionic HOCM by 2:1. For ionic and nonionic contrast media the most common ADEs (urticaria, dyspnea, vomiting, facial edema, and hypotension) ranked similarly, and the intravenous and intraarterial routes (97.4%)were the predominant routes of administration. The serious nonfatal ADEs were most often related to serious cardiorespiratory symptoms. Myelographic ADRs (e.g., seizures, headaches) from intrathecal administration of contrast media differ from the most common ADRs mentioned above and were treated separately. The authors noted the prevalence of severe nonfatal ADEs to be 0.044% for nonionic LOCM and 0.26% for ionic HOCM, comparable with the published values (537, 538) and also noted that the number of ADEs reported did not significantly decrease since the introduction of nonionic LOCM.

Mortality as a direct consequence of use of iodinated contrast media is a rare occurrence. Hatayama et al. (535) observed two deaths among 337,647 cases in which iodinated contrast media were injected intravenously. Spring et al. (542) showed that 37% more deaths were reported each year in 1987–1994 than in 1978–1986. Most of the increase was associated with the use of nonionic contrast media. In 1978–1987, 376 deaths were reported and in 1987–1994,474. In 1987–1994, 220 deaths were associated with use of ionic HOCM alone, 32 with ionic LOCM alone, and 214 with nonionic LOCM alone. The calculated occurrence of 1 death per 200,000 or more examinations was comparable with published data. The authors remarked that the availability of LOCM in the United States did not lead to a marked decrease in the contrast media-related deaths.

Lasser et al. (543) compared the patient reactions to ionic and nonionic contrast media based on data on reactions to X-ray contrast materials collected under the supervision of the FDA Division of Epidemiology and Surveillance and manufacturer data from 1990 through 1994. In 1992–1994 there were more than 16 million examinations performed ***in toto*** each year in angiography, CT, and urography. The ionic HOCM, nonionic LOCM (iohexol, iopamidol, and ioversol), and ionic LOCM (ioxaglate) had the following incidences per million intravascular examinations for total reactions 193.77:44.44:142.52; for severe reactions, 37.44:10.52:33.61; and for deaths, 3.90:2.07:6.39, respectively. The incidence of severe reations to total reactions was higher with nonionic LOCM (23.7%)and ioxaglate (23.6%)than with ionic HOCM (19.3%). The incidence of death to severe reactions was 19.7% with nonionic media, 19.0% with ioxaglate, and 10.4% with ionic media. In patients with pathologic cardiac conditions, the incidence of renal failure was 3.6 times higher with LOCM than with HOCM.

Bettmann et al. (544) created a prospective registry for surveillance of incidence of adverse events related to ionic and nonionic contest media in angiography, to identify patients likely to benefit from use of low osmolality contrast agents (LOCAs) and to ascertain risk factors for increased incidence of an adverse event. The registry consisted of data of 75,616 studies from 60,891 patients in angiography at 26 high volume institutions, 56% with nonionic LOCAs, 8% with the ionic LOCA, and 36% with HOCAs in a 2-year study (July 1, 1990 to June 30, 1992). Most major risk factors correlated with an increased incidence of adverse events, but varied with the type of procedure, with a higher incidence associated with cardiac and interventional procedures. The incidence of adverse events was significantly increased with use of HOCAs, but severe adverse events were not much different between ionic and nonionic contrast agents except for cardiac procedures. Patients with a previous reaction or more than one other ma-

jor risk factors are most likely to benefit from use of LOCAs.

Several hypotheses were postulated on the pathogenesis of contrast mediareactions. Lalli (545), after reviewing a large number of severe reactions to contrast media and mortality in cholangiography, angiography, and urography, proposed that all reactions are based on the effect of contrast media on the central nervous system. Contrast media are 400–1400 times more toxic intracisternally than intravenously (213,546). Penetration of the blood-brain barrier and direct stimulation of the brain by contrast media can produce abrupt responses leading to immediate and severe contrast media actions. Lasser (508, 547) noted that the nonionic contrast media have substantially reduced the local toxicity in animal and human studies, although the impact on systemic toxicity is less clear. The author postulated an allergy diathesis to prime a patient for a contrast media reaction and believed that bradykinin may contribute significantly to the reactions. Contrast media disrupt endothelium surfaces, causing degranulation of mast cells, releasing histamine and substances capable of contact activation (548). These substances are listed as an endogenous heparin-like material (EHM), a cryptic soluble surface (CSS), or a negative surface (549) and a complex of the plasma inhibitor a,-macroglobulin with kallikrein (508), that can cause complement activation to release the Hageman factor XII, inducing sequentially the cleavage of prekallikrein to produce kallikrein and the cleavage of kininogen to produce bradykinin. Bradykinin is a more persistent and potent mediator than histamine in anaphylaxis and may mobilize arachidonic acid to produce leukotrienes and vasoactive prostaglandins. Contrast media inhibit angiotensin-converting enzyme, responsible mainly for *in vivo* bradykinin hydrolysis. Premedication with corticosteroids may reduce the concentration of complement activators. Lasser et al. (512, 549) examined and evaluated contact system dynamics in patients' plasma and compared the plasmas of asthmatic patients with that of the controls. Moreau et al. (550) discussed Lalli and Lasser's theories and showed the biological chain reactions induced by contrast media and those blocked by premedication with drugs.

Brasch (541, 552) reviewed the severe, immediate reactions to contrast media and suggested that reactions, often designated as anaphylactoid, may have an antibody basis. A prospective study by Laroche et al. (543) on the mechanisms of severe, immediate reactions to iodinated contrast material gave support to this theory. Laroche et al. measured the levels of plasma histamine, tryptase, and urinary methylhistidine by radioimmunoassay or immunoradiometric assay in blood and urine samples from controls and from patients who experienced allergic-type reactions after the administration of contrast material. The serum level of specific immunoglobulin E (IgE) against ioxitalmate or ioxaglate was determined by the radioactive anti-IgE antibody techniques of Guéant et al. (544). The human anaphylatoxins C3a and C4a were measured in plasma using radioimmunoassays, and serum C3 and C4 levels measured by nephelometry. The patients with severe reactions all showed higher measured values than those of the controls, and the values increased with severity of reactions. Histamine concentration decreased rapidly with time and reached normal within 1 h after the reaction, but the tryptase level decreased more slowly than histamine. The half-life of histamine in healthy volunteers is less than 2 min. Tryptase and histamine levels are useful for the diagnosis of anaphylactoid reactions during anesthesia. The study showed that the frequency of immediate reaction is higher with ionic contrast material of high or low osmolality than with nonionic contrast material, and that patients with risk factor of a previous reaction to ionic contrast material react less frequently to nonionic contrast material. The study pointed out the involvement of IgE-mediated reactions.

6.5.4 Toxic Effects on Red Blood Cells. Contrast media produce morphological changes of red blood cells by altering their size and shape and also increase their tendencies for aggregation (555, 556). The change in morphology of red blood cells is caused by chemotoxicity and hyperosmolality of the contrast medium (557, 558). The chemotoxic effect affects the red blood cell membrane and transforms the normally biconcave disc-configured red blood cell into a crenated cell known as an

echinocyte. Ionic and nonionic contrast media, such as iohexol, iopentol, metrizamide, and metrizoate in solution isotonic to blood produce echinocytes, but high concentrations of iopamidol, iohexol, and iopentol also produce stomatocytes. Nonionic dimer iodixanol transforms the normal red blood cells entirely to stomatocytes. These two types of cells differ from each other in relation to the sites of contrast medium binding on the bilayer cell membrane. The exact mechanism is unclear, but shift from echinocytes to stomatocytes may occur by transbilayer diffusion, a well-known phenomenon in drug-erythrocyte interaction (557, 559). In addition to the chemotoxic effect, the hyperosmolality of the contrast medium also affects the morphology of the red blood cells by pulling water, as it were, out of the normal cells to produce a shrunken red blood cell, known as a desicocyte. Among the nonionic contrast media that cause echinocyte formation, metrizamide shows the most chemotoxic effect. Iohexol, iopamidol, and iopentol are comparable to one another and show the least chemotoxic effect on cell membrane (557,558). Bucherer et al. (560) used a micromanipulation technique to visualize the deformation of individual erythrocytes and found that the low osmolar monoacidic contrast agent ioxaglate makes the erythrocyte membrane less rigid and the nonionic iopamidol makes the membrane more rigid compared to the control. Hardeman et al. (427) compared the *in vitro* effects of ioxaglate, iohexol, and iopamidol in different iodine concentrations on whole blood, collected from healthy adults and uremic patients, with respect to RBC morphology and aggregation and found that RBCs in the presence of 50% iohexol and iopamidol were considerably rigidified, whereas the addition of 50% ioxaglate resulted in only moderate RBC rigidification. Iohexol gave a mixture of normal biconcave erythrocytes and echinocytes, iopamidol mostly echinocytes, and ioxaglate mostly normal RBCs with a high degree of rouleaux formation in all different samples tested. Parvez and Patel (561) observed by transmission electron microscopy that ioxilan affected the erythrocyte membrane less than iohexol and iopamidol; the latter two produced acanthocytes, whereas ioxilan had no effect on erythrocyte morphology.

Human erythrocytes at rest have a diameter of approximately 7.5 μm with a higher degree of deformability (558, 562). The normal disc-shaped erythrocytes are aggregated in rouleaux formation and retain a smooth surface, allowing the cells to bend and deform to pass through capillaries with diameters ranging from 3 to 5 μm (563). A high percentage of crenated and deformed cells inhibits rouleaux formation and causes disaggregation. Normal red blood cells have an average water content of 66.46 $\pm$ 1.12% by weight and may lose up to 11% of internal water on coming into contact with a hyperosmolar solution of contrast medium (564). The loss of internal water increases the red blood cell viscosity, which results in reduced deformability and rigidification of red blood cells, causing increased resistance to flow and disturbance of the rheology of blood flow in microcirculation (565, 566). The rigidified erythrocytes can block the pulmonary capillaries and cause an increase in pulmonary arterial pressure. The high viscosity of the contrast medium may prolong its passage through the vessels compared to that of less viscous solutions. Increased contact of contrast medium with the vessel wall can cause endothelial cell contraction, leading to endothelial damage and patches of denudation, which can be the location of thrombus formation (567).

Nonionic contrast media, although more biotolerant than ionic contrast media, pose a serious risk of thromboembolism during clinical cardiac procedures. A critical review shows that both ionic and nonionic contrast media prolong whole blood and plasma clotting times in a dose-dependent manner and inhibit *in vitro* aggregation of platelets, with the nonionic contrast media exerting less interference in coagulation and platelet aggregation than the ionic contrast media (568). Kurisu and Tada (569) show that in a canine model, the ionic low osmolar ioxaglate has a greater anticoagulant effect than that of the nonionic contrast agent iopamidol, which is about the same as the saline control in its inhibition of clot formation. Levi et al. (570), by use of ^{125}I-labeled fibrinogen as a marker to study the effect of contrast media on thrombus (aggregated platelets) growth in a rabbit jugular vein thrombosis model, showed that

the ionic ioxitalamate and ioxaglate exhibit marked inhibition of thrombus growth, whereas the nonionic contrast agents as a group show prothrombotic effect with iopamidol promoting more thrombus growth than iopromide and iohexol. The thrombogenic effect of iopamidol can be inhibited by simultaneous administration of 80 IU/kg heparin. According to Hwang et al. (571), the optimal heparin concentration to achieve a complete anticoagulant effect is 5 IU/mL.

Thrombus formation is a part of the coagulation cascade, complicated and involving many factors, and occurs when a nonionic contrast medium is mixed with whole blood (572, 573). According to Ing (574), ionic and nonionic contrast media exert their actions at different stages of the clotting process. Ionic contrast media inhibit thrombin activity and fibrin polymerization, whereas nonionic contrast media inhibit only fibrin polymerization. Ioxaglate inhibits both the coagulation sequences before thrombin generation and the final stages of fibrin monomer formation and polymerization, whereas the nonionic iopamidol and iohexol inhibit coagulation primarily after the generation of thrombin. This difference in inhibition may lead to serious complications for nonionic contrast media because some unclotted mixtures of blood and nonionic contrast agents, which may contain thrombin, may cause clotting on reinjection. Grabowski et al. (575) showed that both nonionic and ionic contrast media retard clotting in plastic and glass angiography syringes in comparison to saline control, and ionic contrast agents are stronger anticoagulants. Container and syringe surfaces influence the formation of thrombus in mixtures of nonionic contrast media and blood, and plastic syringes were found to retard clotting better than glass ones (573, 575). By use of scanning electron microscopy Corot et al. (576) investigated the surfaces of polyethylene (PE) and polyurethane (PU) and inside surfaces of catheters after contact with mixtures of blood and contrast media, such as diatrizoate, ioxitalamate, ioxaglate, iopamidol, and iohexol. The PE surface was relatively heterogeneous and the PU surface was smooth and homogeneous. Iopamidol and iohexol, when mixed with the blood, formed thick fibrin fibers on the PE surface and not on the smooth PU surface. In comparison, the ionic contrast agents showed no significant fibrin formation on either of these surfaces. The blood-catheter interface at the internal surface of catheters after injection of ioxaglate or iohexol or iopamidol, collected from different European clotting centers, showed signs of blood activation, platelet activation, fibrin formation, or occurrence of red thrombi. These findings show that the clotting process is procedure dependent and varies between these centers and that ioxaglate is significantly better than the low osmolality nonionic contrast media in retarding clotting.

Krause and Press (577) studied the effect of nine contrast media and four gadolinium chelates on blood coagulation and measured their thromboplastin time (TPT). All contrast media influence TPT, but the driving force is ionic charge. The authors found that these agents induced a stastistically significant increase in TPT, listed in the following ascending order of the increase: plasma = saline < gadobutrol < iodixanol = iotrolan = iohexol (different from iodixanol) = iopental (different from iodixanol and iotrolan) < iopromide < iomeprol ≪ Gd-DTPA ≪ ioxaglate ≪ diatrizoate. Ionic contrast agents interact with the coagulation cascade to a greater degree than nonionic contrast agents and are able to influence TPT and to combine with calcium ions. The TPT in human plasma was found to be longer than that in other species, and the interspecies ranking for TPT was dog < rabbit < rat < human. The addition of heparin to contrast media significantly prolonged TPT.

In a human endothelial cell culture, ioxaglate appears to be more potentially toxic to endothelium than diatrizoate, iohexol, iopromide, ioversol, iotrolan, and gadolinium-DTPA (578). Nitric oxide, known earlier as "endothelial-derived relaxing factor," may also play a role in mediating the chemotoxicity of contrast media, given that infusion of iothalamate accompanied by L-arginine analogs could protect the rats and raise the completely lethal dose (LD_{100}) significantly (579).

Wiesel et al. (580) observed that slight but significant platelet activation and thrombin generation in patients during coronarography occurs with both ionic and nonionic contrast

media, and that both types are potentially thrombogenic and behave as activators of platelet and blood coagulation. These authors observed only signs of biological activation of hemostasis without clinical symptoms. In patients with atherosclerotic lesions, contrast media may interfere with homeostasis by facilitating platelet adhesion. The risk factors for nonionic contrast media-induced thrombosis are listed (538). Koza et al. (581) evaluated platelet activation by ionic and nonionic contrast media in native heparinized or hirudinized human blood by use of flow cytometric analysis techniques, measurement of platelet factor 4 and thromboxane B_2, and antibodies to identify platelets (CD-61) and activated platelets (CD-62), to establish that dose- and time-dependent platelet activation was observed in nonionic, but not in ionic contrast media. The study suggests that contrast media use is associated with significant levels of platelet activation and that the anticoagulant heparin or combinant hirudin (where heparin is contraindicated) is preferable for use with nonionic contrast media to reduce contrast media-induced platelet activation.

6.5.5 Cardiovascular Effects. To visualize blood vessels and other structures of small thickness, a sufficient radiopaque dose must be used. Contrast media for angiography are usually administered intravascularly in high concentrations and large volumes (582). During coronary arteriography, the blood is replaced for a short period of time with a contrast medium solution (583). The effects that a contrast medium exerts on the central cardiovascular function will be determined by the iodine concentration, osmolality, viscosity, and the rate of injection. In angiocardiography the contrast medium can cause (1) electrophysiologic effect, such as heart rate alterations, atrioventricular conduction delay, and ventricular arrhythmias; (2) hemodynamic effect, such as hypotension, inhibition of left ventricular coronary contractile site, and alterations in coronary blood flow; and (3) metabolic effects, such as hypocalcemia and alterations in myocardial metabolism (469, 584).

Contrast media can cause fluid shifts upon mixing with blood, "pulling" out water from the red blood cells, and blood vessel endothelial cells within a few milliseconds of exposure. The mixture of blood and contrast medium, when entering and passing through the lungs, can cause changes in pulmonary pressure (582,585). In addition, contrast media can exert a direct effect on the myocardium, depressing myocardial contractility (584). Thus, systemic hypotension, changes in the activity of the smooth muscle cells in vessel walls, aggregation and crenation of red blood cells, and hypervolemia are examples of vascular reactions induced by the administration of contrast media. Agglutination and crenation of red blood cells cause blood sludging and reduce the cells' ability to undergo deformation, thus blocking the blood flow to capillaries and leading to pulmonary hypertension and related physiological responses (495–498). The hypervolemia resulting from a shift of water elevates the ventricular filling pressure and increases the cardiac output from increased ventricular stroke work. The potassium ions released from shrinking and crenating red blood cells contribute to the lowering of systemic vascular resistance and blood pressure (499).

Sheu et al. (586) compared the effects of ionic monomer diatrizoate and nonionic monomer iohexol on coronary hemodynamics and myocardial metabolism. In dog hearts, the intracoronary injection of iohexol produced an increase in myocardial contractibility, aortic systolic pressure, and myocardial oxygen consumption. In contrast, the injection of diatrizoate produced a decrease in these parameters. This difference between ionic and nonionic contrast media was significant. Nevertheless, the degree of increased coronary blood flow after coronary injection was similar for both agents.

In coronary arteriography, ionic monomers, because of their high osmolality and binding to calcium and magnesium in plasma, elicit more physiologic effects than nonionic monomers. The nonionic molecules do not combine calcium; the chelators used in the formulation have less calcium binding capacity; and the osmolality of nonionic monomers is only half that of the ionic monomers. Thus, nonionic contrast media cause fewer fluid shifts, less vasodilation, arrhythmia and fibrillation, and minimal cardiac depression. In an-

imal experimentation, injections of short duration (2–5 s) produce fewer incidences of myocardial fibrillation than prolonged injections (25 s) (482). The nonionic dimers or bis compounds, such as iotrolan and iodixanol, further reduce the clinical adverse reactions compared to those of the nonionic monomers. The dimers are isotonic to blood but have viscosities (>10 mPa·s) more than double the viscosity of blood (4 mPa·s) and more than 8 times the viscosity of plasma (1.2 mPa·s), with no apparent adverse effects (582). Ventricular fibrillation and serious arrhythmia have been linked to hyperosmolality and the absence of sodium ions. The safety of iotrolan is related to its isotonicity and the small amount of sodium ions it contains (482).

The safety, tolerance, and diagnostic efficacy of iopentol, iopromide, iohexol, ioversol, and iopamidol have been compared in different cardiac angiographic procedures (587–590). In a canine heart model, ioversol with added sodium in the form of NaCl can reduce the propensity for spontaneous ventricular fibrillation and arrhythmias but it also causes an increase in the QT interval in the electrocardiogram. The QT interval is associated with cardiac conduction and depolarization and repolarization. The prolongation of the QT interval, unlike that caused by ionic contrast media, is not an indication of arrhythmogenicity (591). The influence of sodium on electrocardiography during coronary angiography with iopromide has also been investigated (592).

Other factors may exhibit synergism in reactions with contrast media. In mice, contrast media with cardiac glycoside cause higher mortality than contrast media alone (593). Incompatibility of contrast media with other pharmacological agents has also been noted (594). Anaphylactoid reactions from contrast media can also be associated with β-blocker exposure, cardiovascular disorders, or asthma (595).

Muschick et al. (596) compared the hemodynamic effects of 11 iodinated contrast media, including ionic (diatrizoate, ioxaglate), nonionic monomers (iohexol, iopromide, iopamidol, iopentol, ioversal, iomeprol), and nonionic dimers (iotrolan. iodixanol) in rats. The animals were injected with contrast medium at a high dose of 1.2 g iodine/kg into the left ventricle. Transient changes in blood pressure (systolic and diastolic), heart rate, cardiac output, left ventricular end diastolic pressure, total peripheral resistance, and electrocardiogram within a few minutes post-injection were observed. In a healthy rat, the authors noted, all contrast media tested are safe but generate a few side effects. Low osmolar nonionic monomeric contrast media have a clear benefit over high osmolar contrast media. Nonionic isotonic dimers are best, with minimal side effects, and are likely to provide a lower risk if used in ventriculography and coronary angiography.

6.5.6 Nephrotoxicity. In urography and arteriography more than 90% of the injected dose of contrast media is excreted through the kidney (597). Contrast media adversely affect the excretory function of the kidneys, partly by osmolality and partly by chemotoxicity. Diuresis, changes of renal blood flow, osmotic nephroses, albuminuria, enzymuria, and changes of glomerular filtration rate are reported in animal studies of contrast media-induced nephrotoxicity (598). In patients with normal and abnormal kidneys, contrast media can cause acute deterioration in renal function, such as reduced glomerular filtration rate, medullary necrosis, and transient proteinuria of glomerular origin, associated with the increased elimination of β_2-microglobulin (599, 600). Individuals with preexisting renal insufficiency, diabetes mellitus, dehydration, cardiovascular disease, advanced age, multiple myeloma, hypertension, hyperuricemia, and several contrast examinations within 24 h have a high-risk of kidney malfunction (598, 601). Low osmolality monomers and dimers, such as iohexol, iopamidol, iopromide, ioversol, ioxaglate, and iotrolan, are less nephrotoxic in high risk patients than high osmolality ratio 1.5 agents, such as diatrizoate, for example (602, 603, 607, 608). The severity of the nephrotoxic effects is determined by the dose and rate of injection (463, 609, 610). In the clinical setting patients with normal renal functions respond similarly to ionic and nonionic contrast media (611–614), and the renal effects are transient only in these patients (600).

Contrast media cause damage to the vascular, glomerular, and tubular structures of the kidneys and changes in urine profiles, which may lead to acute renal failure (463,597,615, 616). In the canine model, renal ischemia potentiates nephrotoxicity (603). Deray et al. (604)studied renal hemodynamics in a normal and ischemic dog kidney and found that iodixanol induced a greater decrease in renal blood flow than did ioxaglate and produced a marked intrarenal vasoconstriction. Rauch et al. (605) compared the effects of contrast media (diatrizoate, iohexol, and iopamidol) on renal vasoconstriction in human, rabbit, dog, and pig arteries and found that all contrast media elicited dose-dependent reversible contractions in human, rabbit, and dog but not in pig renal arteries. All the contrast media tested exerted a vasorelaxing effect on pig renal artery. Nygren et al. (606) found that intravenously administered ioxaglate and iopamidol enhance the microvascular obstruction, as a consequence of red-cell trapping evoked by ischemic injury, and that iopamidol may induce local impairment in renal medullary microcirculation in a normal kidney.

The response of renal vasculature to contrast media exposure is biphasic. Immediately after intraarterial or intravenous injection of the contrast medium peripheral resistance decreases (vasodilation) and renal blood flow increases, followed by renal vasoconstriction and decreased renal blood flow. The size of the kidney changes and reaches its maximum size within the first 5 min after injection (598). The contrast media within the renal vascular bed attract water from the interstitial space and possibly from the renal tubules. In this renal hemodynamic and fluid shift, contrast media and some low molecular weight substances are freely filtered by the glomeruli and remain in the tubular lumen in a high concentration for a relatively long time, causing tubular damage (609). Water, contrast media, and urinary components that are not reabsorbed in the tubules are excreted as hyperosmolar urine in diuresis (597,609, 615).

Contrast media cause damage to the endothelia of renal vasculature and release vasoactive peptides, such as renin, prostaglandins, and kallikreins. Other associated events are deformation and rigidification of red blood cells and platelet accumulation in the areas of denuded blood vessels (598). The relation of the vasoactive peptides and associated events to vascular hemodynamic effects is not well understood.

Urographic and uroangiographic contrast media cause glomerular damage by altering membrane permeability, thus allowing protein leakage into the urine, resulting in proteinuria. After renal angiography, protein excretion in the urine may increase by a factor of more than 1000 (598, 617). In normal rats, albumin levels return to normal after 2 h (616). Massive proteinuria from glomerular damage may cause protein precipitation in tubular lumen, decreased urine flow, and may produce acute renal failure (463). It has been shown that nonionic iohexol produces less, if any, albuminuria in animals and humans than ionic contrast media or metrizamide (598). Iohexol has been proposed for use as a marker for glomerular filtration rate (618, 619).

One of the nephrotoxic effects of contrast media is the vacuolization of the proximal tubular cells (598). Iohexol and iotrolan induce more significant and longer-lasting vacuolization than the ionic diatrizoate and nonionic monomer iopromide (620). Iohexol also causes significantly more tubular vacuolization and capillary congestion than another nonionic contrast agent, iobitridol (301). This vacuolar response reflects lysosomal changes and the chemotoxicity of contrast molecules. Tubular vacuolization is also known as osmotic nephrosis. Wasaki et al. (621)compared the toxic effects of iobitridol and iohexol on the acute renal failure (ARF) kidney in a rat model and found that iobitridol produced a lower degree and incidence of renal tubular injury of renal proximal and distal tubules compared with iohexol. In the rat renal slice system, iobitridol had significantly less effect on the *p*-aminohippuric acid accumulation compared with that of iohexol. There were no changes of intracellular potassium content.

Tubular damage causes enzymuria. Urinary excretion of the lysosomal enzyme *N*-acetyl-β-D-glucosidase (NAG), the cytoplasmic enzyme lactic dehydrogenase (LDH), and the brush-border enzyme gamma glutamyl transpeptidase (GGT), caused by contrast media, has been studied in rats with drug-in-

duced nephropathies (622, 623). These enzymes with molecular weights greater than 70,000 are not filtered by the glomerulus (624). The level of enzymuria appears to correlate with the degree of proximal tubular cell injury. This may suggest that the urinary enzyme levels might be a more sensitive parameter than the serum creatinine levels to assess the renal toxicity of iodinated contrast media (616,625,626).

Edée and Bonnemain (627) reviewed the reliability of experimental models, from methodological considerations to pathophysiology, of iodinated contrast media-induced acute renal failure and nephropathy. Deray and Jacobs (628, 629) reviewed radiocontrast nephrotoxicity and renal tolerance of nonionic dimers. Nonionic dimers (iotrolan and iodixanol) are iso-osmotic and have high viscosities. Iotrolan at 300 mg I/mL has a viscosity of 17.8 mPa·s at 20°C and at 320 mg I/mL, 24.6 mPa·s. In comparison, iobitridol at 300 mg I/mL has a viscosity of 11.2 mPa·s at 20°C. These chemical characteristics that cause an increase of the reflection coefficient, characterized as "the tendency of solutes to be retained in the vasculature with resulting prolongation of the phase of osmotic expansion of plasma volume," may be responsible for the toxic symptoms. Vergare and Sequel (630) recommended warming the contrast media to 35°C before injection to improve tolerance.

6.5.7 Neurotoxicity. Contrast agents are toxic to the central nervous system (631). Their presence there is brought about either by direct injection into the cerebrospinal fluid or by leakage through the blood-brain barrier. Contrast agents are more toxic when administered intracisternally than intravenously (214). The ratio between intravenous and intracisternal LD_{50} values in the rabbit can be as high as 1450 to 1 for diatrizoate sodium (213, 546). Adverse effects caused by ionicity, chemotoxicity, and hyperosmolality of contrast media can range from discomfort to the patient to the disruption of the blood-brain barrier (BBB), epileptogenicity, and late arachnoiditis.

The transport of materials from the blood to the tissues (or extravasation) in the brain, unlike that in all other organs, is restricted by the BBB (632). Contrast media administered by intrathecal and intracisternal injections will bypass the BBB and come in direct contact with the neurons and glial cells (633). The BBB refers to the tight junctions between the cerebrovascular endothelial cells in cerebral blood vessels, which form a physical barrier to the exchange of ions and large molecules between blood and brain (634–636). Concentrated solutions of electrolytes and nonelectrolytes such as urea can reversibly open the barrier by separating the tight junctions through osmotically shrinking the endothelial cells (637–640). Barrier opening may be evaluated by the entry of dyes (e.g., Trypan blue, Evans blue) (634,641) or radioactive ions (e.g., ^{99m}Tc, ^{32}P) (642) into the brain parenchyma. The percentage penetration of ^{131}I-tagged iothalamate and diatrizoate in the rat cerebral cortex, studied by use of historadioautographic techniques, correlates well with the degree of severity of neurotoxicity (643). When there was little or no penetration of contrast medium into the brain tissue, no convulsions were observed. These experimental methods when applied together with those of induced convulsions (641–643) and damage to tissue cultures of neurons (644), to determine the neurotoxicity of contrast agents, may or may not necessarily measure the same molecular property responsible for neurotoxicity. In fact, cytochemical studies show that the BBB damage induced by contrast medium differs from the BBB damage caused by other types of insult, in that the plasma membrane-bound enzyme Ca^{2+}-ATPase and the negative charge that resides on the endothelial luminal surface are not affected (645). Damage from different contrast media may also differ. For example, the nonionic dimer iotrolan causes more BBB damage in the basal ganglia than in the cortex, whereas the reverse is true for the ionic diatrizoate (646). To predict the entire spectrum of neurotoxicity of contrast media, Sovak et al. (647) proposed the use of a number of methods for assessment of neurotoxicity in testing new nonionic contrast media.

The neurotoxicity of contrast media is determined by their lipid solubility or partition coefficient (631). Lipid solubility regulates the passage of these agents through the unaltered BBB. The partition coefficients of iothalamate,

ioxitalamate, diatrizoate, metrizoate, iodamide, diprotrizoate, and 2,4,6-triiodobenzoate have been calculated (631), using Hansch's rule for additive constitutive properties (648–651). This study shows that the neurotoxicity of these agents increases with increase in the combined partition coefficient P and is related to the brain uptake index. The relation is similar to that observed by Oldendorf (652) between the chain length of short-chain monocarboxylic acids and their transport across the blood-brain barrier, which reaffirms the dependency of neurotoxicity on lipid solubility. Nonionic contrast molecules designed with 4–6 hydroxyl groups in the molecule to maximize hydrophilicity have been shown to have a good correlation between partition coefficient (octanol/water) and neurotoxicity (653). For example, the hydrophilicity increases as the partition coefficient decreases, in the case of four monomeric nonionic contrast agents according to the ranking: metrizamide > iopamidol > iohexol > ioversol (654, 655). Their neurotoxicity, measured in terms of lethality, EEG effects, and motor effects, decreases proportionately with the increase in hydrophilicity.

In addition to osmolality and lipid solubility, the cations, anions, and chemotoxicity of contrast media also contribute to neurotoxicity. The sodium salts of diatrizoate and iothalamate are about three times more neurotoxic than the meglumine salts when compared at comparable doses in rats by intracisternal application, and the diatrizoate anion is about 10 times more neurotoxic than the iothalamate anion (656). In both hypertonic and hypotonic ranges, neurotoxicity increases with increasing concentration of contrast medium. When comparing the neurotoxicity, not only the molal concentration of the contrast medium but also the brain weight of the experimental animals should be considered. Evill et al. (657) compared the chemotoxic effects of nonionic contrast monomers iohexol and ioversol and dimers iodixanol and iotrolan in the rabbit using ^{99m}Tc-pertechnetate as a marker for BBB damage and mannitol as osmolality control. At comparable osmolalities, mannitol causes no brain ^{99m}Tc uptake, whereas the contrast agents cause significant ^{99m}Tc uptake according to the order: iohexol > iodixanol > ioversol > iotrolan, with iohexol causing more BBB damage than iotrolan. Motoji et al. (658) studied the neurotoxicity of contrast media using [8-^{14}C]dopamine as a marker for BBB damage in Monogolian gerbils and found that both meglumine sodium amidotrizoate and iohexol caused damage to the BBB, as manifested in cerebral cortex, brain stem, and hippocampus sections in the left hemisphere. Iopamidol caused a much smaller but significant effect, and ioversol caused no damage to the BBB. Michelet (659) used iothalamate and iohexol as reference contrast agents and ^{99m}Tc-DTPA, ^{125}I-labeled human serum albumin and Trypan blue as tracer to evaluate degrees of damage to the BBB in male albino rabbits. The ionic monomer iothalamate caused marked BBB damage, based on a large extravasation of tracers, the nonionic monomers iopentol and iohexol caused some BBB damage, and the dimer iodixanol caused only minor changes to the barrier. The author recommends iodixanol as a safe contrast agent for computed tomography of the brain. Harnish et al. (660) found that iohexol or ioxilan at a solution concentration of 175 mg I/mL caused no BBB damage in anesthetized Wistar rats. This strain of rats has been reported to be more sensitive than other strains to the intrathecal effects of contrast agents (187). Whisson et al. (661) studied acute hypertension (HT) as a risk factor for damage to the BBB in carotid angiography with nonionic contrast media (iopamidol) in groups of rats. Anesthetized animals received intravenous injections of ^{99m}Tc-pertechnetate and horseradish peroxidase as tracers for quantitation and histological examination. Two groups of rats received metaraminol to raise their blood to between 165 and 190 mmHg peak systolic and then received intracarotid injection of saline and iopamidol at 300 mg I/mL. Two groups of normotensive rats received normal saline and iopamidol as controls. The authors found that the extravasation of both tracers were significantly greater in the hypertensive group that received contrast media than in the other groups and that acute hypertension potentiates the BBB-damaging effects of nonionic contrast media during carotid angiography in rats.

Luzzani et al. (426) assessed the neurotolerability of six monomers (iomeprol, iopam-

idol, iohexol, iopentol, ioversol, and iopromide) and three dimers (iofratol, iotrolan, and iodixanol) of nonionic X-ray contrast media in rats and mice by their median lethal dose (LD_{50}), compared to controls. The contrast agents were administered by intracerebroventricular (i.c.v.) injection to mice and by intracisternal (i.c.i.) injection to rats. The iodine contents of monomers and dimers injected were 350–370 and 300–320 mg I/mL, respectively. The toxic effects for all contrast media included convulsion, dyspnea, hypoactivity, and sedation. In mice iohexol was significantly more neurotoxic than other monomers, and the dimers had similar neurotoxicity but were more toxic than the monomers; this difference became more evident when the concentrations of LD_{50} values were expressed in mmol/kg instead of mg I/mL. In rats iopentol and iopromide were sufficiently more neurotoxic than others that their LD_{50} values could be accurately determined. The authors attributed the observed differences in neurotoxicity of the contrast media to differences in chemical structures, that is, to chemotoxicity rather than hydrophilicity or physicochemical characteristics of pharmaceutical formulations of the contrast media. Mice and rats had different sensitivities toward contrast media, apparently from drug actions targeted at different areas in the brain as a result of different sites of injection.

Damage to the spinal cord by contrast medium also causes neurotoxicity (456,457,662). Antoni and Lindgren (663) first observed spinal cord damage after aortography. A survey conducted by Killen and coworkers (664,665) showed that 90% of the reported spinal cord injuries was caused by acetrizoate, especially at high concentrations. Others (666–670) have shown that all water-soluble angiographic agents can inflict damage on the spinal cord. Contrast agents such as sodium iodide, acetrizoate, iodomethamate, diprotrizoate, diodone, diatrizoate, and thorium oxide have neurotoxicity decreasing in that order (670). Foster et al. (671) found that diodone, acetrizoate, and diprotrizoate have higher toxic effects on kidney and spinal cord after aortography than thorium dioxide, diatrizoate, and iothalamate. Damage to the spinal cord is highly likely in any procedure that concentrates the contrast agent in this region.

Metrizamide was the first-generation nonionic contrast agent for the central nervous system found to be almost free of neurotoxic effects (672). Metrizamide is often used as a standard of comparison in animal experimentation when newer nonionic contrast agents are evaluated for neurotoxic effects. Adams et al. (673) used electroencephalography (EEG) recording to detect neurotoxic effects of contrast media after i.c.i. administration in the rat. These authors found that ionic meglumine iothalamate produces profound seizure-like patterns in EEG at 30 mg I/mL. In comparison, metrizamide produced minimal EEG abnormalities at this concentration. Ioversol and ioglunide produced mild to slight EEG abnormalities in doses ranging from 60 to 240 mg I/mL. Ioglunide was well tolerated in total spinal column myelography in a group of 86 patients but was considered to be at a competitive disadvantage in manufacture when compared to iohexol and iopamidol, because ioglunide contains a sugar moiety and is as unstable in solution as metrizamide (673). Iopamidol provoked seizure-like patterns in EEG at a considerably lower dose than metrizamide when injected as highly concentrated solutions (400 mg I/mL) into the subarachnoid space of the anterior fossae of guinea pigs (674).

Nonionic contrast media in either hypertonic or hypotonic solution, when injected intracerebrally or into the subarachnoid space of rats, will cause distinct depression of the central nervous system (CNS) and associated brain functions but no excitation (675). Such depressive action can obscure the excitatory action caused by ionicity or chemotoxicity of myelographic agent. When an isotonic contrast medium is injected and mixed with the normally produced cerebrospinal fluid (CSF) in the subarachnoid space of the rat, the resultant mixture is hypertonic with higher levels of sodium and chloride. This movement of sodium and chloride into CSF without accompaniment of water is unexpected. Mennini et al. (672) noted that contrast enhancement of brain parenchyma is never achieved by direct intracarotid or intravenous injection of nonionic contrast media, unless the BBB is spontaneously or experimentally broken (646).

This may suggest specific sites for neurotoxicity other than just BBB disruption. In search of such specific receptors, Mennini et al. (672) found that iohexol and iopamidol at concentrations as high as 100 μM did not displace the tritium-labeled ligands from their neurotransmitter binding sites and suggested that the cell responses may be mediated by the phosphatidylinositol second-messenger system (676). According to Ekholm (633) and Marinetti (677) nonionic contrast media (iohexol, iopamidol, metrizamide, iodixanol, and iotrolan) can influence the uptake of ^{45}Ca and the incorporation of [^{32}P]phosphate into phospholipids (PA), phosphatidylinositol (PI), phosphatidylinositol-4-phosphate (PIP), and phosphatidylinositol-4,5-biphosphate (PIP-2) in isolated rat brain synaptosomes, but it is not known whether these reactions are important in clinically observed neurotoxic effects of contrast media.

Injection of vasodilators, vasoconstrictors, or other agents that change the relative flow of blood and contrast medium toward or away from the spinal cord or alter its response to the contrast medium can modify the neurotoxic effects (666, 678). After intra-aortic injection, the neurotoxicity of contrast agents may be increased by the presence of chemicals such as heparin (679), which increase the permeability of the blood-brain barrier. Hypothermia, procaine (prior-injected), 20% glucose, and low molecular weight dextran can exert a protective effect against spinal cord damage (671, 681, 690). Diazepam can prevent clonic convulsions and muscular twitchings after myelography with iocarmic acid (682). These side effects can also be reduced in patients receiving levomepromazine (683). The mechanism of these protective effects is not fully understood.

6.5.8 **Protein Binding.** Protein binding is reputed to affect the toxicity and selective excretion of contrast media (414,417,684). Ionic contrast media were shown by equilibrium dialysis to combine with serum albumin at a number of strong and weak binding sites on the protein (414, 416, 417, 685). Acetrizoate, iodipamide, and iopanoate with an open position 5 in the substituted benzene nucleus combine strongly with albumin, whereas the fully substituted triiodobenzoates such as diatrizoate and iothalamate bind only weakly. Acetrizoate and iodipamide bind to bovine serum albumin strongly at one site and weakly at several other sites, whereas iopanoate binds strongly to three sites on the protein. The difference in the strength of protein binding sites may account for the species specificity of the toxicity of acetrizoate observed in 15 species of animals. The oral cholecystographic agent iophenoxic acid, which is no longer used, has a strong affinity of binding to plasma albumin with a half-life in humans of about 3 years (686). Its structure is identical to that of iopanoate, with the exception of an OH instead of a NH, group at position 3 of the benzene nucleus. Iophenoxic acid is tightly bound to human serum albumin at site I as compared to iopanoate at site II. Nonionic contrast media, such as iohexol, iopamidol, and iodixanol, for example, in spite of their fully substituted benzene nucleus and high water solubility, also interact with proteins. According to Lang and Lasser (685, 687), the binding between the contrast material and protein is attributed to hydrophobic interaction.

Protein binding may affect the selective excretion by the kidney or liver of a contrast medium (414, 688, 689). The cholegraphic agents iodipamide and iopanoic acid show strong binding to serum protein. Although the relationship between the affinity for protein binding and the preferential uptake and excretion by the liver of contrast agents has not been clearly defined, two hypotheses have been advanced to explain the phenomenon. One hypothesis is that the binding with albumin prevents glomerular filtration of the contrast medium, thus providing a continuing concentration to liver parenchymal cells (689, 690), and the other hypothesis is based on the free penetration of serum albumin in the Disse's space, which allows preferential access of the bound contrast medium to liver cells (691). Neither of these hypotheses is tenable because (1) it is not possible to force greater quantities of acetrizoate into the bile by increasing the serum-binding capacity of acetrizoate to the level of iodipamide by priming with serum albumin (692), and (2) some substances such as Evans blue that are extensively bound to serum albumin are not ex-

creted in the bile to any significant extent, whereas other poorly bound compounds such as p-aminohippuric acid are efficiently excreted by the liver (414). These findings point to the involvement of additional mechanisms besides serum protein binding in determining the biliary excretion of contrast medium. Glucuronidation can increase the bile excretion of contrast media (693).

Sokoloff et al. (694) showed that iopanoic acid and iodipamide, but not iothalamate, bind to the Y and Z proteins found in the liver and the mucosa in the small intestine. Song et al. (695) studied the role of serum albumin in the hepatic excretion of iodipamide and found that the contrast agent establishes an equilibrium between serum protein and the intrahepatic anion-binding Y protein, also known as ligandin, with the latter controlling the process of hepatic uptake and excretion in which serum albumin plays no role. Goldstein and Arias (696) have studied the interaction of ligandin with iopanoic acid, diatrizoate, iodipamide, and iodopyracet. It is apparent that the role of ligandin in the transfer of contrast medium from blood to liver is to bind the contrast material in the liver cell for intracellular accumulation.

Contrast media inhibit enzyme activity, and the degree of inhibition parallels their binding affinity to serum albumin. Iopanoate, iodipamide, acetrizoate and isofamic acid, and diatriazoate and iothalamate exert, in descending order, an inhibitory effect on the following enzymes: lysozyme, glucuronidase (two types), alcohol dehydrogenase, glucose-6-phosphate dehydrogenase (414, 687), adenosine triphosphatase, carbonic anhydrase (698), and cholinesterases of human red blood cells and plasma (672,673). Cholecystographic agents can adversely affect the glucuronidation of p-nitrophenol more than urographic agents (700). Sodium ipodate strongly inhibits the microsomal p-nitroanisole demethylase and aniline hydroxylase activity (701). Metrizamide binds reversibly with catalase (702) and exhibits weaker inhibition of cholinesterase, glutamic-oxalacetic transaminase, and glucose-6-phosphate than that of either diatrizoate or iothalamate (703).

Cholegraphic agents such as iodipamide and iopanoic acid are about 10–100 times more potent inhibitors of cholinesterases than the angiographic agent diatrizoate (698). Toxic reactions manifested as gastrointestinal symptoms and hypertonicity of bladder from the use of oral cholecystographic agents may be attributed to their anticholinesterase activities. The hypotensive effects induced in experimental animals by ioglycamic acid and iodipamide are not completely blocked by atropine sulfate, indicating that not all the toxic effects of cholegraphic agents are attributed to inhibition of acetylcholinesterase activities (704).

Contrast media induce serum complement activation (705). The degree of activation is a function of concentration and chemical structure of the contrast agent. The order of serum complement activation by contrast agents is metrizamide < iothalamate < diatrizoate < acetrizoate < iodipamide and iopanoic acid, which is also the order of enzyme inhibition. Activation of serum complement results in structural and functional alterations of cell membranes because of the release of small active peptides such as the anaphylatoxins, bringing about a wide variety of reactions including contraction of smooth muscle, increased capillary permeability, release of histamine from mast cells, directed attraction of polymorphonuclear leukocytes, and release of hydrolases from these cells. Contrast media affect the normal process of fibrin and plasma formation in the coagulation mechanism by changing the fibrin fiber diameter and increasing the size of protein aggregates with an altered spatial arrangement of the clots (706).

Nonionic contrast media have high hydrophilicity, extremely low toxicity, and an insignificant level of protein binding of less than 3%, and may induce serum complement activation (477, 707). At a contrast medium concentration of 1.2 mg I/mL, the extent of binding to human plasma proteins is about 1.5 ? 0.3% for iohexol, 0.1 ± 2.2% for iopentol, 2.9% for iopamidol, 4.3 ± 0.3% for metrizamide, and 7.6 ± 1.5% for ioxaglate (708, 709). Dawson (653) ranked the relative protein binding capacity of iohexol:iopamidol:ioxaglate:iothalamate as 1:1.5:3.5:4 and correlated the partition coefficient (butanol/water) of metrizamide (0.37):iopromide (0.14):iopamidol (0.11):iohexol (0.07) with their respective ap-

proximate LD_{50} values: 14:16:22:24 g I/kg in the mouse. Reactions of serine proteinases (e.g., thrombin) will activate the coagulation cascade, but nonionic contrast media may bind to the proteins to inhibit the clotting (710). X-ray analysis of crystals of iohexol and serine proteinase (pancreatic porcine elastase) reveals that three molecules of iohexol are associated with elastase, with one close to the active site (subsite S1), the second in the vicinity of Ser^{214} in subsites S2/S3, and the third located in a pocket at the surface of the protein. The association is a result of the affinity of iohexol directed toward the hydrophobic regions of the enzyme and supports the hypothesis of the contrast medium's potent inhibition of thrombin. Another example of the contrast medium-protein interaction is between iopamidol and fibrinogen or lysozyme (711). Isothermal calorimetry revealed a weak endothermal effect at high concentrations of iopamidol for both proteins. This endothermal effect does not appear to be attributable to a direct protein-iopamidol association but suggests the alterations of protein solvation after protein unfolding in the presence of contrast medium. This altered solvation is confirmed by Raman spectroscopy on two amino acids in the presence of iopamidol, which showed that the spectrum of alanine is unchanged at any iopamidol concentrations studied, whereas the spectrum lines attributed to the thiol group of cysteine are shifted in a manner consistent with altered solvation. A recent report on the binding chemistry of 2,3,5-triiodobenzoic acid (TIB) with the crystal form of human serum albumin (HSA) revealed that the ligand TIB is bound in a hydrophobic crevice in a cavity in subdomain IIA of the HSA structure (712).

6.5.9 Histamine Release. Contrast media administered to laboratory animals by either intravenous injection or infusion or local application caused histamine-like reactions: endothelial injury (713), changes in vascular permeability (714, 715), and perivascular edema (716). These agents release histamine from tissue mast cells by degranulation (717–720). Leite et al. (721) reported that meglumine iodipamide and a mixture of sodium and meglumine salts of acetrizoate were more effective than iodamide in histamine release in the rat, and diatrizoate was ineffective. Others have reported the release of histamine from rat peritoneal mast cells by acetrizoate, diatrizoate, and iothalamate (722, 723). Histamine was found in the plasma of dogs after pulmonary artery injection of meglumine iodipamide (722) and in the perfusion of canine lung with meglumine salts of acetrizoate, diatrizoate, iothalamate, and iodipamide, or meglumine chloride alone (527). The ability or inability of diatrizoate to release histamine reported in these experiments is probably caused by the difference in injection rate. Meglumine salts of contrast media, with the exception of diatrizoate, release much greater quantities of histamine than do the sodium salts. Histamine release is not caused by hyperosmolarity, given that the injection of isosmotic sodium chloride (721) or dextrose (724) solutions produces negligible effects. Administration of diphenhydramine hydrochloride and burimamide (i.e., *N*-methyl-*N'*-[4-[4(5) imidazolyl]butyl]-thiourea), which are H_1 and H_2 receptor antagonists, respectively (725), abolishes the histamine release properties of the contrast agents in *vivo* (721, 726).

In humans the allergic reactions from contrast media resemble those elicited by histamine injection, and the unpleasant side effects are rarer after fast injection than after slow injection of the same dose of contrast medium (489). Seidel et al. (724) showed that usual doses of chemically different contrast media were able to affect the plasma histamine levels in venous blood of healthy persons. Histamine release ceases when the dose rate is reduced to 0.17 g kg^{-1} min^{-1}. This may explain the advantage of administering urographic agents by infusion to avoid reactions.

Patients with a history of allergy react more frequently to injection of contrast media than those with no history of hypersensitivity (455, 456). Blood of patients with radiocontrast idiosyncrasy and atopic individuals release higher levels of histamine than that of normal individuals. Siegle et al. (727) incubated ^{125}I-diatrizoate with blood from atopics and nonatopics and found significant retention of radioactivity by the leukocytes and not by the erythrocytes. Histamine is released from human leukocytes by contrast media.

The low osmolality contrast media, such as iohexol, iopamidol, metrizamide, and ioxaglate, release less histamine on the basis of iodine concentration than the ionic contrast media, such as diatrizoate, sodium, and meglumine iothalamate. The meglumine salt releases more histamine than the sodium salt, and ioxaglate releases more histamine than iohexol, iopamidol, and metrizamide (728). Because the exact mechanism relating histamine release to adverse effects of contrast media is unclear, cholinergic mechanisms have been proposed to play an important role also. These mechanisms are activated by the inhibition of cholinesterase. Again, iohexol and iopamidol cause less enzyme inhibition than ionic ioxaglate, iothalamate, and ioglycamide (729). An *in vitro* assay based on histamine release to pretest patients at high risk before infusion with contrast medium has been proposed (730), but the rise of plasma histamine levels cannot be correlated with allergic-like effects in patients in a conclusive manner (731). Studies by Pinet et al. (732) showed that the values of plasma histamine and blood histamine in patients receiving ioxaglate, iohexol, iopamidol, and ioxithalamate for intravenous pyelography were within the limits of statistical fluctuation. Reactions to contrast media are in some cases precipitous, resembling anaphylactoid reactions of immunologic nature, which led Brasch (551,552) to suggest an antibody-type reaction to contrast media. Krause et al. (733), from the results of Brasch's study, suggested that iotrolan is probably not immunogenic.

6.5.10 Pharmacokinetics. The quality of roentgenograms in cholangio-cholecystography and urography is determined by the localization of the contrast media applied. In oral cholecystography, the radiographic quality is determined by the absorption and excretion of contrast medium, which is controlled by many factors (734, 735). In intravenous cholangiography and urography, the excretion of contrast medium alone determines the opacification of the organ under examination (736–739).

Absorption of oral cholecystographic agents occurs by passive diffusion of the nonionized compound across the lipoidal barrier of intestinal mucosa (734) and is determined by the rate of solubilization of contrast medium, affected by pH, the presence of bile salts, mixing, the effective surface area, and the physical state of the compound (735). The rate of absorption of iopanoic acid and iosumetic acid is increased if the contrast agent is administered concomitantly with sodium bicarbonate (300, 740, 741). Bile salts or a fatty meal have been recommended with the administration of iopanoic acid, to ensure its solubilization and absorption in the duodenum and to improve gall bladder visualization (735, 742, 743). The fatty meal releases cholecystokinin, which promotes the enterohepatic circulation of bile salts, thus enhancing both intestinal absorption and hepatic excretion (734). The ability of bile salts to increase the excretion of iopanoic acid is attributed to an allosteric interaction between the bile salts and a carrier for the contrast medium in the canalicular membrane (744, 745).

Because of the often incomplete absorption of oral cholecystographic agents, detailed analytical approaches to pharmacokinetics studies have not been available. Goldberg et al. (735) studied the biopharmaceutical factors influencing the intestinal absorption of iopanoic acid and attributed the frequent failure of the first dose of the agent to afford diagnostic visualization of gall bladder to impaired intestinal absorption. Uncertainty in absorption is avoided when intravenous agents are used.

Water-soluble dimers such as iodipamide, ioglycamide, iodoxamate, and iotroxic acid are intravenous cholangiographic agents and can be injected to achieve a desired plasma concentration. After injection, the pharmacokinetics of these contrast media including dilution and mixing with body fluid, uptake by the liver, and excretion into the bile and urine can be fitted to multicompartmental kinetic models. A logical kinetic model for the transfer of a substance through a secreting cell may consist of three phases: uptake (passage from the plasma into the cell); passage through the cell (with or without accumulation); and passage from the cell to the tubular lumen (excretion) (746,747). Corman et al. (748) have proposed several pharmacokinetic models for ioglycamate. Although a simple, two-compartment model can account for approximately 90% of

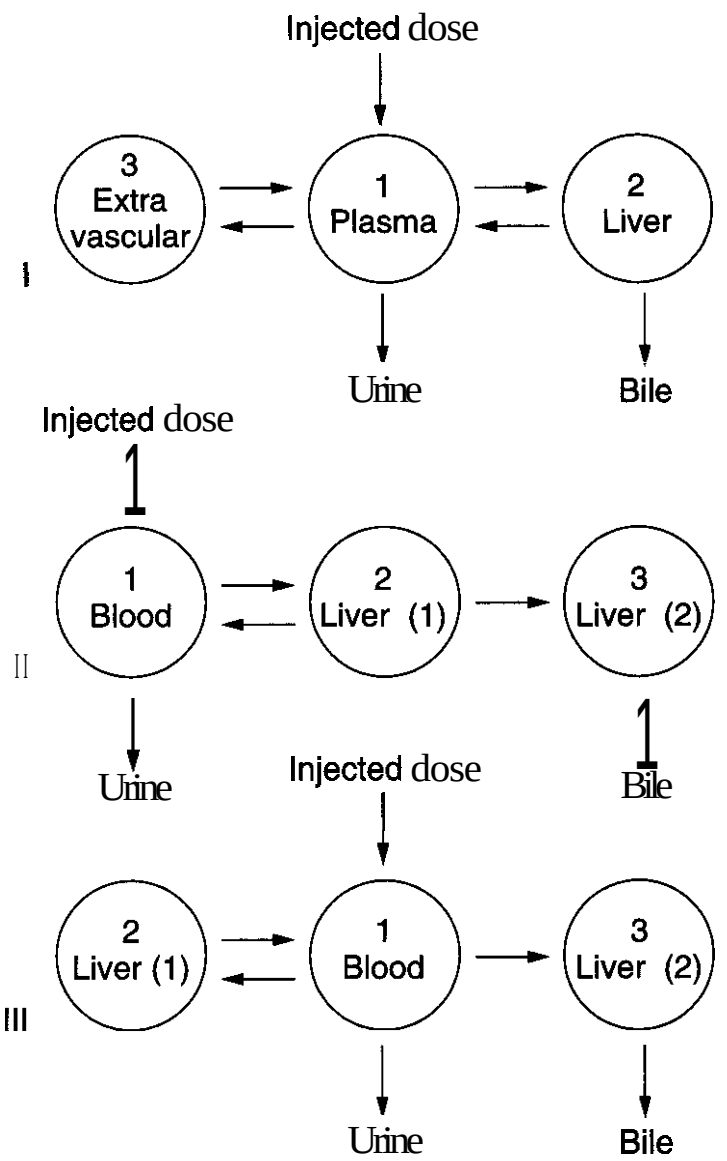


Figure 10.1. Pharmacokinetic models for the excretion of iodipamide, iodoxamic acid, and ioglycamide in the dog (747–752).

the administered drug, the experimental data are better fitted to three-compartment models. The introduction of an extravascular space with which the plasma contrast medium can rapidly exchange would avoid the difficulty of requiring a considerably larger initial volume of dilution for the contrast medium than the plasma volume in a two-compartment model (748) and is based on the observation that the distribution volume of iodipamide in the dog is similar to the extravascular fluid volume in size (749). Pharmacokinetic models involving three compartments have been proposed for ioglycamic acid by Corman et al. (747), for iodipamide by Shames et al. (748), and for iodoxamic acid by Strata et al. (750). Segre and Rosati (751) have proposed nonlinear kinetic models for the disposition of iodipamide, ioglycamide acid, and iodoxamic acid in dogs. These models are shown in Figure 10.1. In the nonlinear kinetic model, the contrast medium is excreted into the bile through the liver compartment 3, which is not in direct communication with the plasma compartment 1. For ioglycamic acid at doses above 175.5 μmol/kg, an alternative model has been proposed in which the passage of the contrast medium from blood to the liver is irreversible.

The relationship between infusion rate or plasma concentration on the one hand and biliary concentration (B), urinary concentration (U), B/U excretion ratio, and excretory deficit (i.e., the amount of contrast medium retained in the body) on the other have been studied in the case of iodoxamate and iodipamide by Berk et al. (752) in unanesthetized dogs. Biliary excretion depended on bile flow and erythritol clearance (which serves as an index of canalicular bile flow). The rate of biliary excretion and the concentration of iodine in the bile increased according to first-order kinetics at low plasma concentration of the contrast agents and leveled off when the plasma concentration reached 0.4 mol/mL. A reasonable fit of these data to equations for hyperbolas can be made, indicating saturation kinetics, which is characteristic of an active transport process presumably occurring at the biliary canaliculus. The rate of urinary excretion of these contrast agents varied linearly with the plasma concentration, indicating a passive transport process. The B/U excretion ratio decreased sharply with increasing plasma concentration (i.e., above 0.4 mol/mL). At an infusion rate of approximately 1 mmol/kg min, a biliary concentration of about 25 and 28 mmol/mL has been achieved for iodipamide and iodoxamate, respectively, which exceeds the concentration (approximately 13–16 mmol/mL) required for visualization of the biliary tree. From these data, the authors suggested that iodoxamate is a better agent than iodipamide in patients with impaired biliary excretion, high basal bile flow, or when a small dose of the contrast medium is infused. Lin et al. (753) have investigated the saturation kinetics of iodipamide. Moss et al. (754) found that iopanoate and iodoxamate, when co-administered intravenously in rhesus monkeys, compete for the binding sites on serum proteins as well as on intrahepatic tissue and that biliary excretion is directly related to the unbound contrast medium in the blood. Staubus (755)

simulated the nonvisualization of gall bladder in the patients by studying the effects of infusion on the pharmacokinetics of iopanoate in the dogs and attributed the nonvisualization to poor intestinal absorption or to poor hepatic/biliary function.

Pharmacokinetic studies of urographic agents such as water-soluble derivatives of 2,4,6-triiodobenzoic acid and 2,6-diiodopyridone showed that the renal excretion of these agents proceeds through glomerular filtration, active excretion in the proximal tubules, and passive reabsorption in the distal tubules of the nephron (756). Iodopyracet and acetrizoate are largely excreted by the proximal tubules in the dog, whereas iodamide is only moderately excreted; and diatrizoate, iothalamate, and metrizoate are not excreted at all by the tubules but are eliminated by glomerular filtration. Studies with aglomerular fish or ureterally obstructed dogs confirmed that there is no tubular excretion or reabsorption of sodium iothalamate (757).

The plasma concentration curve obtained after a single injection of sodium diatrizoate reflects the initial mixing in the vascular compartment, equilibration in the fluid space, and concomitant excretion through the kidneys. The initial volume of mixing is of a size comparable to the measured extracellular space. Based on the excretion data of ^{131}I-labeled sodium and meglumine diatrizoate, a three-compartment kinetic model, analogous to that proposed for iodipamide excluding the biliary exit has been developed using analog computer simulation. The model is composed of a blood compartment, a tissue compartment, and a "deep" tissue compartment (738). The deep tissue compartment corresponds to the extracellular fluid space and is characterized by a very small entry constant and a large entry-to-elimination constants ratio, usually exceeding 10. This ratio was found to be 9.4 ? 4.5, with a half-time of elimination of 11.7 ± 1.15 h for sodium diatrizoate compared to 17 ± 4.5 and 14.5 ± 1.5 h for the meglumine salt. These values show that the pharmacokinetic model for the diatrizoic acid remains the same, unaffected by its salt form.

The plasma concentration C_p of the nonionic monomer iohexol, as shown in two studies in human volunteers, was best represented by a three-compartment model, $C_p = Ae^{-\alpha t} + Be^{-\beta t} + Ce^{-\gamma t}$, where A, B, and C are the feathered intercepts of the plasma concentration curve; a, β, and y are the rate constants; and t is the time (758, 759). In one study the mean apparent first-order terminal (y-phase) elimination half-life for iohexol was estimated to be about 12.6 h; the half-life of initial distribution (a-phase, $t_{1/2\alpha}$) about 22 min, and the half-life of disposition and elimination (β-phase, $t_{1/2\beta}$) about 2.1 h. The mean ± SD apparent volume of distribution (V_d) was calculated to be 165 ± 30.7 mL/kg body weight. The magnitude of the value suggests that iohexol was distributed in a volume comparable to that of extracellular water. The renal clearance (CLR) was 120 ? 18.6 mL/min and the total body clearance (CLT) was 131 ± 18.6 mL/min. In a separate study, the $t_{1/2\alpha}$ value of iohexol was estimated to be an average of 21.8 rnin and the $t_{1/2\beta}$ value an average of 121 min; the CLR was calculated to be 122 (118–128) mL/min and the CLT, 120 (116–118) mL/min. Nonionic monomers, such as iopamidol (195, 760), ioversol (761, 762), iopromide (763), and iopentol (764) have similar pharmacokinetics profiles in healthy subjects. The mean plasma concentration curve of iopamidol at three dose levels in healthy volunteers was best fitted to an open, linear, two-compartment model with an average $t_{1/2\alpha}$ value of 28.2 min and an average $t_{1/2\beta}$ of 2.14 h (699). Ioversol given to healthy volunteers by intravenous injection at two dose levels (50 and 150 mL) showed the $t_{1/2\alpha}$ and $t_{1/2\beta}$ values to be 15.5 min and 2.06 h for 50-mL dose group and 18.9 rnin and 2.04 h for 150 mL dose group, respectively (762). The CLR and the CLT were 178 and 156 mL/min for the 50-mL dose group and 190 and 184 mL/min for the 150-mL dose group, respectively.

The pharmacokinetics of iopromide was fitted in a two-compartment model in healthy volunteers at low dose (15 g iodine150 mL) and high dose (80 g iodine150 mL) levels and their $t_{1/2\alpha}$ and $t_{1/2\beta}$ values were 1.95 and 2.6 h for the low dose group and 1.90 and 2.5 h for the high dose group, respectively (763). The CLR and the CLT were 104 and 110 mL/min for the low dose group and 100 and 103 mL/min for the high dose group, respectively. The pharmacokinetic parameters of iopentol were 23 min for $t_{1/2\alpha}$ and 121 rnin for $t_{1/2\beta}$. The apparent vol-

ume of distribution was 250 mL/kg of body weight; the CLR was 104 mL/min, and the CLT, 106 mL/min. The nonionic dimer iodixanol showed the same profile in pharmacokinetics as that of the nonionic monomers. The serum concentration of iodixanol declined biexponentially after intravenous administration of the dose. The $t_{1/2\alpha}$ and $t_{1/2\beta}$ values were 25.6 and 130.6 min, respectively. The average apparent volume of distribution was 0.275 L/kg body weight. The renal clearance and the total body clearance were 104 and 106 mL/min, respectively.

Urinary excretion of water-soluble contrast agents is by glomerular filtration without any tubular reabsorption (689). The CLR for the contrast medium was compared with the clearance of ^{51}Cr-EDTA, injected alone or co-injected with the contrast medium, given that ^{51}Cr-EDTA is known for its excretion by glomerular filtration only (759, 765). The elimination half-life $t_{1/2\beta}$ usually expresses urinary excretion in a comparable term for water-soluble contrast agents. In addition to the values given above, iotrolan has a $t_{1/2\beta}$ of 106 min and diatrizoate, 108 min. According to Olssen et al. (759),

> [T]he apparent volume of distribution is an important pharmacokinetic parameter relating serum concentrations of a drug to the total amount of drug in the body. The parameter usually has no direct physiologic meaning and does not refer to a real volume. However, it is generally accepted that a volume of distribution of 15–27% of body weight (0.15–0.27 L/kg) indicates the distribution in the extracellular fluid only.

The volumes of distribution reported for contrast media in the dogs are generally higher than the reported human values, such as 0.42 L/kg for ioxaglate, 0.37 L/kg for iocarmate, 0.64 L/kg for ioxithalamate, 0.39 L/kg for mitrizamide, and 0.38 L/kg for diatrizoate.

Absorption and excretion of metrizamide and diatrizoate after suboccipital injection in the cat followed first-order kinetics. Golman and Dahl (766) determined the rate constants for absorption and excretion and observed that 99% of the absorbed dose disappeared from cerebrospinal fluid during the 3 h after suboccipital injection. The difference in the total amount of metrizamide excreted during 48 h after intravenous and suboccipital injection in the rabbit was negligible, indicating a rapid removal of the agent from cerebrospinal fluid to blood (767).

6.5.1.1 **Excretion.** Contrast media are excreted in both the bile and urine; the route that predominates is determined by their chemical structure and route of administration. In general, contrast molecules with an unsubstituted position in the benzene nucleus will be sufficiently lipophilic to bind to the proteins and will be absorbed in peroral administration and excreted in the bile and urine. On the other hand, if the benzene nucleus is fully substituted, the contrast molecules will be highly hydrophilic, will not bind to the proteins, and will be rapidly excreted in the urine. The nonionic contrast media are highly soluble in water and will not be adequately absorbed per *os*. Hepatic and urinary excretions of ionic contrast agents and their rates of excretion have been reviewed by Catell (688), Knoefel and Carrasquer (768), McChesney (769), and Sperber and Sperber (749).

Oral contrast agents bind serum protein at physiological pH (417, 688). Protein binding prevents glomerular filtration but does not impair hepatic excretion. Transport of these agents from the blood to the liver is an active saturable process (770) and involves the Y and Z proteins found in the liver as hepatic acceptors (694, 695). These acceptors show affinity for cholegraphic agents, such as iopanoic acid and iodipamide, but exhibit little or no affinity for urographic agents such as iothalamate. The liver transport capacity of contrast medium, expressed as maximum transport rate or velocity T_m, is defined as the amount of compound going into the bile per unit time, which cannot be influenced by additional increases of dose (190). Rosati et al. (190) reported the T_m values for ioglycamic acid, iodipamide, and iodoxamic acid after rapid intravenous injection in the dog to be 0.768 ± 0.101, 0.530 ± 0.072, and 1.226 ± 0.109 μmol kg^{-1} min^{-1}, respectively. These values remain essentially the same for slow infusion (738, 771). Iopanoate has a T_m value varying from 0.16 to 0.74 mol kg^{-1} min^{-1}, depending on the presence of bile salts (772). In bile salt-

depleted dog, less contrast agent is excreted. Julian et al. (773) reported the maximum transport rates of iodipamide and iodoxamic acid in humans.

Contrast agents with strong affinity for serum protein show a threshold plasma value below which little or no excretion through the liver or the kidney occurs (771), and in addition, a species difference exists in the excretion of cholegraphic agents (774). The threshold values of ioglycamic acid and iodipamide in the dog were shown to be 0.16 and 0.6 μmol/mL, respectively. Iodoxamic acid, iodipamide, and ioglycamic acid were excreted in the rabbit at higher concentrations to the extent of about 77, 38, and 42% of the dose in the bile and about 17, 38, and 35% of the dose in the urine, respectively (190). Iocetamic acid is excreted in the rat predominantly in the intestine but in human subjects, 41 and 62% of the administered dose appear in the urine in the first 24 and 48 h, respectively (774). Kiyono et al. (775) studied the distribution and excretion of iodipamide in mice and man, using ^{131}I-labeled material. Earlier, Fischer (776) derived from biliary and urinary excretion studies an optimal dose of 205 μmol/kg for iodipamide.

Biliary excretion of contrast medium is affected by the bile flow (688, 734, 745, 777, 778). Bile is isosmotic with plasma and is produced from the transport of water from the liver cell into the bile canaliculi (canalicular bile flow) and from the excretion and reabsorption of water and electrolytes in the bile ductules (ductular bile flow). Bile flow is increased by taurocholate and dehydrocholate; their presence in the canaliculi creates an osmotic gradient that produces the flow of water and solute. There is a positive correlation between the canalicular bile flow stimulated by taurocholate and the amount of iopanoic acid excreted by the liver. Feeding the patient a fatty meal or taurocholate at the time that iopanoic acid is administered can improve the quality of cholecystograms (734).

In addition to bile salts, contrast media (779) and other agents (752, 777) may also increase bile flow and cause choleresis. Iodipamide produces 0.025 mL bilelpmol excreted compared with 0.009 mL bile/μmol for ipodate, but both agents are able to achieve similar maximum bile iodine concentration. The reason for this phenomenon is that ipodate is excreted as glucuronide conjugate, incorporated in the bile salt micelles, which exert a small choleretic effect, whereas iodipamide is excreted unchanged (780).

Drugs such as phenobarbital and cinchophen alter the bile flow and the biliary excretion of cholegraphic agents. Pretreatment with phenobarbital decreases iodipamide bile excretion and increases iopanoate bile excretion (752). Cinchophen increases both biliary and urinary excretion of iophenoxic acid but has no effect on iopanoic acid (777). It has been reported recently that a high level of prostaglandin-like substance in the mucosa and muscle wall of gall bladder caused nonvisualization of the gall bladder in cholecystography (781).

Urinary excretion is the principal means of elimination for diatrizoate, iothalamate, metrizoate, metrizamide, and other urographic agents (688, 767, 782, 783). Diatrizoate, iothalamate, and metrizamide were excreted in the urine of the rabbit to the extent of more than 80% 24 h after injection (768, 783–785). The total recovery including the amount excreted in feces 96 h after injection was about 91.5% of the dose for metrizamide, 93.8% for diatrizoate, and 86.0% for iothalamate (782).

The nonionic contrast media are highly hydrophilic. When given orally, iohexol is absorbed from normal bowel (785). In dogs, approximately 2% of the oral dose is absorbed and excreted in the urine within 6 h, and about 4% of the dose is absorbed and excreted in cats. Using ^{125}I-labeled iohexol, Mützel and Speck (267) found intestinal absorption to be about 2% of the intravenous dose given at 60 mg I/mL body weight to male rats. The urinary excretion from the rat was 91.5 ± 3.6% of the administered dose of ^{125}I-labeled iohexol, and the fecal excretion 6.8 ± 2.7% of the dose 24 h after intravenous injection. In the dog the urine contained 81 ± 9% of the administered dose within 3 h after injection. Total recovery from urinary excretion in the dog during the 7 days after injection was 98 ± 4% of the dose and total recovery from fecal excretion was 0.95 ± 0.45%. Healthy volunteers excreted 84% of the dose in urine within 4 h and 100% of the dose within 24 h after intravenous injection at the rate of 25 mL/min of iohexol at a

solution concentration of 350 mg I/mL at one of the dose levels (i.e., 100, 200, 375, and 500 mg I/kg body weight) (733). Another group of healthy volunteers were administered iohexol at higher dose levels (i.e., 500, 750, 1000, and 1500 mg I/kg body weight) and excreted 92.3 ± 4.44% of the dose in urine 96 h after injection, with 89.9 ± 7.0% of the dose appearing in urine within the first 12 h (758).

The biodistribution of ^{125}I-iobitridol was studied in rats using whole-body autoradiography (786). Radioactivity was found mainly in the kidneys, skin, and thyroid. The accumulation of radioactivity in the thyroid was attributed to free ^{125}I-iodide formed in radiolabeling. Iobitridol, a marker of extracellular fluid, was excreted totally in the urine by glomerular filration within 48 h after intravenous injection. The CNS showed no uptake. Bourrinet et al. (787) studied the excretion of iobitridol or iohexol in goat milk after an injected dose of 300 mg I/kg and found 0.7% of the administered dose in the milk for iobitridol, compared with 1.6% for iohexol. In the gestating rabbit concentrations of both contrast agents fell rapidly below the detection limit (0.2 mg I/mL) 180 min after injection. Concentrations in fetal plasma and amniotic fluid were not measurable up to 24 h for both contrast agents.

The excretion of nonionic iopamidol was studied in healthy subjects at several dose levels by use of two different solution concentrations of 200 and 300 mg I/mL, which were administered intravenously at a rate of 20 and 39 mL/min, respectively (760). Urinary elimination of iopamidol was rapid at all dose levels, with more than half of the dose excreted by the kidneys in the first 2 h after injection. Total urinary recovery within 72–96 h after injection was more than 90%, and the fecal recovery for the same period was about 1% of the administered dose. ^{14}C-Labeled iopamidol was also included in this study for quantitation and the search for metabolic products of iopamidol, of which none was found. In dogs the urinary and fecal excretion of iopamidol at 72 h were 94.8 ± 1.2 and 0.63 ± 0.32%, respectively, of the injected dose at the dose level of 50 mg I/kg body weight. At a higher dose level of 200 mg I/kg, these excretions were 93.4 ± 1.5 and 3.17 ± 1.39%, respectively (195). The excretion of another nonionic contrast medium ioversol, labeled with iodine-125, was studied in rats. The elimination of radioactivity from the blood was extremely rapid. Urinary excretion was 95.33% and fecal excretion was 3.8% of the administered dose within 48 h after intravenously injection. Urinary excretion of ioversol in healthy volunteers was complete at all dose levels within 24 h after intravenous administration (762). The excretion of a dimeric nonionic contrast agent iodixanol was investigated in healthy volunteers at four dose levels (i.e., 0.3, 0.6, 0.9, 1.2 g I/kg body weight). Within 24 h after injection, 97% of the dose was excreted unmetabolized in the urine and 1.2% in the feces (765).

These water-soluble contrast agents are excreted as rapidly as glomerular filtrate can be formed (688). Their elimination may be impaired by primary and secondary glomerular dysfunction or reduced renal blood flow that diminishes the filtration rate (788). After clearing the blood, the contrast medium in its passage down the nephron can affect the salt and water reabsorption in the proximal tubule and water reabsorption in the distal tubule. The osmotically active contrast medium can produce salt and water diuresis, and its presence in the nephron may still cause renal toxicity (789). In experimental animals (790) and in humans (688), the sodium salt of diatrizoic acid produces less diuresis than that of the meglumine salt. The renal extraction of *p*-aminohippurate is reduced by the contrast anion and the meglumine cation (791). Iodamide, an intravenous pyelographic agent, is rapidly and almost completely excreted in the urine; in patients with moderate to severe renal impairment, it is still excreted in the urine but at a much slower rate (792).

The excretion of contrast agent by the liver or the kidney is not exclusive; when either of these organs is in a dysfunctional or diseased state, heterotopic excretion by the other organ will occur to compensate for the impediment (688, 781, 783). In patients with renal failure or advanced renal disease, contrast media may still be used to produce nephrograms (689); if the tubular obstruction prevents the passage of urine along the nephron, back-diffusion of the contrast medium into the interstitial volume will occur (791,793,794). During the pe-

riods of ureteric stasis, metrizarnide is excreted faster than diatrizoate, thus producing a higher urinary iodine concentration (685, 795). Extrarenal clearance of water-soluble contrast media is very low and can be practically neglected (796).

When suboccipitally injected, metrizamide was recovered in the urine and feces of rabbit, rat, and cat to the extent of more than 90% of the dose (787), similar to the recovery of iothalamate (797) and iocarmate (798) in dogs.

In peroral administration, metrizamide and diatrizoate are not well absorbed from the GI tract; the urinary recovery of both agents in the rabbit was below 5% of the administered dose, 24 h post-administration (767). The nonionic contrast media are highly hydrophilic and cannot cross the lipophilic cell membranes in the intestine to be absorbed as an oral dose. Iophendylate, when given to rats, guinea pigs, or rabbits, was eliminated from the body in 5 h (799). It was transported along the lymph system.

6.5.1 2 Biotransformation. Contrast media may be excreted either metabolized or unchanged, depending on their molecular properties. McChesney (800) has reviewed the subject of biotranformation of contrast media.

The fully substituted 2,4,6-triiodobenzoic acids used for angiography and urography have high water solubility and are stable chemically. Because of their low pK_a values, they are highly ionized at physiological pH. These contrast agents are poorly absorbed from the gastrointestinal tract and rapidly excreted unchanged within 5–6 h after intravenous injection (776). Their metabolic inertness is attributed to their inability to cross cell membranes and to penetrate liver microsomes to undergo biotransformation (800). Their rapid excretion is caused by the absence of tubular reabsorption, thus allowing as rapid an excretion as the glomerular filtrate can be formed. Diatrizoate (3,5-diactamido-2,4,6-triiodobenzoate) can be partially deacetylated to 3-amino-5-acetamido-2,4,6-triiodobenzoate (I) and 3,5-diamino-2,4,6-triiodobenzoate (II) by liver microsomes of the rabbit and guinea pig (801). The deacetylated products are rapidly excreted. In the urine of patients only the presence of (I) is confirmed.

In contrast to the 2,4,6-triiodobenzoic acids, substituted alkanoic and alkoxyalkanoic acids containing a 2,4,6-triiodophenyl or a 2,4,6-triiodophenoxy group with open position 5, such as iopanoic acid, tyropanoate, ioglycamic acid, and iopronic acid, have both lipophilic and hydrophilic properties. These contrast acids have an acidity constant about two units higher than that of the water-soluble, fully substituted 2,4,6-triiodobenzoic acids (263) and are not completely ionized at the physiological pH. In the nonionized form, the contrast molecules can be absorbed and transported across cell membranes after oral administration.

The oral cholecystographic agents are in general more toxic than angiographic and urographic agents. Detoxification of the former may involve glucuronidation, N-acetylation, *O*-acetylation, esterification, deiodination, and hydrolysis. These metabolites appear in the bile and urine. The metabolites of many cholegraphic agents have been characterized to some degree but are not chemically identified.

Conjugation with glucuronic acid occurs with most oral cholecystographic agents before excretion. Bunamiodyl (802, 803), iodoalphionate (804, 805), iolidonic acid (806), iophenoxic acid (807–809), and tyropanoate (802, 803) are excreted as glucuronides. McChesney and Banks (803) showed that 90% of the contrast agents in the urine appeared as glucuronides. Because glucuronides are usually poorly reabsorbed from the intestine, glucuronidation can thus circumvent extensive enterohepatic recirculation of the contrast medium.

The fate of iophenoxic acid (809) and the efficacy of iopanoate (800, 802) are uniquely linked to glucuronidation. Iophenoxic acid is retained in the body over a period of many years after administration and is slowly excreted. It forms acyl and ethereal monoglucuronides and the diglucuronide. These glucuronides have the uncommon property of being freely lipid soluble and can undergo hepatic, intestinal, and renal tubular reabsorption. Wade et al. (809) pointed out that the rate of formation of glucuronide conjugates may be an important factor in the persistence of iophenoxic acid in the body. Iopanoate glucuro-

nide, when administered orally, gave no visualization of gall bladder but when injected intravenously yielded an excellent cholecystogram 1 h after administration; however, an equivalent dose of iopanoate when injected required a delay of 8–24 h for an image of diagnostic quality to become apparent, indicating that the iopanoate was converted to glucuronide before being excreted in the bile (800, 802).

Bornschein et al. (297, 810) reported the appearance of the unchanged iomeglamic acid, the methyl ester, the deiodinated derivative, the N-desmethyl derivative, the methyl ester of the latter derivative, the N-acetylated derivative, and the deiodinated methyl ester of iomeglamic acid in human urine 72 h after a 3 g dose. Lindner et al. (293) showed evidence of biotransformation of iobenzamate in the mouse, rat, and human, which involved conjugation with glucuronic acid, hydroxylation of the unsubstituted phenyl ring, or hydrolysis of the amide linkage, for example, but isolation and identification of the metabolic products were not carried out. Harwart et al. (286) studied the metabolic fate of ipodate and found that there was unchanged ipodate in the bile, and the major product exereted in urine was soluble in butanol. One of the metabolites was identified as 3-amino-2,4,6-triiodohydrocinnamic acid, a reduction product of ipodate.

The hexaiodinated dimeric intravenous cholangio-cholecystographic agents, such as iodipamide, ioglycamide, iodoxamate, and iotroxic acid, are excreted mainly unchanged (788, 776, 811–814). Strickler et al. (815) reported an unidentified metabolite of iodipamide, probably formed from hydrolysis of the amide linkage. Miitzel et al. (816), by use of ^{131}I-labeled tracers, found that ioglycamide, iodoxamate, and iotroxic acid each yielded two urinary metabolites, one of which was more polar and the other less polar than the parent molecule. It was shown that neither of these two metabolites was a deiodinated product, and that the bile and plasma contained no metabolites. Pitre and Felder (817) reported three metabolites of iodoxamic acid in human urine, one of which was identified as 3-[3(α-hydroxyethoxy)-propionamido]-2,4,6-triiodobenzoic acid, a product formed from symmetrical scission of the dimer.

Deiodination is not an important reaction in the biotransformation of contrast media, in spite of the presence of deiodase in the mammalian liver (818). The rat and pig liver, however, can convert 2,3,5-triiodobenzoic acid into 2,5- and 3,5-diiodobenzoic acids (819–821). Iodoalphionate (822, 823), iothalamate (824), iodipamide, and ioglycamide (812) were reported to undergo deiodination to a small degree, never exceeding 1% of the dose. Because most organic iodine compounds are sensitive to light, the observed deiodination may be a result of both enzymatic reaction (821) and photolytic degradation (825). Wong (826) studied the kinetics of deiodination of the water-soluble radiopaques diatrizoate and iopamidol, and found that deiodination proceeds by a Cu(II)-catalyzed S_N1 mechanism and that the copper complexes specifically with the *ortho*-carboxylic acid group. Nonionic iopamidol without the o-carboxylate group undergoes deiodination by S_N2 mechanism involving hydroxyl ion, and the rate is extremely slow. Kinetic data show that the S_N1 mechanism may still be participating in deiodination. Deiodination in both contrast agents is prevented by the presence of sodium edetate (0.04%).

Deiodination of contrast agents may affect the thyroid ^{131}I uptake. Iodoalphionate (827, 828), iodipamide (827, 829), and iodopyracet (830) affect the thyroid uptake by a slow release of the iodide. In thyroid patients, the thyroid uptake returns to normal 4 days after the administration of acetrizoate, 58 days after iopanoic acid (831), and many years after iophenoxate (832). Iophenoxate binds avidly to plasma protein and is transported across the placental barrier as long as 5 years after administration. The cholecystographic agents may have a direct effect on the thyroid. Iodide-induced hyperthyroidism may result from ipodate administered for cholecystography (833) or from iophendylate injected for myelography (834). Intravenous administration of ioxitalamic acid to euthyroid patients increased their protein-bound iodine (PBI) and hormonal iodine levels, which returned to normal between 2 and 8 days (835).

6.6 Uses

Roentgenographic procedures requiring the use of radiopaques are listed in Table 10.10.

Table 10.10 Uses of Contrast Agents: Radiographic Procedures for Visualizing Organs After Administration of Contrast Agents

Procedure	Organs Visualized
Angiography	Blood vessels
Arteriography	Arteries
Aortography	Aortas
Arthrography	Joints
Bronchography	Lungs
Cholangiography	Gall bladder and bile ducts
Cholecystography	Gall bladder
Esophagography	Esophagus
Hepatography	Liver
Hepatolienography	Liver and spleen
Hysterosalpingography	Uterus and oviducts
Lymphography	Lymph nodes and vessels
Lymphadenography	Lymph nodes
Lymphangiography	Lymph vessels
Myelography	Spinal cord, subarachnoid space
Pelviagraphy	Pelvis
Phlebography	Veins
Pyelography	Kidney and ureter
Splenography	Spleen
Splenohepatography	Liver and spleen
Urography	Urinary tract
Venography	Veins
Ventriculography	Ventricles of the brain

Before the development of other medical imaging systems, the roentgenographic procedures listed in Table 10.10 were performed with radiocontrast agents. MRI with superparamagnetic contrast agents, ultrasonography with echogenic contrast agents, and nuclear medicine with radiopharmaceuticals, labeled with ^{11}C, ^{18}F, ^{99m}Tc, and other short-lived radio-nuclides for example, are additional modalities for organ imaging and organ function studies. These systems have their own merits, and their use led to the replacement of some of the above roentgenographic procedures. Progress in medical instrumentation has brought CT, helical CT, and the multidetector-CT scanner into practice; these fast instruments greatly facilitate the use of radiocontrast agents. The textbook of contrast media by Dawson, Cosgrove, and Grainger (836), published in 1999, contains up-to-date discussions of X-ray contrast agents, MR contrast agents, and ultrasound agents for medical imaging. The strengths and problems of the currently available imaging techniques in their book are compared, to which the reader may refer. Illustrative examples of the use of radiocontrast media for some roentgenographic procedures, current or dated, are given below.

6.6.1 Computer-Assisted Tomography (CT). Unlike the conventional roentgenographic system in which the transmitted X-ray image is directly projected onto a fluorescent screen or a photographic plate, in the CT system an object is scanned with a narrow beam of X-rays from a multitude of angles, and the absorption values of the material contained within the object are computed from the X-ray transmission readings and presented as a series of pictures of slices of the object (837). The image reconstruction requires fast computing. The speed of the CT scanners has improved from the first-generation 4.5-min head-only scanner to the fourth-generation 2-s head or body scanner (838, 839). The ultrafast scanners that may be categorized as the fifth-generation X-ray CT scanners are necessary for the imaging of moving organs, such as the heart.

The first CT system was shown to be approximately 100 times more sensitive than the conventional X-ray system (837). Contrast enhancement in CT is measured in terms of a density unit. In the original article, water was arbitrarily set at a value of 0 unit; air, 500 unit; fat, 50 unit, and so forth. On this scale, a number of tissues in the body fell within 20 units above water or "4% window" in scanning. This unit was later named the Hounsfield Unit (HU) in honor of the author (840). Using HUs, air has a value of −1000, water a value of 0, and dense bone a value of +1000. The white tone on the picture represents dense material with enhanced contrast. Materials such as tumor will be less enhanced by the contrast medium because of limited blood supply and will appear hypodense, gray, or less white. Organs or tissues not contrast enhanced will appear black on the picture. A similar unit, known as the CT number, for measuring the image contrast or conspicuity, discussed by Dawson (836), is based on a com-

parison of the attenuation coefficient (μ) of the region of interest (ROI) to that of water and is defined as:

$$CT\ Number = \frac{\mu_{ROI} - \text{hater}}{\mu_{water}}$$

The increase in density in HUs or CT numbers is linearly dependent on the iodine concentration (mgI/mL) of the injected contrast medium. CT has two advantages over projection radiography. One is the significant increase in contrast resolution and sensitivity, which reduces the contrast detection threshold, thereby allowing a lower level of concentration of contrast media to be administered; the other is the cross-sectional imaging, which permits successive visualization of structures without the superposition of other tissues to affect the image quality (632).

Computed tomography (CT) can detect small differences in X-ray absorption at approximately one-fifth, perhaps as much as one-eighth, of the concentration of contrast media, if one uses lower kilovolt (kVp) techniques, compared with that of conventional radiography (841).Contrast media for CT may be administered as bolus, or bolus followed by infusion (biphasic), or infusion alone (842, 843). The pharmacokinetics is such that within 1 min after bolus injection the contrast enhancement reaches maximum in the extravascular space, followed by a washout. Computer-assisted tomography has been applied to angiography, arteriography, cardioangiography, phlebography, and numerous other X-ray procedures. The sensitivity of CT to minimal density differences between tissues allows a variety of contrast agents, such as xenon, air, saline, particulates, microemulsions, liposomes, and iodinated oils, to be explored for contrast enhancement. These in combination with dynamic CT, in which conventional water-soluble nonionic iodinated contrast media are rapidly administered in incremental amounts, are powerful means in obtaining diagnostic information (92,844, 845).

6.6.2 Spiral (or Helical) CT. Conventional CT scans the body or organ volume by slices, whereas spiral CT scans the patient continuously during a single breath hold, thus affording contiguity of slices and uninterrupted scanning information. The spiral geometry in CT scanning is achieved by continuously transporting the patient through the gantry in synchrony with continuous data acquisition over a multitude of 360" scans. Kalender et al. (846, 847) used a standard continuous rotating CT scanner that produces up to 12 consecutive 1-s scans and a modified table feed mechanism with a stepper motor to allow continuous transport of the patient at low, but accurately controlled, speeds (0.1–11.0 mm/s) during scanning. The images are mathematically reconstructed from the spiral geometry. Many physical parameters, such as spatial resolution, image uniformity, linearity, and contrast that determine the image quality of spiral CT, are not affected, but noise is slightly reduced and section sensitivity profiles are widened as a result of transport during scanning. In spiral CT the scan times for a complete volume are at a minimum, and the scannings are contiguous, independent of patient breathing and motion (847). Spiral CT provides speed, breath hold protocols for less motion, fewer geographic misses, and easier timing of contrast peak distribution (841). Hidajat et al. (848) showed that spiral CT can lead to a reduced radiation integral dose exposure to the patient compared with that of conventional CT.

6.6.3 Ionics versus Nonionics. Contrast media are foreign substances, just like other drugs, and thus when given in a large dose within a short period, adverse reactions, accompanied by delayed symptoms perhaps, will inevitably occur (849). McClennan (850) emphasized that mild and moderate reactions should be given recognition and response because a minor side effect may be a prelude to a more serious adverse reaction. Siegle (514) compared the rates of idiosyncratic reactions in patients receiving ionic and nonionic contrast agents and found that 4.4% of patients receiving ionic agents experienced a reaction as compared with 0.59% of patients receiving iohexol. The percentage of patients requiring treatment was 1.2% for ionic agents and 0.24% for iohexol. Patients receiving ionic agents plus steroids did not fare better. Cohen (851)

reviewed the toxicity of nonionic contrast agents in children and found that moderately severe reactions occur less frequently with nonionic agents and that the incidence of adverse reactions in children and in adults appears to be similar, contrary to observations by others (553, 536, 630).

Bettmann (852) discussed the safety and efficacy of iodinated contrast agents from the viewpoints of use, regulation, and adverse events and noted that an intra-arterial digital subtraction angiography of the pelvis and lower extremities performed with a contrast agent, diluted to an iodine content of 100 mg I/mL, yielded useful images for diagnosis. Dilution lowers the osmolality of contrast media and improves tolerance. Dean et al. (853) studied the effect of dilution of diatrizoate (ionic monomer), iopamidol (nonionic monomer), ioxaglate (ionic dimer), and iodecol (nonionic dimer) in a rat model at a dose of 500 mg I/kg administered at two different concentrations of 300 and 150 mg I/mL. The authors observed that the dilution of the contrast medium did not lower iodine tissue concentration, iodine distribution volumes, plasma volumes, or hematocrit, and should have no effect on CT contrast enhancement. Vergara and Sequel (630) found that warming ionic contrast agents to 35°C before injection significantly decreased the incidence of adverse reactions and that ionic contrast agents without a sodium cation caused significantly fewer reactions than with a sodium cation. According to these authors a nonionic, warmed contrast medium was the best option for the outpatient CT examination because no severe reactions resulted from its use.

The cost differential between ionic and nonionic contrast agents is substantial and is a consideration for cost containment in health maintenance. In 1994 in the United States this cost differential is estimated to be about 10- to 20-fold, and it is far smaller in Europe (852). The cost differential between nonionics and ionics has been reduced to 5 or 6 to 1 in 2002 in the United States, according to one of the reviewers of this manuscript. This reviewer also indicated that ionic contrast media are no longer administered intravenously, but used instead to fill lumens (i.e., bladders, fistulas, etc.).

6.6.4 Angiography. Methiodal sodium, sodium iodomethamate, and iodopyracet were the earliest water-soluble organic iodine compounds available for angiography (10). From the incidence of patient reactions and from laboratory investigations, it became evident that these compounds were not entirely suitable for angiography use. In about 1950, substituted triiodobenzoic acid derivatives were introduced as contrast agents (219,490). Acetrizoate was the first of the series introduced, followed by diprotrizoate and then by diatrizoate, iothalamate, metrizoate, iodamide, and ioxitalamic acid. Acetrizoate and diprotrizoate were found to have certain toxic effects but diatrizoate, iothalamate, and metrizoate were better tolerated and have found extensive use in angiography (490). Iodamide and ioxitalamic acid are widely used in Europe. Benton et al. (854) found that meglumine calcium diatrizoate is superior to meglumine diatrizoate and meglumine sodium diatrizoate. In 1970, dimers and trimers of contrast agents and nondissociable compounds were introduced for angiographic use (9). Newer agents such as iocarmic acid (bisiothalamate), iozomic acid, and tris(iothalamate), as well as the nondissociable metrizamide, have fewer side effects and are better tolerated. Metrizamide, the first-generation nonionic contrast medium, has been the choice for cardioangiography and coronary angiography (855). The low osmolality ionic ioxaglate and the second-generation nonionic iopamidol and iohexol are better tolerated than the ionic contrast media. with no measurable clinical disadvantage in diagnostic and therapeutic cardiac radiology, particularly in high risk patients (856). Coronary angiography with iopamidol is less painful and has fewer deleterious effects on myocardium metabolism and cardiovascular system than the ionic media (857–859).

6.6.5 Cholangiography. Intravenous cholangiography was introduced in 1954 as a radiographic technique in the diagnosis of biliary tract disease and is practiced in cases in which conditions causing obstruction to passage and resorption are present and prevent the use of oral cholecystography (737). The sole contrast agent used in the procedure in the United States is iodipamide, which is

available as sodium and meglumine salts. The difference between the two salt forms is in the volume of the dosage required for examination, with the volume of meglumine salt less than that of sodium salt. Adverse reactions after a single bolus injection are reduced if the contrast medium is administered by slow-infusion technique.

Many other cholangiographic agents are available. Brismar et al. (860) studied ioglycamide or ioglycamic acid in relation to iodipamide. Ioglycamide is widely used in Europe and has an excellent contrast property for examinations of bile ducts after intravenous injection but offers no advantage over iodipamide in examinations of gall bladder. An intravenous cholangiographic agent, iodoxamic acid, introduced in 1973, apparently has lower toxicity (190, 861).

Cholangiography agents are mostly dimers of triiodobenzoates, such as iodipamide, ioglycarnide, iodoxamate, iotroxate, and iosulamide, with high water solubility and high biliary excretion (862). Iosulamide with a fully substituted triiodophenyl ring was found experimentally to cause less severe reaction and more rapid opacification than iodipamide (323).

6.6.6 Cholecystography. Graham et al. in 1924 (863) obtained the first cholecystogram in dogs with tetrabromophenolphthalein. Tetraiodophenolphthalein was used for cholecystography until 1943, when iodoalphionic acid was introduced (175), which produced better radiographic visualization of the gall bladder with fewer toxic reactions. Its use was superseded by iopanoic acid, a substituted triiodobenzoic acid introduced in 1951 (864). Iopanoic acid is better tolerated and has a greater opacity than iodoalphionic acid. The N-butyryl derivative of iopanoic acid, tyropanoate, was introduced in 1963 and was found to be more readily absorbed from the GI tract than iopanoic acid. This compound, in addition to rapid oral absorption, gives excellent opacification of the gall bladder under conditions of clinical use and has a low incidence of side effects. Opacification with tyropanoate occurs with diagnostic quality on the 4-h film and the 8-h film in 64 and 78% of the patients examined, respectively (865,866).

A number of substituted 2,4,6-triiodobenzoic acid derivatives were introduced as cholecystographic agents. Among them, bunamiodyl and iophenoxic acid were later withdrawn by the manufacturers because bunamiodyl caused renal shutdown and iophenoxic acid caused high protein-bound iodine values persisting over many years.

Ipodate, introduced in 1961, can yield visualization of both gall bladder and bile ducts. Tyropanoate, iopanoic acid, and ipodate are widely used in oral cholecystography. They show about the same efficacy but differ in the intensity of opacification, the frequency of dim and absent shadows, and the frequency of side effects. Russell and Frederick (867) compared these three agents but failed to demonstrate the superiority of any one of them. Iocetamic acid was favorably compared with and preferred to the above three agents, but skin reactions were reported in a few cases (775,868, 869).

6.6.7 Myelography. Oil-soluble and water-soluble contrast agents have been used in myelography. These agents are administered intracisternally. The absorption of oily and water-soluble contrast media from the subarachnoid space is completely different. The oily media remain in the subarachnoid space for years after injection, whereas the water-soluble media are eliminated within a few days (799).

The water-soluble iodopyracet was introduced for myelography in 1931 (870). Because of the extreme irritant effects as well as occasional production of some long-term disabilities, it never gained wide use. Iodized oil Lipiodol was used for myelography with less irritation. Its aftereffects were avoided if the oily medium was removed by aspiration from the spinal canal (871). Iophendylate was introduced by Ramsey et al. (872) for myelography in 1944 and proved to be an excellent contrast medium for the entire spinal canal and the basal cisterns. Like the iodized oil, it had to be aspirated to avoid aftereffects (873). Arachnoiditis did develop in some cases in the subarachnoid space because of the presence of residual iophendylate.

Iothalamate meglumine was used for myelography in 1964. Melartin et al. (656) showed

that meglumine and iothalamate ions are less neurotoxic than sodium and diatrizoate ions. The irritating effect of meglumine iothalamate is so slight that its use in myelography requires no spinal anesthesia (682). Iocarmic acid (i.e., dimerized iothalamate), with a higher iodine content and a lower toxicity than those of iothalamate, has been used in myelography (214, 874). Both iocarmic acid and iothalamate tend to be spasmogenic, causing clonic convulsions or muscular twitchings in the leg (875). Nonionic, water-soluble metrizamide was introduced for myelography in 1973 (876). It is less irritating than available myelographic agents and is capable of visualizingsmall structures such as nerve roots, root pockets, and blood vessels. Nonionic, water-soluble iopamidol causes fewer and less severe adverse reactions particularly in terms of behaviorial changes and compares favorably to metrizarnide (857). Iopamidol also induces less discomfort to the patients in cerebral angiography than iothalamate and diatrizoate (877).

6.6.8 Urography. Contrast agents rapidly excreted by the kidney are used in excretion urography. Iopax, or Uroselectan, was first introduced for urography in Germany in 1929. Many other agents such as methiodal sodium, hippuran (sodium oiodohippurate), sodium iodomethamate, and iodopyracet were also introduced. These agents were the earliest available, and iodopyracet was the most widely used (490).

With the introduction of modern contrast agents beginning in about 1950, urography has been conducted by use of the water-soluble acetrizoate, diatrizoate, iodamide, iodipamide, iothalamate. and metrizoate. These agents, because of their higher radiopacity and better tolerance, have superseded the earlier agents, although their individual toxicities vary. Among these, diatrizoate is the least toxic and the most favored in urography (490).

Contrast agents cause osmotic diuresis. Studies show that sodium diatrizoate is excreted in higher concentration in the urine than is meglumine diatrizoate because of resorption of sodium ions in the renal tubules. Benness (790, 878) reported a difference in roentgenographic quality in pyelograms in favor of sodium contrast agents.

Dimeric and trimeric contrast agents with more iodine atoms per molecule improve the opacity of urine by their high iodine concentration and cause a decrease in osmotic diuresis. In sheep, these new large molecules produced smaller urinary solute output, less diuresis, and higher urinary concentration than the same parameters of diatrizoate (879, 880, 809). These agents are clinically useful.

Urine iodine concentration, after the injection of nonionic metrizamide. is about twice as high as after sodium diatrizoate injection. Golman et al. (795) reported that during the periods of ureteric stasis, metrizamide was excreted faster than diatrizoate. Nonionic iopamidol reduces the incidence of adverse reactions in excretory urography and produces equal quality urograms with less iodine than diatrizoate (881).

7 MISCELLANEOUS AGENTS

7.1 Perfluoroctyl Bromide

In addition to organic iodinated compounds, perfluorocarbons are also potential contrast agents. The least toxic one among them is perfluoroctyl bromide ($C_8F_{17}Br$), which has been introduced by Long et al. (882–884) for bronchography, myelography, splenography, lymphangiography, and special gastrointestinal studies requiring small volumes of nontoxic radiopaques.

Perfluoroctyl bromide (PFOB) has a high degree of chemical and physical stability and nonreactivity, a biologic inertness comparable to that of Teflon, a high oxygen solubility, and a low surface tension. Liquids with low surface tension wet surfaces readily and flow freely into tiny folds and orifices. This property in a contrast agent improves the quality of the roentgenogram. PFOB has a radiopacity about 50% of that of iothalamate, and may be administered as neat liquid or emulsions. Emulsions in normal saline can be prepared with the aid of the emulsifying agent Pluronic F-68. Perfluorocarbon emulsions have a non-Newtonian viscosity that can be varied over a very wide range by changing perfluorocarbon and surfactant concentrations and emulsifying technique (882,883).

Perfluoroctyl bromide, when given tracheally either as neat liquid or as a 10:1 emulsion, was cleared roentgenologically from the lung within 24 h. Evaporation and mucociliary clearance play an important role in the elimination of this radiopaque from the lungs. When the tissue samples were analyzed by gas chromatography, approximately 1% of the neat liquid and 8% of the emulsion were found still remaining in the tissue. The lungs had the largest amount, followed by the intestine, adipose tissue, and lymph nodes. When injected intrathecally into the dog, a dose of 0.5–1.0 mL/kg caused no toxic manifestation. It is much less irritating than iodophendylate to the arachnoid tissue.

Perfluoroctyl bromide is especially useful roentgenographically for patients who are allergic to iodine compounds and who have had meningitis before myelography. Emulsions of PFOB have been investigated for contrast enhancement of tumors (845), lymph channels and nodes in animals (840), and as an arthrographic contrast agent in the knee joints of dogs and a human cadaver (885). The PFOB emulsion is less viscous and easier to inject than Ethiodol for lymphography. The high sensitivity of computed tomography to minimal density differences between tissues allows PFOB to be experimented in the rabbits for contrast enhancement of malignant neoplasma (886), reticuloendothelial systems (liver, spleen, and macrophages), and VX2 tumors (887), and in CT imaging of septic and aseptic arthritis (888). PFOB produces in CT prolonged opacification of the blood pool and subsequent selective enhancement of liver and spleen in the pig for days (889). The fluorocarbon causes no acute hemodynamic effect in comparison to ionic contrast media. The LD_{50} value for intravenously administered 48% PFOB emulsions in rats was found to be approximately 25 or 30 mL/kg, and the agent is eliminated as vapor from the organism through the lungs and skin (887). PFOB may activate the immune system and stimulate macrophage migration to the sites of infection and neoplasia. Information on details of the whole-body toxicity of PFOB and on the mechanisms of vacuole formation in macrophages has been scanty.

7.2 Iodinated Particulate Suspensions

After bolus injection, water-soluble contrast media, routinely used in angiogrphy, are rapidly distributed throughout the intravascular space and diffuse across the capillary wall into the interstitial space. A brief time window exists for imaging the differential contrast between organs and adjacent tissues before nonspecific total body opacification occurs. In comparison, particulate contrast media are not hyperosmolar, remain longer in the intravascular space, and are specifically targeted to organs, such as blood pool, liver, and spleen (890). Particulate contrast media, when administered in the form of emulsions, liposomes, microcapsules, and nanocrystals, for example, exert no osmotic pressure and are rapidly taken up by the macrophages and the Kupffer cells in the reticuloendothelial (RE) system and accumulate in the liver and spleen. The RE system is saturable, and overflow from high doses will persist in the circulation. Particulate agents produce high contrast between normal and pathologic tissues at a fraction of the dose required of conventional agents and maintain the density difference for CT imaging over prolonged periods of time. The adverse effects of the particulates, including the clearance, the effect of long-term retention, Kupffer cell activation, disturbances in'the microcirculation of the liver (891), and vacuoles in the RE cells (892), have yet to be further studied. Violante and Fisher (893) discussed the complexities and problems in designing and formulation of RE-selective contrast media. Later advances in producing liposomes and encapsulated vesicles entrapping water-soluble iodinated contrast media and other particulate suspensions are briefly mentioned below:

7.2.1 Liposomes. Liposomes are vesicles consisting of one or more bilayers of lipid encapsulating an aqueous core, carrying water-soluble contrast agents. The weight ratio of iodine to lipid (I/L ratio) is a measure of the amount of iodine per unit weight of lipid delivered to the target organ (894). For hepatic enhancement in CT this ratio needs to exceed 1:1 (895). The leakage and the I/L ratio of liposomes depend to a large extent on the lipid

components, the microemulsification process, and the nature of water-soluble contrast agent. Methods for producing contrast-carrying liposomes may be categorized as (1) the microemulsification method using a high pressure microfluidizer (894, 896, 897), (2) the dehydration-rehydration method (891, 898), and (3) the reverse-phase evaporation method (899,901).

Cheng et al. (894) produced liposomes by microemulsification through use of a high pressure microfluidizer from a contrast agent and a mixture of egg phosphatidylcholine, phosphatidylglycerol, and cholesterol in the molar ratio of 0.4:0.1:0.5. The liposomes carrying nonionic contrast agents have higher entrapment and weight ratio than those carrying ionic contrast agents; the decreasing order is as follows: iotrolan > metrizamide > iohexol > iopamidol > ioxaglate > diatrizoate > iodipamide. For diatrizoate the mean I/L ratio was 0.062 ± 0.01 mg/mg and the entrapment value, 0.12 ± 0.02 L/mole. The weight ratio for nonionic contrast agents increased significantly in the liposomes with increasing initial concentrations of the contrast agent. The leakage rate of encapsulated diatrizoate was over 20% in physiological saline. Encapsulated iohexol gave greater and more prolonged opacification of the liver and spleen than plain iohexol. Maximum contrast was reached within 30 min after injection and maintained for more than 60 min. The results indicated that osmotic pressure, charge, viscosity, and lipophilicity of contrast media might influence the encapsulation process.

Leakage of water-soluble contrast agents from the encapsulated liposomes can be eliminated, using an interdigited lipid phase consisting of hydrogenated soy phosphatidylcholine or distearoyl phosphatidylcholine to produce "interdigitation-fusion" (IF) vesicles. Janoff et al. (897) found that the IF vesicles carrying iotrolan, ioversol, ioparnidol, ioxaglate, and diatrizoate indeed have higher I/L ratios varying from 5.5 to 8.9, and vesicle sizes ranging from 1 to 5 μm in diameter. Leakage by incubation of the liposomes *in vitro* with fetal calf serum at 37°C for 4–24 h showed that iotrolan and ioxaglate liposomes retained almost all of the iodine, whereas iopamidol and diatrizoate liposomes lost about 50 and 80% of the encapsulated iodine, respectively. In dogs and rats, 1 h after injection at dose levels ranging from 25 to 250 mg I/kg, the liver retained an average of 93.7% of the injected ^{125}I-labeled iotrolan IF-vesicles and the blood retained less than 5% of the dose (895). At 24 h the initial dose in the liver fell to about 10% and decreased thereafter with a half-life of about 6 h. By 115 days, 0.2% of the initial dose still remained in the liver. The high liver uptake was attributed to less lipid in the IF-vesicles so that the RE system was not prematurely saturated.

Krause et al. (899,900) studied the characterization of iopromide liposomes, produced from a lipid mixture of phosphatidyl choline, cholesterol, and stearic acid at a molar ratio of 4:5:1 by a dehydration-rehydration method. The liposome had an average diameter of 0.5 ± 0.1 pm. Encapsulation efficiency was between 30 and 40%, with an iodine-lipid (I/L) ratio of approximately 1. The iopromide-carrying liposomes had a leakage of 4.6% in rabbit plasma and 9.2% in human plasma in the 0- to 4-h period and a leakage of 9% in rabbit plasma and 20% in human plasma in the 0- to 24-h period. The LD_{50} value of iopromide liposomes was approximately 3 g I/kg in rat and mouse. The pharmacokinetic parameters of the liposomes in rat, such as total clearance and terminal half-life, were dose dependent. The terminal half-life in blood was increased from 0.8 h for a 250 mg I/kg dose to 2.9 h for 1000 mg I/kg dose, suggesting a longer circulation of liposomes after higher doses. When the liposomes were given to rats at a dose of 250 mg I/kg by mouth, recoveries (% of dose) from excretion in 0–72 h were approximately 93% (feces) and 3% (urine); by subcutaneous injection, recoveries in 0–48 h: 7% (feces), 61% (urine), and 31% (injection site); by intramuscular injection, recoveries in 0–72 h: 9% (feces), 69% (urine), and 6% (injection site). In day 7, a total of 0.36% of the administered dose was recovered in organs and tissues. Thus, iopromide-carrying liposomes proved to be stable and highly tolerable vesicles for the targeting to specific organs in animal model. Fifteen minutes after intravenous injection of 100 mg I/kg, rat blood showed the highest proportions of iodine, followed by the liver, the spleen, and the kidney. Significant proportions of iodine

were found only in the liver and spleen, after total clearance from the blood and the kidneys. In the rabbits with VX2 carcinoma, the iopromide liposome showed strong contrast enhancement of the liver. In the dog, interdigital injection of iopromide liposomes enhanced the contrast of ipsilateral lymph nodes (899). Ioxaglate-carrying liposomes with a lipid component consisting of egg phosphatidylcholine alpha-tocopherol had a mean liposomal diameter of 220 nm and an encapsulation efficiency of 85% and at larger doses (≥250 mg I/kg bw), showed a 10-fold greater enhancement for the spleen than for the liver and a sustained intravascular contrast enhancement of the aorta (902).

Petersein et al. (903) evaluated in healthy rabbits two new liposomal contrast agents aimed at the reticuloendothelial system for liver CT: BR2 and BR21 (from Bracco Research SA, Geneva, Switzerland). Both are suspensions of iomeprol-containing liposomes in an iomeprol solution; BR2 has twice the liposome concentration of BR21, that is, 40 vs. 20 mg lipid/mL, at an iodine content of 260 vs. 320 mg I/mL, respectively. The liposomes are 0.4 mm unilamellar vesicles, made of a phospholipid bilayer surrounding an aqueous phase. The membranes consist of phospholipids (phosphatidyl choline and dipalmitoyl phosphatidic acid) and cholesterol in a 2:1 molar ratio. The authors studied the time course of contrast enhancement in liver CT with the liposomal contrast agents, by use of extracellular iomeprol at 300 mg I/mL as the control. In healthy rabbits, at doses of 1.5 mL/kg or greater, the two liposomal agents induce a significantly stronger and more prolonged enhancement of the liver than that of the extracellular iomeprol and provide a larger imaging window for optimizing CT examinations of the liver. Dick et al. (904) evaluated a new contrast agent, liposomal iodixanol (LI), made of a 1:1 mixture of 10% glucose and iodixanol (50% encapsulated iodixanol: 1 mL of that mixture contained 50 mg encapsulated iodine) for examinations of pyrogenic liver abscess in a rabbit model by CT. The experimental group of animals received the LI at a dose of 200 mg I/kg, and the control group received iopentol at a dose of 600 mg I/kg. Results showed that the LI exceeds the extracellular iopentol in overall abscess contrast and duration of the diagnostic interval, in that the liposomal iodixanol gives higher hepatic vessel contrast and better abscess localization.

Activity of encapsulated liposomes is closely related to the method of preparation. Musu et al. (890) prepared large unilamellar vesicles (0.3–1 μ) carrying iopamidol from phosphatidylcholine and dipalmitoylphosphatidic acid in a 9:1 ratio and iopamidol solution (300 mg I/mL) and extruded the vesicles through a polycarbonate membrane of different pore sizes (0.8–2.0 μ). Extrusion above the transition temperature (75°C) of the lipids reduced the average size and size distribution of the vesicles and increased their I/L ratio. Distribution studies of extruded and unextruded iopamidol-carrying liposomes in rats showed that extruded liposomes gave higher spleen uptake than did unextruded, whereas the liver uptake was comparable. Lung entrapment was significant with unextruded but almost eliminated with extruded liposomes.

After intravenous injection conventional liposomes are rapidly taken up by cells of the mononuclear phagocytic system (MPS). The inclusion of glycolipids and hydrophilic polymers in the liposome membrane modifies the surface characteristics to evade the MPS system and increases the liposomal circulation time (905). Sache et al. (906) prepared iopromide-containing liposomes by the continuous high pressure extrusion method, and used the liposomes without prior removal of unencapsulated contrast agent for surface modification. The liposome membranes were made from soy phosphatidylcholine (SPC), cholesterol (CH), and soy phosphatidylglycerol (SPG) in a 6:3:1 (SPC/CH/SPG) molar ratio, and with the original lipid concentration at 120 mg/g total suspension. The liposome membranes were modified by inclusion of lipid derivatives of polyethylene glycol (PEG) by coating, simply carried out by mixing the preformed iopromide-containing liposomes with 5 mol % of either of the two coating agents (DSPE-PEG2000 or CHHS-PEG2000) for 16 h at room temperature with stirring. The DSPE-PEG coating increased the mean diameter of the vesicles to approximately 200 nm, probably attributable to aggregation and fusion of the vesicles, and decreased the zeta po-

tential of the negative surface charge. The stability of the unmodified and surface-modified iopromide liposomes in human plasma was determined by equilibrium dialysis of a liposome/plasma mixture against the respective plasma to be stable over a period of 6 h. The biodistribution of modified and unmodified iopromide liposomes was studied in rats, and no significant differences in blood concentration could be found 1 h after injection between different formulations at a dose of 250 mg I/kg body weight, corresponding to 500 mg lipid/kg. Computed tomographic images were studied in rabbits. The unmodified and DSEP-PEG-modified liposomes displayed prolonged blood concentration with CT density differences above 70 HU units (aorta) for up to 20 min and proved to be useful for CT imaging, displaying favorable imaging properties.

Liposomes may also serve as a vehicle for carrying an oil-soluble contrast agent such as Ethiodol to the liver (907). To form Ethiodol liposomes, a chloroform solution of Ethiodol and a mixture of egg phosphatidylcholine and phosphatidic acid in a molar ratio of 7:1 and a chloroform solution of Ethiodol are mixed in a proportion of 3:2, followed by solvent evaporation and addition of water with stirring and sonication. The Ethiodol is contained within the liposomes, probably in the hydrophobic region. The liposomes have diameters of 0.015 to 0.2 μ and show rapid liver uptake in the rabbits and long retention times. Unlike the normal liver tissue the tumor tissue in the rabbits with implanted VX2 carcinoma accumulates no Ethiodol. The dose of Ethiodol liposomes needed for contrast enhancement of the liver is about 1/13th of that required of water-soluble diatrizoate.

Caride et al. (908) incorporated brominated phosphatidylcholine with or without cholesterol into brominated radiopaque liposomes. The liposomes were 1–5 μ in diameter and showed contrast enhancement of the liver in the dog 1 h after intravenous injection. A few hours after injection brominated liposomes were found inside the hepatocytes. Because the bromine atom is not as effective as the iodine atom in attenuating X-rays, a correspondingly large dose of the brominated liposomes had to be administered for imaging. No information on chemical characterization of brominated phosphatidylcholine or its fate and biotransformation was given.

Yang et al. (909) used poly(D,L-lactide), a polymer with molecular weight in the range of 5000–50,000 Da, for microcapsulation of ethyl esters of iopanoate and diatrizoate and Ethiodol by a solvent evaporation method. The microcapsules were about 1 μm in diameter. Particles of this diameter or smaller can safely traverse the pulmonary capillary bed to be available for the liver and Kupffer cell phagocytosis. In *vivo* microscopic examination revealed the activity of Kupffer cells phagocytizing the microcapsules. These particles were essentially macrophage-imaging agents. Microcapsules of all three contrast media increased the density of the liver in the rabbits. Maximum opacification of the liver parenchyma appeared 15–30 min after injection, leaving any focal hepatic lesions as defects.

7.2.2 **Emulsions.** Ethiodol may also be emulsified by mixing with a small amount of phospholipid as emulsifying agent (910). The oil droplets of size 2 to 3 microns in the emulsion are rapidly and efficiently taken up by the RE system of the liver. The imaging quality of ethiodol emulsions is comparable to that of ethiodol liposomes.

Microemulsions of a series of polyiodinated triglycerides (ITG), labeled with iodine-125 and processed in a microfluidizer, were investigated for their contrast enhancement of the liver and the tumor in normal and tumor-bearing (Walker 256) rats, in rabbits bearing VX2 carcinoma, and in normal dogs in CT (911). The mean particle diameter of microemulsions was less than 300 nm. These preparations were stable and autoclavable. Thirty minutes after intravenous injection, from 66 to 78% of the injected dose remained in the liver of the rats. After 3 h the liver still retained from 46% to 93% of the dose. At dose levels ranging from 20 to 70 mg I/kg, the increase in density was reported to be about 40 HU in the rats. In a female pig, the contrast enhancement within 1 h of injection was 90 HU. The liver uptake of ITGs was partially dependent on the formulation vehicle, but the metabolism and clearance from the liver were

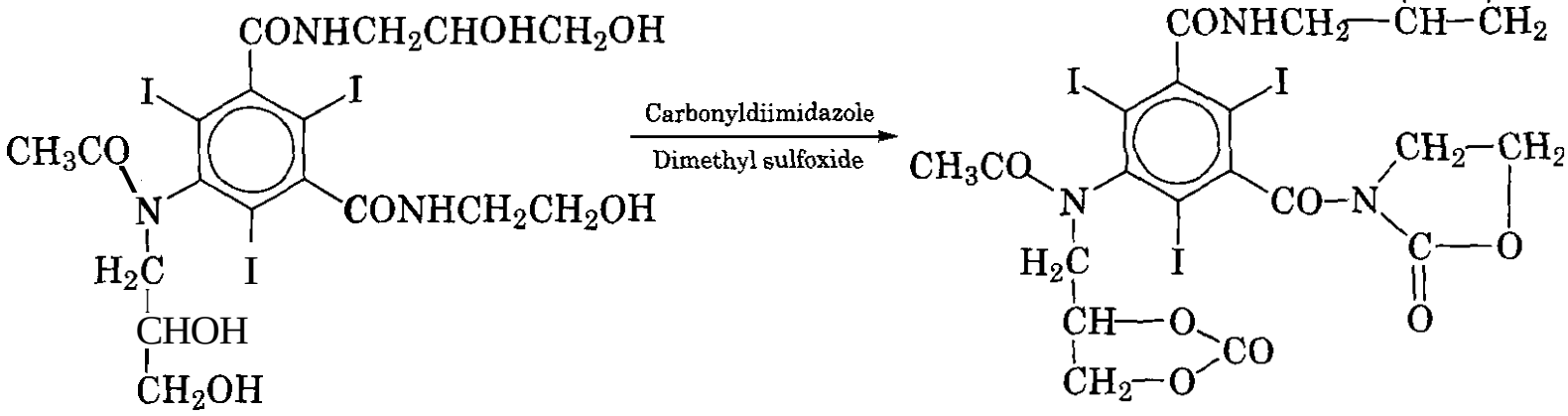
dependent on the chemical structure and the alkyl chain length (911).

7.2.3 Particulates. Particulates are prepared from insoluble derivatives of contrast agents, finely milled to uniform size, and suspended in water in the presence of small amounts of surfactants and stabilizers. These particles have sizes between 200 and 400 nm in diameter and are referred to as nanoparticles. Nanoparticles can be formulated as blood-pool and liver-spleen CT contrast agents for injection at concentrations of 15–20% (w/v), corresponding to 89–118 mg I/mL. The insoluble ethyl esters of ionic contrast media in nanoparticles will be taken up by the RE system upon injection and hydrolyzed by esterases into ethanol and water-soluble ionic contrast agent and excreted. Rubin et al. (912) prepared nanoparticles from the water-insoluble diatrizoate ethyl ester and investigated the effect of different types of surfactants on contrast enhancement of aorta, liver, and spleen in the rabbits, and used iohexol as comparator. In the presence of a high molecular weight nonionic polymeric surfactant, the nanoparticles produced excellent and prolonged enhancement of aorta and vena cava, but in the presence of a low molecular weight anionic nonpolymeric surfactant, the nanoparticles markedly opacified liver and spleen. This difference in targeting to different organs was attributed to the shielding of the nanoparticles from opsonization by the high molecular weight nonionic polymeric surfactant, causing a decrease in the uptake by the RE system. Gazelle et al. (913) tested more than 50 insoluble derivatives of diatrizoic acid, iothalamic acid, urokonic acid (acetrizoic acid), and metrizoic acid, formulated as nanocrystals for blood-pool and liver-spleen imaging. Most of these agents demonstrated either blood- or liver-dominant enhancement patterns in normal rabbits and rabbits with VX2 carcinoma. Some of the nanoparticles gave improved liver-to-lesion contrast compared to that of iohexol. The chemical identity of these insoluble derivatives was not divulged.

Violante et al. (914,915) prepared particles of iothalamate ethyl ester with a diameter of 2 ± 1 pm by injecting a solution of ethyl iothalamate in dimethylformamide at a rate of 3 mL/min into a 5% aqueous solution of polyvinylpyrrolidone (PVP), chilled to 0–2°C and circulated at a rate of 1000 mL/min. The particles precipitated out from solution as white suspensions after being warmed at 40°C for 30 min and were centrifuged at 3000*g* for 5 min, repeatedly washed with water, and resuspended in saline for injection. These particles were stable in normal saline and showed no particle aggregation when mixed with rat, rabbit, dog, or human serum but formed aggregates up to 80 pm in diameter in plasma. It was found that fibrinogen interacts with the particles, forming aggregates. Aggregation was prevented by preincubating the particles in human serum albumin before introducing them into plasma. The authors also found that in solution, iothalamate has a higher intravenous LD_{50} value in mice than that of iodipamide (13–19 g/kg versus approximately 4 g/kg), whereas as nanoparticles, iothalamate ethyl ester has a lower intravenous LD_{50} by rapid injection in mice than that of iodipamide ethyl ester (550 mg I/kg versus 1200 mg I/kg). Lee et al. (916) noted that in computed tomographic portography bolus injections of iodipamide ethyl ester particles were consistent in detecting all the lesions in pathologic liver in a canine model. This demonstrated that cholegraphic agents could be used to produce particulate contrast agents.

Li et al. (917, 918) synthesized ioxilan carbonate by reaction of ioxilan with carbonyldiimidazole in dimethyl sulfoxide. The reaction is specific for nonionic contrast media, protecting all the hydroxyl groups in the molecule and rendering it water insoluble. The reaction is given on the following page.

The ioxilan carbonate particles were prepared as a contrast medium by solvent extract/evaporation method. The preparation involved emulsification of a methylene chloride solution of the carbonate, removal of solvent, and washing and sizing the particles. The iodine content of the particles was 45%. The average diameter of the ioxilan carbonate particles was 1.1 pm, 95% of them ranging from 0.6 and 2.0 μm. Electron microscopy showed the particle surface to be smooth, and the particles showed practically no aggregation when mixed with rat plasma. The LD_{50} value for the ioxilan carbonate particles was 1.4 g I/kg for



Ioxilan

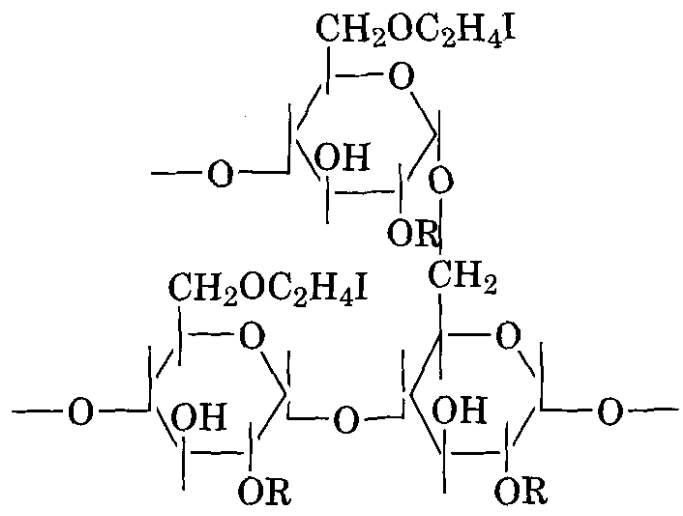
Ioxilan Carbonate

male and 1.2 g I/kg for female mice, corresponding to a particle mass of 3.1 and 2.6 g/kg body weight, respectively. Liver attenuation enhancement was 38 HU after intravenous injection of 200 mg I/kg of the carbonate particles in normal rabbits and rabbits with implanted VX2 carcinoma. Attenuation enhancement was dose dependent and increased with the dose. At a dose of 270 mg I/kg the attenuation enhancement was 110 HU. Liver enhancement reached maximum approximately 30 min after injection, and the spleen enhancement was many times higher than that of the liver. Tumors in the liver of rabbits bearing VX2 carcinomas were detected. Ioxilan carbonate particles were rapidly cleared from the blood. Opacification of the gallbladder and the kidneys was observed, indicating that the particles and their degradation products were excreted through hepatic and urinary pathways.

7.3 iodinated Polymers

Soluble starch treated with ethylene oxide and iodinated with N-iodosuccinimide yields 6-iodoethylated starch (IES). The repeating unit of IES is shown in the following column.

Sako et al. (919) prepared two iodinated polymers, IES-200 and IES-40. IES-200 has an average molecular weight of 200,000 Da, an iodine content of 12.5%, a water solubility of 0.8 g/mL, and a CT number of 1510 HU. IES-40 has an average molecular weight less than 40,000 Da, an iodine content of 20%, a water solubility of 1.0 g/mL, and a CT number



of 1900 HU. Both preparations have biocompatible viscosity and osmolality, show no acute toxicity in mice at dose levels of 5 and 10 g/kg body weight, and also no serological toxicity at 5 g/kg in rabbits. On intravascular injection the iodinated ethylated starch is retained in the blood for a considerable length of time with minimum leakage from the capillary wall. On intraparenchymal injection the iodinated starch opacifies the regional lymph nodes. The iodinated soluble starch is a potential contrast agent for blood-pool and vascular bed opacification and for indirect lymphography.

Other polymers have also been investigated as blood-pool contrast agents. Revel et al. (920) used an iodinated polymer consisting of a carboxymethyldextran with a triiodinated benzoic acid substituent. The iodinated polymer had a mean molecular weight of 32,000 Da, ranging from 10^3 to 10^6 Da and contained 24 mg I per 100 mg of powder. An intravenous

solution for injection had an iodine concentration of 8.7% with an osmolality of 560 mOsm/kg. This iodinated polymer enhanced the contrast differentially, up to 10 min, between normally perfused and ischemic liver and showed blood-flow differences at the capillary level in normal rabbits and rabbits suffering segmental portal ischemia. The polymeric material, because of its high molecular weight, was confined to the vascular space and was able to achieve vascular enhancement with about one-fourth of the iodine concentration required for diatrizoate, a water-soluble contrast agent. Doucet et al. (921) also reported the use of an iodinated carboxymethyldextran containing an unidentified triiodinated benzoic acid substituent group of a molecular weight of about 32,000 Da as a blood-pool X-ray contrast agent. Lautrou et al. (922) studied the pharmacokinetics of iodinated polymer P509 (mol. wt., 47,000) as a blood-imaging product and compared it with ioxaglate. The iodinated polymer P509 showed a considerably higher plasma concentration, a lower urinary excretion, a considerably lower biliary excretion, and an elimination half-time twice as long as that of ioxaglate. These studies demonstrate that iodinated polymers are potentially useful blood-pool contrast agents.

The above-mentioned blood-pool contrast media are polymers into which are grafted iodinated groups, ranging widely in molecular size and prone to be antigenic. As an alternative, Sovak et al. (923) proposed and synthesized blood-pool radiopaque polymers by copolymerization of triiodinated isophthalmic acid species containing acrylic or methacrylic acid groups with a nonradiopaque component consisting of acrylic or methacrylic acids, with or without their hydroxylated amides to form polymers of about 50,000 in molecular weight. These polymers with suitable physicochemical properties, intravenous tolerance, and the capability of being excreted were selected and further linked by biodegradable bis-acrylic linkages to form large polymers, in excess of 50,000 in molecular weight. These polymers were uniform in size as compared to the grafted radiopaque polymers and were biodegradable and excretable after degradation. The molecular configuration of these polymers is such that they contain extended hydrophobic regions and triiodophenyl rings with carboxylic acid groups, which is contrary to the design theory so successfully applied to the design of nonionic radiopaque agents. The authors remarked that in view of this observation, the development of radiopaque polymers may need to proceed on a trial-and-error basis.

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CHAPTER ELEVEN

Microarrays and Gene Expression Profiling Applied to Drug Research

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1 INTRODUCTION

The sequencing of the human genome represented a milestone accomplishment of the 20th century. The 23 pairs of human chromosomes that comprise almost three billion base pairs of deoxyribonucleic acid (DNA) have now been established to contain approximately 38,000 genes that encode some 60,000–80,000 different proteins (1, 2). Traditional analyses of genes and proteins are generally directed to one gene or protein at a time. This leisurely approach cannot match the pace of modern genomics and makes it difficult, almost impossible, to unearth complex interactions among genes or proteins. Traditional methods cannot readily reveal multigene relationships, the complex temporal symphony of gene action, or reveal the "whole picture" of gene functions and interactions. The study of the interactions among so many cellular factors requires the development of complex analytical tools and the use of sophisticated computer methods in the management and analysis of the data derived from such tools.

The start of the 21 century is witnessing the embryonic development of a new bioanalytical technology for the analysis of gene expression and genotyping—DNA microarrays. DNA arrays using oligonucleotides were first developed for a hybridization-based DNA sequencing method (3). DNA microarrays have more recently been developed as tools for studyinggene expression (4). DNA microarray technology translates what the cell is displaying at the genomic level into interpretable data. This technology enables researchers to obtain instantaneous snapshots of the expression levels of all genes. It also offers the potential for understanding complex regulatory pathways, deriving the distinguishing genetic identities of individuals, and discovering and understanding the molecular basis for disease states.

Modern genomics and DNA microarrays have been described as tools for revolutionizing drug development (5). However, promises of the genomics-driven revolution in drug research come with few details. How does knowing the sequence of all genes in our cells help us discover or develop drugs? In the fields of drug discovery, drug design, and drug development, microarrays are becoming the instruments for displaying complex drug interactions in the cells at the genomic and proteomic level. This chapter describes principles of DNA microarray technology and basic analysis of DNA microarray data, followed by discussions of recent ventures in applying DNA microarray technology to drug research.

2 DNA MICROARRAY TECHNOLOGY AND METHODS IN GENE EXPRESSION ANALYSIS

2.1 DNA Microarray Fabrication

DNA microarrays fall under the category of DNA chips. The term DNA chip is used to generally describe a solid platform cut from a larger substrate by dicing (hence chip) that incorporates a molecular component derived from deoxyribonucleic acid, ribonucleic acid, or biomimetic versions of these genetic molecules. An array used in biological studies is an orderly arrangement of known biological entities displayed in two dimensions on a flat surface. These entities may be (1)proteins, such as with antibody microarrays, (2)nucleic acids used to study gene expression or gene copy number in cells, or (3) tissues where the tissue-specific level of expression of a protein or gene is being explored. There can also be combinatorial microarrays aimed at materials development. DNA microarrays are thus far the most developed and widely used of the microarray devices. The array of DNA material may be printed on glass, supported hydrogel, nylon, or metal substrates. In its simplest manifestation, the only requirement is that the substrate be flat, smooth, chemically uniform, and provide a physicochemical means for the attachment of the several DNA sequences. In more complex manifestations, the substrate may present three dimensionally such as a substrate-supported hydrogel layer, a microporous membrane, an etched-roughened surface, or a channel-bearing substrate.

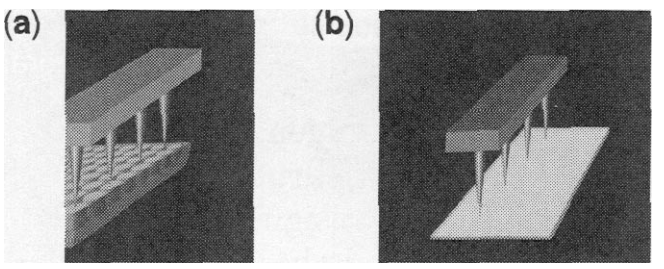


Figure 11.1. Pin tool for spotting DNA on a glass slide. Panel a shows a four-pin device acquiring DNA solution from a microtiter well plate. The DNA solution is printed onto a glass slide in panel b. The DNA identities, well positions, and the spot positions are determined in a pattern program used by the spotter arrayer.

Currently, the most widely used substrate is glass, with dimensions of the familiar 3.0" × 1.0" microscope slide.

There are two general platforms for DNA microarray fabrication. The most versatile of these platforms uses high-speed robotics (spotter arrayer) with DNA solutions. In a spotter arrayer, precision robots are combined with nanoliter liquid transfer capability. The nanoliter liquid transfer device may be of two generic types: contact or non-contact transfer of liquid to create the spots of the microarray. Contact methods include pin-tools and quills. Pin-tools are typically pencil-like devices with laser sharpened titanium tips. Quills are similar but possess a laser-drilled capillary running from the tip up the center of the shaft. Both exploit the surface tension forces of water to create a uniform drop of DNA solution that is transferred to the substrate on physical contact (Fig. 11.1). Non-contact methods use electromechanical actuation to propel a fixed volume of the DNA solution to the surface. This method can use inkjet heads, micro-solenoid valve dispensers, or piezoelectrically driven liquid dispensers. The DNA being arrayed can be bound to the substrate through a variety of fixation chemistries. Pin-tool robots can print greater than 25,000 spots on a glass microscope slide, given a feature size as small as 100 μm with 200-μm center-to-center spacing.

The second platform for DNA microarray fabrication involves controlled *in situ* synthesis of oligonucleotides (on-chip). This technology, implemented by Affymetrix in the manufacture of GeneChips, uses photolithographic masks to expose selected spots to light, resulting in deprotection of reactive groups needed for nucleotide coupling (Fig. 11.2). Each nucleotide added is itself protected, requiring light exposure to deprotect for subsequent addition of the next nucleotide. A different mask is used for each nucleotide added, and the process is repeated until each spot has oligonucleotides with a specific DNA sequence. The oligonucleotide length for GeneChips is 25 bases. Using this photolithographic technique, the synthesis of 25-base oligonucleotides requires 100 chemical synthetic steps and can potentially give rise to 39,635 (25^4) unique probe sequences. Because of the high precision of the photolithography process, a feature size of 20 μm is obtained, allowing the production of chips with as many as 400,000 spots.

2.2 Applications of DNA Microarrays

DNA microarrays can be used in a variety of experimental protocols to reveal different types of data about the genome. The type of data desired determines the kind of DNA that is arrayed. Cloned genomic DNA can be arrayed for the analysis of DNA or gene copy number in cells, for example, in tumor cells that may have undergone gene loss or amplification (6). Oligonucleotide arrays have been particularly useful in DNA sequence analysis and single nucleotide polymorphism (SNP) scanning (7). The arraying of both oligonucleotides and cDNA sequences has been extensively used in analysis of gene expression. Arrays allow the parallel determination of the expression level for all genes in a cell, thus providing a complete gene expression profile. Two general applications for DNA microarrays in gene expression analysis are to compare gene expression patterns between cells of different tissues and to compare gene expression patterns in a single cell type under different conditions. An example of the latter application is the comparison of gene expression profiles between drug-treated cells and untreated control cells. In contrast, gene expression profiling of different tissues does not allow the luxury of control cells for comparison, and thus the profiles are only compared to each other. Table 11.1 lists a number of studies that have employed DNA microarray tech-

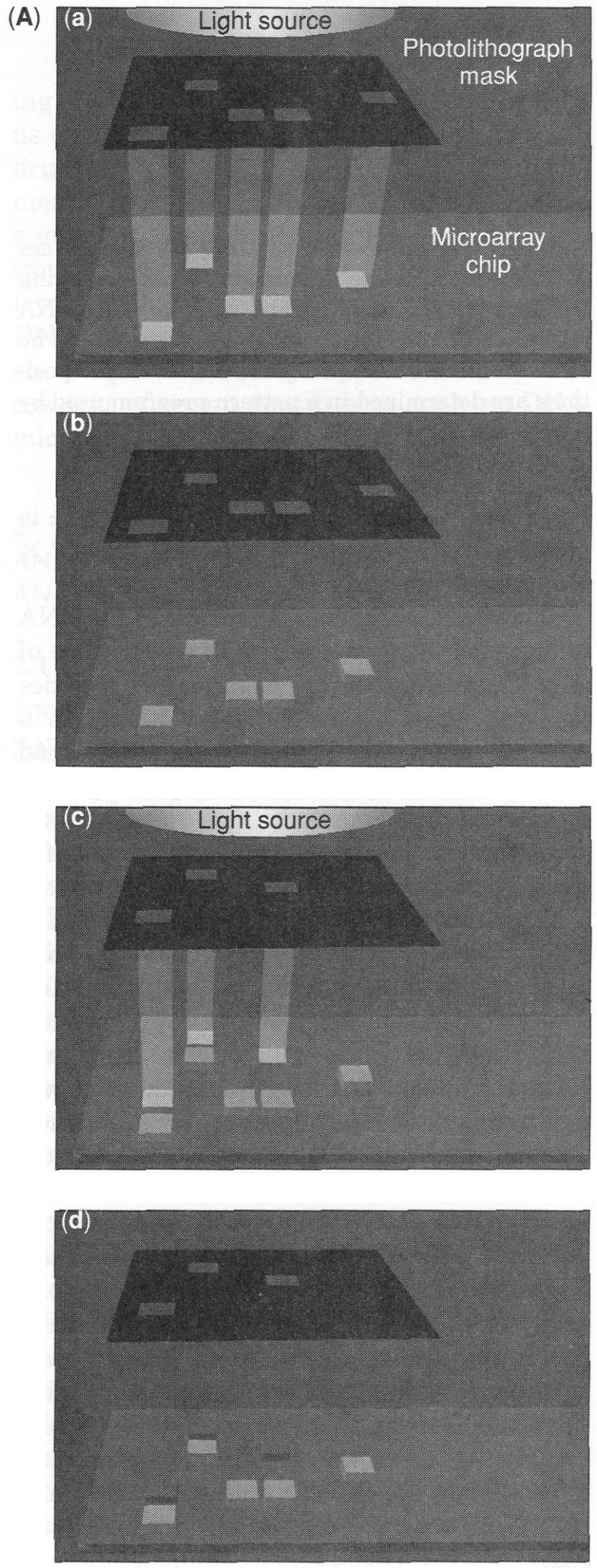
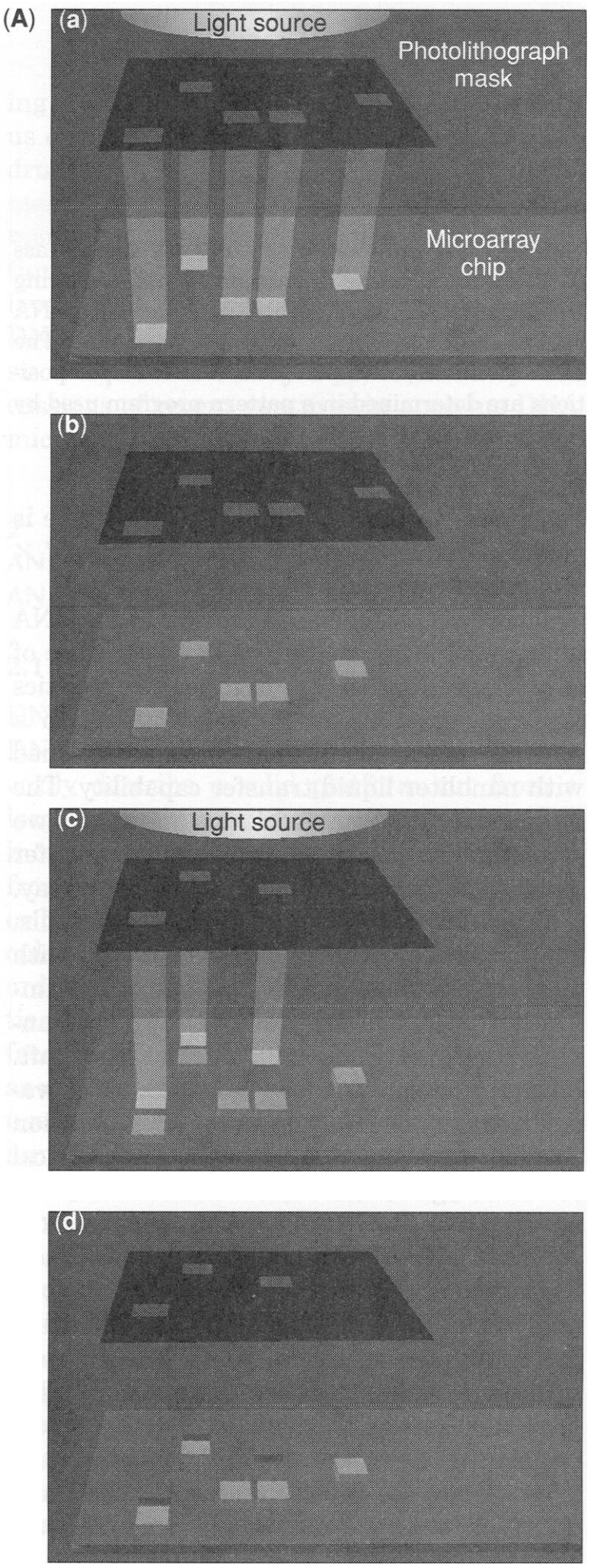


Figure 11.2. Photolithographic process for on-chip synthesis of oligonucleotides. **A:** the steps in this process in two cycles of nucleotide addition. A lithographic mask and light source are shown in panel a that results in exposure of specific spots on the microarray chip. The light activates reactive groups on the chip such that nucleotide coupling can occur only on the specific spots as shown in panel b. The nucleotide added is shown by green blocks and is thymidine for this example. Panel c shows a new mask allowing exposure of new spots to light in a second round of light activation of reactive groups. Panel d shows the next round of nucleotide addition in which the red blocks represent the next nucleotide, for example, adenosine. This process is repeated until all spots have the desired DNA sequence. B and C show schematic illustrations of the many steps in the solid-phase synthesis of the oligonucleotides **(A)** using MeNPOC chemistry (B). In this example, 4 masks are needed to produce the first base of each oligonucleotide, and 80 masks are needed to produce a 20-base oligo chip. See color insert.

nology for gene expression profiling. To date, the field of cancer research has gained the most from using microarray technology, with other fields rapidly catching up.

The expression level of a cell's genes, and in turn its proteins, is the major determinant of cell type and function. One traditional method for quantitating the gene expression level or



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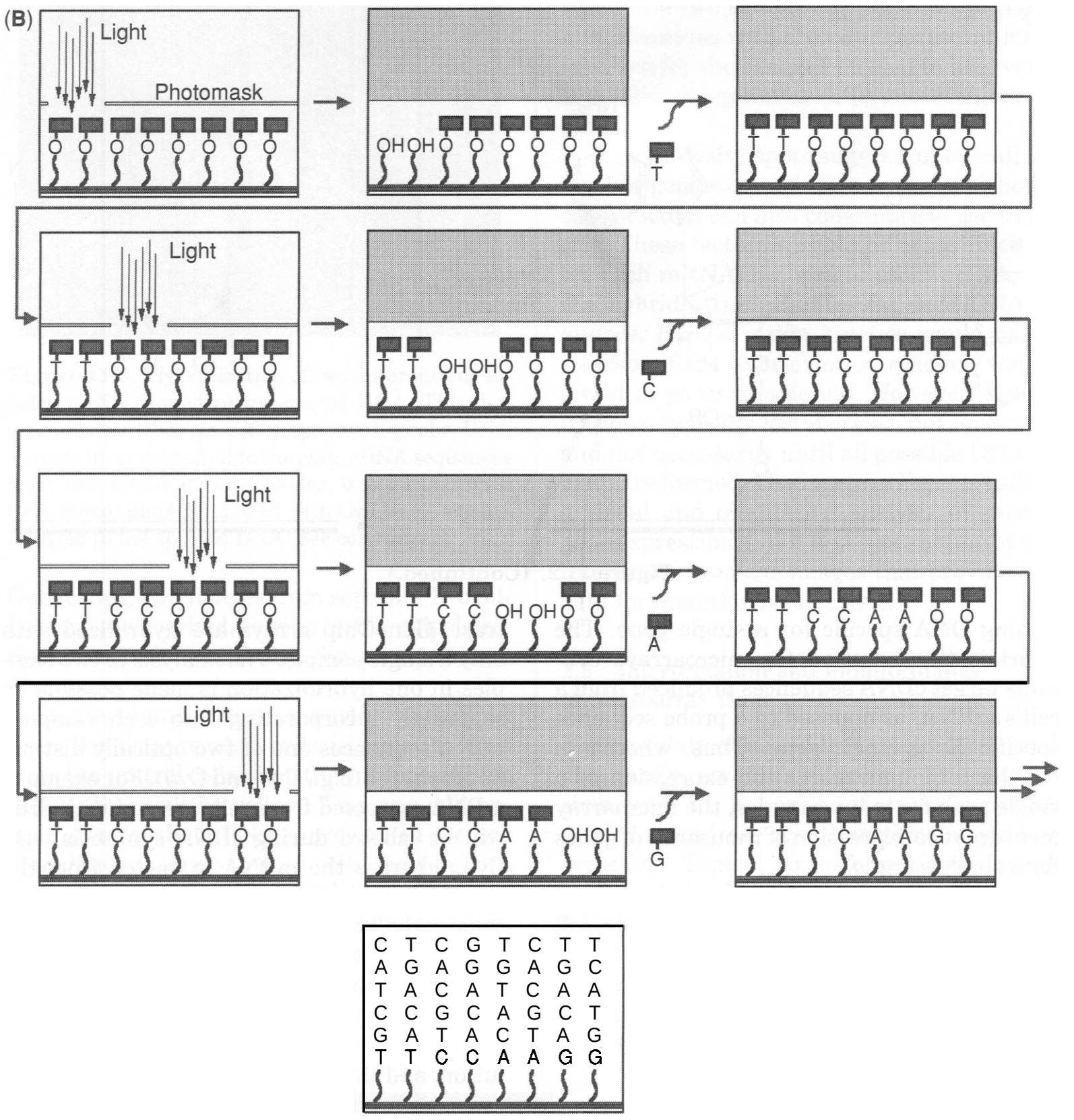

Figure 11.2. (Continued.)

concentration of a specific mRNA within a cell is Northern blot hybridization. This method involves fixing total mRNA (target sequence) from cells of interest to a membrane substrate and hybridizing with a labeled DNA probe specific for the gene of interest. The relative level of mRNA in the cells is calculated by normalizing the label's signal to the signal from an internal control mRNA, such as a housekeeping gene detected by a separate hybridization. Using this method, the relative gene expression level for a single gene can be determined for multiple cell samples (e.g., 10–20 samples).

Whereas microarray analysis of gene expression employs similar steps as Northern blot hybridization, it is radically different in principle. The major difference is that the roles of the nucleic acid bound to the substrate and the labeled nucleic acid in the hybridization solution are reversed for microarrays. In microarray analysis, the DNA probe is fixed to the substrate with each spot on the array con-

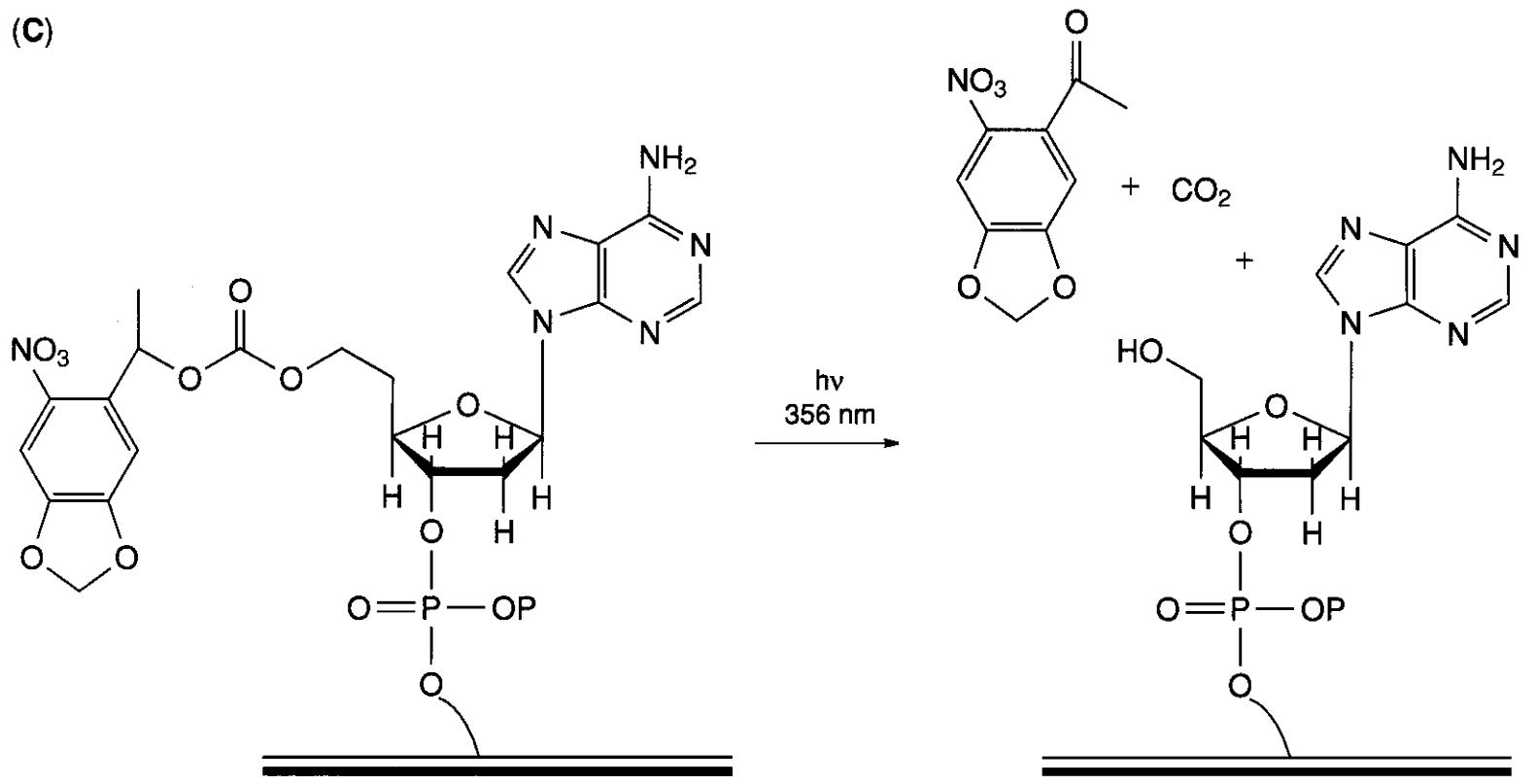

Figure 11.2 (Continued.)

taining DNA specific for a single gene. The hybridization solution for microarrays contains target cDNA sequences produced from a cell's mRNA, as opposed to a probe sequence specific for a single gene. Thus, whereas a Northern blot measures the expression of a single gene for many samples, the microarray measures the expression of thousands of genes for a single sample.

Spotted arrays are routinely used to simultaneously analyze gene expression in two cell samples using one hybridization. In many cases, one of the two samples represents a control cell, providing an internally controlled reference for each spot hybridization. In contrast, GeneChip arrays are hybridized with only a single sample. The analysis of two samples in one hybridization is made possible by separately incorporating into each sample's cDNA sequences one of two optically distinct fluorophores (e.g., Cy3 and Cy5). For example, mRNA extracted from cells treated with drug will be labeled during cDNA synthesis with Cy3, whereas the mRNA extracted from the untreated cells will be labeled with Cy5. The separately labeled cDNA sequences are mixed and hybridized to the DNA microarray chip (Fig 11.3). The number of molecules of probe DNA in each microarray spot is in far excess of the number of labeled cDNA molecules in solution, and thus the two labeled cDNAs do not compete, but hybridize independently to their specific DNA spots. The amount of labeled cDNA bound to a particular gene probe or spot is therefore indicative of the level of expression for that gene's mRNA.

Table 11.1 Applications for Gene Expression Profiling

Studies	References
Categorization of tissues and tumors	11–16
Identification of transcriptional regulatory pathways	26, 38
Disease gene identification	39
Drug characterization	24, 25
Identification of drug interactions and mechanism of action	28
Identification of diagnostic and prognostic markers	40, 41
Toxicologic responses	33, 42
Predicting therapeutic efficacy	22

The amount of Cy3 and Cy5 label bound to the microarray is quantitated by a fluorescence scanner that generates a grayscale image for each label. The two images are typically pseudo-colored, for example green for Cy3 and red for Cy5. The intensities of the two images are normalized, and the images are overlaid to produce a single image that displays the relative expression of each gene based on hue (Fig. 11.4). For studies using

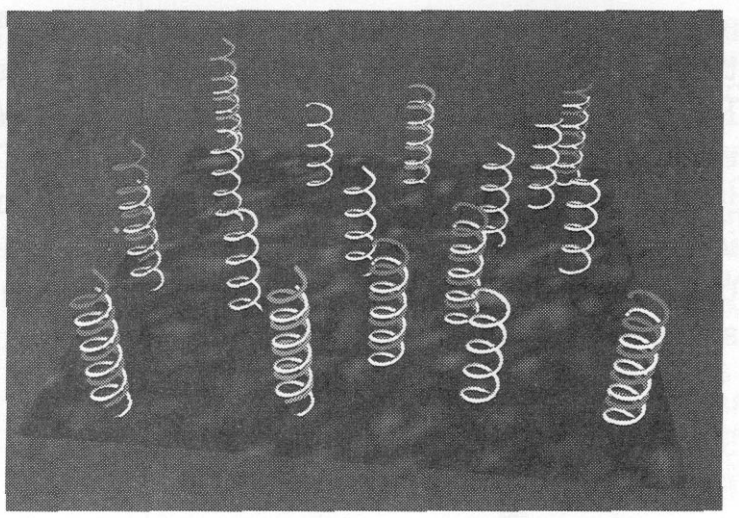

Figure **11.3.** Hybridization of two differentially labeled cDNA sequences to arrayed DNA. This diagram shows a single array spot with probe DNA strands (in gray) bound to the chip. cDNA sequences from two different cell samples, one labeled with Cy3 (green) and one labeled with Cy5 (red), are hybridized to the spotted DNA. See color insert.

GeneChips, two images from separate hybridizations are overlaid, relying on the chips' precision and reproducibility for proper image alignment.

If the abundance of a particular mRNA from the drug-treated cells is equal to that from the control cells, then the spot specific for that mRNA will be yellow. The spot will appear green if the mRNA is present in greater abundance in drug-treated cells than in control cells, and red if the mRNA from the drug-treated cells is lower. A potential anomaly in the analysis is caused by differences in how the two fluorescent labels are incorporated in the sample DNAs, which can result in the appearance of differences in genes expression between the two samples. It is typical to repeat hybridizations with the two fluorescent labels reversed for the samples labeled to help verify and validate legitimate differences in gene expression.

Low intensity spots suggest an overall low level of gene expression for a gene, although other factors can also contribute to the intensity. These factors include labeling efficiency for each mRNA, the size of each mRNA, and the hybridization kinetics for each DNA sequence. Hybridization kinetics would not be an issue if the hybridization reaction was allowed to go to completion. However, hybridizations typically are carried out overnight and not necessarily until all possible DNA hybrids are formed. The image in Fig. 11.4 allows a visual and qualitative analysis of relative gene expression, but it is the extraction of spot intensities from the images that provides the data for quantitative analysis.

2.3 Interpretation and Bioinformatics of Microarray Data

The raw data obtained from microarray analyses rarely remain raw for long. The data are simmered in a series of transformations needed to address basic questions of gene expression. Typical transformations include background subtraction, normalization, ratio calculations, and log transformation. The normalization is required because the scale for fluorescence emissions from different fluorophores are rarely equivalent. In addition, the scale for signal from the same fluorescent label

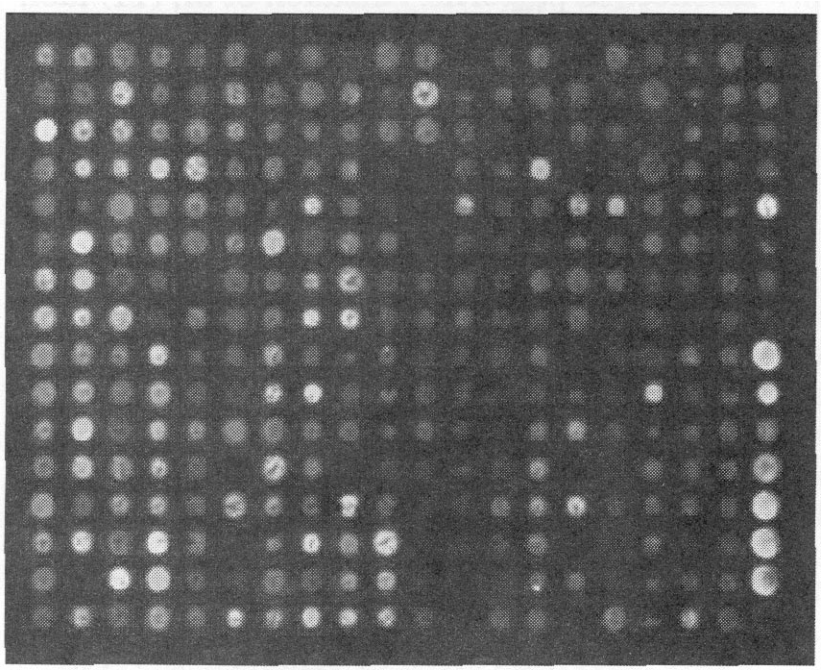

Figure **11.4.** A spotted DNA array with two-color detection of hybridization. An example of a spotted DNA array (16 × 20 elements) is shown after hybridization with two differentially labeled cDNA preparations, Cy3 (pseudo-colored green) and Cy5 (pseudo-colored red). The overlaying of the green and red images produces the image shown. The hue of each spot, ranging from green to red, indicates the relative expression level for the gene specific for each spot (image courtesy Packard Biochip Technologies). See color insert.

may not be the same from experiment to experiment. The validity of the normalization and ratio calculations relies on the bulk of the genes exhibiting the same levels of gene expression in each cell analyzed. If 90% of a cell's genes were to increase threefold in expression level under a specific condition, this event would not be detected. Instead, the interpretation would be that expression of the unaffected 10% of genes decreased approximately threefold compared with the control cells.

The ratio calculation is well suited for two-label hybridization experiments, as shown in Fig. 11.4, where there is an internal control for hybridization. The ratio calculation provides an indication of induction or repression of gene expression relative to the control. The distribution and variance of the ratio data are also usually analyzed to determine thresholds for values significantly higher or lower than one, although in many cases this threshold is arbitrarily chosen and is typically at the level of a twofold change. The log transformation is used to bring the distribution closer to a normal distribution for statistical analyses. Because of the high number of genes analyzed and the few observations or replicate experiments, it can be difficult to demonstrate statistical significance for small changes in gene expression.

A common goal in studying gene expression from microarray data is to identify groups of genes, cells, or conditions displaying similar patterns. A widely used tool for reaching this goal is cluster analysis (8). Cluster analysis organizes items into clusters based on how close the items are to each other using a distance or similarity calculation between items. Hierarchical clustering not only allows clustering of items but clustering of clusters and is usually presented in the form of a hierarchical tree diagram or dendrogram. An example of this analysis is shown in Fig. 11.5 in which 60 cell lines were clustered based on the gene expression levels of 548 selected genes. Most of the cell lines cluster based on tissue origin. The lengths of the horizontal lines connecting the cells relates to the distance between cell line data points or similarity. The two cell lines, MDA-MB-435 and MDA-N, have the shortest distance between their data points or the greatest similarity, and thus have the shortest horizontal lines connecting them. A typical measure of similarity is based on the Pearson correlation coefficient, which was used in the clustering in Fig. 11.4. The significance of these clusters will be discussed later in this chapter. An overview of the basic methods used in microarray data analysis is presented by Quackenbush **(9).**

3 APPLICATIONS OF GENE EXPRESSION PROFILING IN DRUG RESEARCH

3.1 Characterizing Cells by Gene Expression Profiling

Virtually all human gene sequences have been arrayed and are being used to create gene expression profiles in a wide range of cell types. Instead of the noted phrase, "you are what you eat", we might now say "you are what you express." The gene expression profile of a cell currently represents the most precise definition of what a cell is, what it does, and what it can do. Of course, a detailed protein expression profile would theoretically be superior, but this must await advancement in the field of proteomics (10).

Gene expression profiling has been used with great success to categorize or classify cell types, particularly cancers. For example, this approach has been employed to characterize lymphoma cells and identify subtypes of lymphomas from patients with diffuse large B-cell lymphoma. These cells express similar gene expression patterns because of their lymphocytic lineage, and they exhibit only subtle differences in patterns. Alizadeh et al. (11) identified two distinct subtypes of lymphomas using spotted arrays containing ~18,000 genes (Lymphochip), which were otherwise essentially indistinguishable. These two subtypes had subtle differences in gene expression profiles that were in common with two different B-cell lineages, suggesting that they represent two distinct diseases. Furthermore, the two subtypes were found to exhibit a differential response to therapy, that is, patients with one subtype showed significantly better survival than patients with the other subtype, while on the standard anthracycline-based therapy. It's important to note that most genes were expressed at comparable levels in

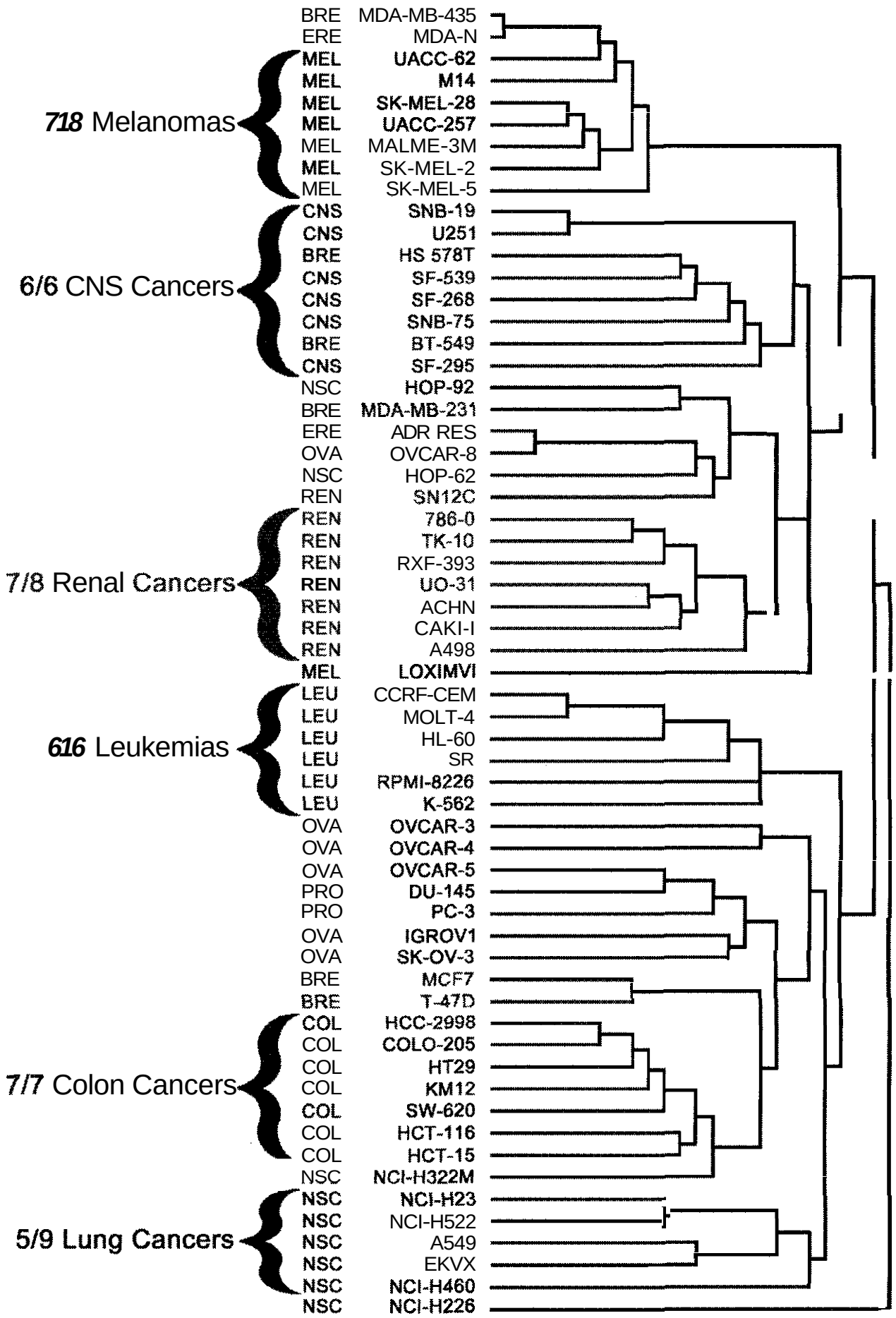

Figure 11.5. Cluster analysis of the NCI 60 cell lines. Gene expression data from the Ross et al. (12) study was filtered to contain only genes with complete data for all cell lines and genes with the highest variance across cell lines. Only 548 genes were selected for this analysis based on genes with variance of greater than twice the variance for the entire dataset. The cluster distance or similarity calculation was based on the Pearson correlation coefficient, and clusters were linked by average linkage using the software SPSS. Most cell lines can be seen to cluster based on tissue of origin, shown by the labels on the left and the fraction of the total number of cell lines for each tissue. The abbreviations for tissue types are as follows: BRE, breast cancers; CNS, central nervous system cancers; COL, colon cancers; LEU, leukemias; MEL, melanomas; NSC, non-small cell lung cancers; OVA, ovarian cancers; PRO, prostate cancers; REN, renal cancers. See color insert.

both lymphoma subtypes, and only a subset of genes displayed differential expression patterns that contributed to the distinction between the subtypes. The gene expression levels of this subset of genes may potentially be useful as a diagnostic tool for distinguishing between the two subtypes and predicting response to therapy.

A variety of tumor specimens have now been characterized by gene expression profiling (11–16). In some cases, the gene expression profiles have confirmed distinctions between tumor types that were already evident by histologic or cytogenetic analysis. In other studies, even finer distinctions between tumors types, not apparent histologically, have been made possible by gene expression profiling. For example, Hedenfalk et al. (17) identified specific gene expression patterns that correlate with BRCAl and BRCA2 mutations in hereditary breast cancer. These findings are complicated by the fact that the BRCAl and BRCA2 mutations correlated with other factors such as tumor grade, mitotic index, and presence or absence of hormone receptors. Many of the genes expression patterns in these cells should also reflect these differences in processes and pathways responsible for tumor characteristics. The power of the microarray/bioinformatics approach is that it offers the potential to identify gene expression patterns that correlate with BRCAl or BRCA2 mutations independent of hormone receptor status, as well as patterns that correlate with hormone receptor status independent of the BRCA1/ BRCA2 mutation status.

Gene expression profiling can also provide insight into a tumor's tissue or cell type of origin. Gene expression profiles were used to determine cell origin for a panel of 60 human cancer cell lines (NCI 60 cells) representing cancers of breast, prostate, lung, central nervous system, leukemia, melanoma, renal, colon, and ovarian origins (12). Gene expression levels were determined in each cell line for over 9000 genes using spotted DNA arrays. Cluster analysis of the 60 cell lines was performed using approximately 1000 genes that showed the highest variance across cell lines. The clustering generally grouped cell lines of the same type together. One exception was the breast cancer cell lines that were scattered among the other cell line groups. Figure 11.5 shows a hierarchical tree diagram of **a** cluster analysis we performed on the data from Ross et al. (12) in which a slightly different variance calculation and cluster distance calculation were used. Again, clusters of cell lines generally coincided with the tissue of origin, similar to the published analysis, but with an improvement in the clustering of lung cancers. The breast cancer cell lines retained a heterogeneous distribution pattern, with some clustering with the melanoma, CNS cancer, and colon cancer clusters. These studies illustrate the use of gene expression profiling for determining tissue origin and suggest how gene expression profiling could be useful in defining cell behavior.

In addition to cell lines with similar gene expression profiles being clustered, the genes can also be clustered based on expression patterns across the 60 cell lines. Cluster analysis of the genes from the 60 cell lines revealed clusters of genes that are part of the same biological process or pathway (12). For example, one cluster represented genes that are highly expressed in melanomas and involved in melanocyte-specific biology. Other clusters contained genes encoding proteins that are involved in cell cycle progression, RNA splicing, and drug metabolism. It is hypothesized from these studies that groups of genes relating to basic cellular processes such as cell proliferation, cell morphology, or drug response could be identified by this type of gene expression analysis. The identification of previously unidentified genes in these clusters may help elucidate the functions of the encoded proteins. Much of this advancement will come from the development of new algorithms for analyzing the data and detecting patterns amidst the considerable noise of a cell's repertoire of expressed genes.

3.2 Cene Expression and Drug Response

The difference between **a** compound and an agent is dictated by the cell just as the difference between an agent and a drug is dictated by the patient. However, at this point, we would like to make a simplification of terms in which a compound, an agent, or a drug will

merely be referred to as a drug even though a compound or agent may not be of known medical value.

The NCI 60 cell lines have been screened for sensitivity to a ~70,000 drug library, resulting in a wealth of data for identification of patterns and relationships between drugs (18).The search for drugs within this library that have sensitivity patterns related to a drug of interest can identify drugs with similar structure and or mechanism of action [See the COMPARE program (19)]. While this data is of enormous use. there is even more data to be obtained relating to the genes and proteins expressed by the cells that dictate how the cells respond to the drugs.

The microarray data for the NCI 60 cell lines can be viewed as a source of ~9000 marker genes for potential correlation with biological properties, including sensitivity to anti-cancer drugs. Scherf et al. (20) explored the relationship between basal gene expression patterns and drug sensitivity in these cell lines using drug activity data for the ~70,000 drugs. We can begin to understand the challenges in bioinformatics when we consider the enormous task of melding the data for these three distinct types of variables: 60 cell lines, 9000 genes, and 70,000 drugs, and looking for relationships and patterns. One straightforward approach for finding relationships in this data is to focus on genes and drugs for which there is a considerable amount of additional information. For example, the activity of 5-fluoruracil (5-FU), a known thymidylate synthetase inhibitor, was found by Scherf et al. (20) to have a significant negative correlation with expression of the gene encoding dihydropyrimidine dehydrogenase (DPYD). DPYD is involved in uracil and thymine degradation as well as in 5-FU degradation, and thus high expression of the DPYD gene is consistent with resistance to 5-FU. Staunton et al. (21) carried this approach further identifying subsets of genes whose expression levels in the NCI 60 cell lines showed good prediction of sensitivity for certain drugs. However, it was not readily apparent what role the identified genes or their proteins played in the sensitivity to the drugs. This level of understanding is likely to require a very detailed knowledge of the pathways with which each drug interacts.

One approach Scherf et al. (20) used for identifying relationships between drug response and gene expression involved clustering the drugs based on their correlation with expression of each gene. A distance calculation between each drug pair was performed using correlation coefficients between the drugs and each gene. This clustering placed drugs in groups sharing similar relationships between drug response and gene expression rather than drug response alone. The results were compared with the cluster analysis, using only drug response data. Drug clusters from the drug response data tended to contain drugs with similar mechanisms of action, such as antimetabolites or tubulin inhibitors. The drug cluster analysis based on gene–drug correlations also tended to group drugs based on mechanisms of action, but clustered certain drugs in groups with distinctly different mechanisms of action. For example, the topoisomerase II inhibitor, etoposide, clustered with other topoisomerase II inhibitors using drug response data, but clustered with DNA alkylators when the drug–gene correlation data was used. These results could reflect a potential difference in mechanisms of action or drug metabolism between etoposide and the other topoisomerase II inhibitors.

The application of gene expression profiling is beginning to pave the way for improved treatment of patients using existing therapeutic agents. First, knowledge of a tumor's gene expression profile may greatly facilitate tumor type identification, leading to more accurate predictions of tumor response to therapies. Second, gene expression analysis can potentially reveal patterns that predict therapeutic response or help identify proteins that play in a specific role in response to therapy. This approach was explored by Kihara et al. (22) in a study of esophageal cancer patients treated with 5-FU and cisplatin. The expression profile of ~9000 genes was determined for patient tumors before treatment, and a subset of genes with predictive value for subsequent therapeutic response was identified.

Another promising application of gene expression profiling is in clinical drug development. For example, a drug that seems to have a poor overall response rate in clinical trials may actually have a significant activity in a

subset of patients whose tumors may share particular gene expression patterns. The ability to preselect a narrower target group of candidates for clinical trial could lead not only to improved trial outcome but also to a cost-saving reduction in trial size. Until now, the number of markers used in this way has been limited, but microarray data is opening a floodgate in the identification of potential markers for drug response.

The use of gene expression profiling to improve therapeutics overlaps with a relatively new field of study, pharmacogenomics. Pharmacogenomics is the study of the genomic or genetic basis for variation in response to drugs observed between patients. This field of study attempts to define and understand differences in drug response at the patient level to enable tailoring of therapeutics to individuals. Much of the field has focused on the study of DNA polymorphisms, particularly single nucleotide polymorphisms (SNPs), and their relationship with drug response. The analysis of SNPs also relies heavily on the use of DNA microarray technology. Information about the field of pharmacogenomics and innovations are presented in this volume by Puckett et al. **(23).**

3.3 Pathways and Targets

The cellular response to a drug depends on both the drug's specific and non-specific interactions in the cell. Whereas a particular drug may be known to inhibit a protein target, inhibition of that target may represent only a fraction of what the drug is doing in the cell. In fact, the primary mechanism of action may not even involve the supposed target. The challenge then, is to record and interpret all aspects of the cellular response to a drug, rather than a single parameter, such as cell growth.

Gene expression profiling offers a much deeper probe into the cellular response to a drug. Two general strategies for use of gene expression profiling are emerging in drug research; the first is involves determining the basal gene expression profile of a cell as a means of obtaining predictive information about a cell's drug response, as described above. The second strategy involves determining the change in a cell's gene expression profile in response to drug treatment. The drug response profile adds an entirely new dimension to understanding of the relationship between gene expression and drug response, because it reflects the targets and pathways with which the drug interacts and the downstream effects of these interactions on gene expression. The cell's response to a drug at the genomic and proteomic level potentially provides a detailed fingerprint reflecting the full range of effects of the drug on the cell.

This concept is illustrated using a simple protein kinase signal transduction pathway as an example (Fig. 11.6). This generic protein kinase cascade translates a signal from a membrane receptor to the nucleus resulting in increased expression of genes A, B, and C, as well as the repressed expression of genes X, Y, and Z. This pathway also has a branch such that PK2 interacts with other pathways and can affect the expression of other genes (genes E, F, and *G).* These interactions or branches may be cell-type specific, and thus the choice of cells for analysis can dictate the genes affected. The goal is to explore drugs that will block the high expression of genes A, B, and C. A panel of drugs (panel 1) previously known to block PK1 is first considered. The effect of each drug in panel 1 is the desired repression of genes A, B, and C as well as the de-repressed expression of genes X, Y, and Z. Each of these PK1 inhibitors also affects the expression of genes associated with the branching pathways. In addition, some of these inhibitors may interact with proteins not connected to the targeted pathway, such as kinases in unrelated pathways. The additional genes affected by the PK1 inhibitors can be very distinct for each inhibitor and can provide part of the fingerprint that distinguishes each inhibitor from the others. This part of the fingerprint may also reveal side interactions of the PK1 inhibitors that were not previously known or anticipated. The same approach can potentially be applied to cases in which little is known about a drug of interest. Clues about the drug's interactions could be deduced from its similarity in gene expression changes with other drugs for which there is more information regarding cellular interactions and mechanisms.

This protein kinase pathway also shows how inhibition of other proteins in the same pathway could give rise to similar changes in

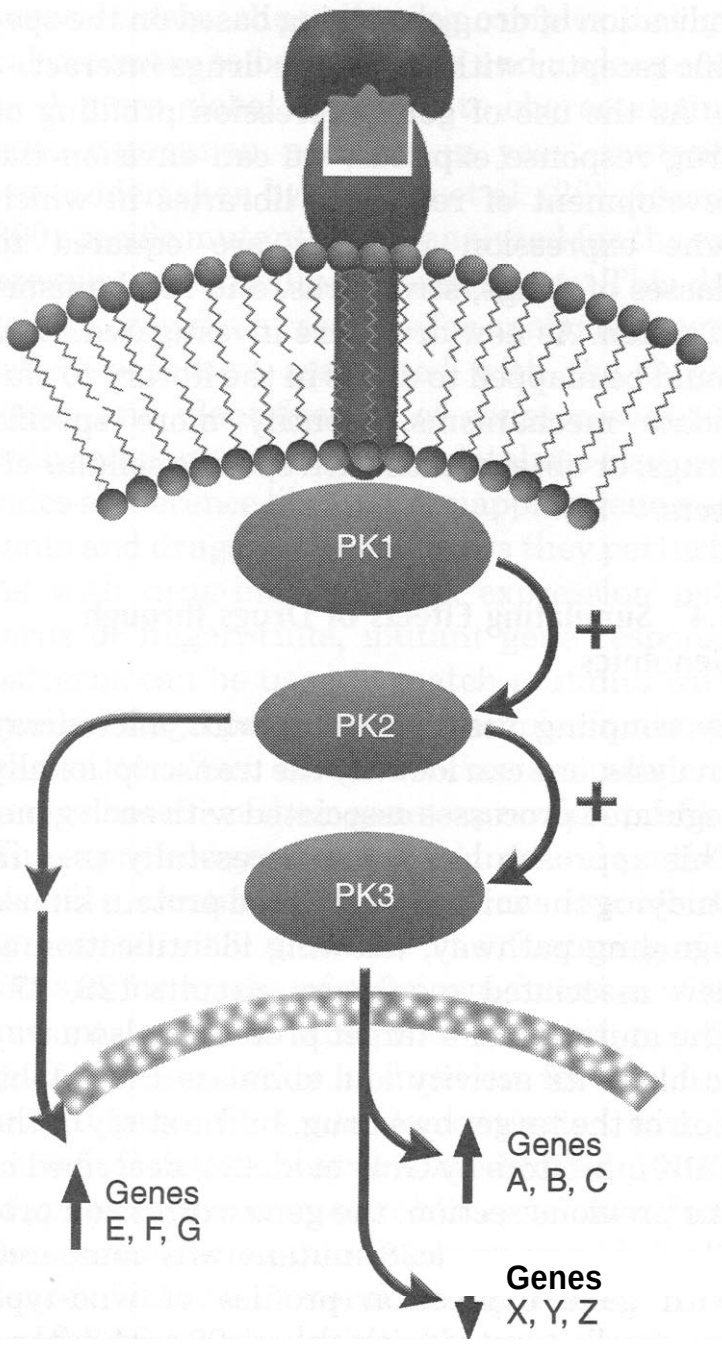

Figure **11.6.** **A** generic protein kinase pathway for cell signaling. This diagram shows a ligand binding to a cell surface receptor, thereby activating a signal transduction cascade. The ligand-induced activation of the receptor leads to activation of protein kinase PK1, which then activates PK2, which in turn activates PK3. PK3 modifies transcription factors in the nucleus that lead to high expression of genes **A,** B, and C, and low expression of genes X, Y, and Z. PK2 also interacts with another pathway that leads to high expression of genes E, F, and G.

downstream gene expression. For example, consider a drug speculated to have some general specificity for protein kinases. This drug has a similar effect on the expression of genes **A,** B, and C and X, Y, and Z, but it has no effect on the expression of genes E, F, and G. We might deduce from this result that the drug blocks a step downstream of the branch at PK2, such as PK3. This drug would be worth pursuing because of its increased specificity compared with drugs in panel 1. Therefore, similarity in gene expression fingerprints may identify a common pathway with which the drugs interact, rather than a common protein. Thus, the definition of a target may be broadened from a single cellular component to a pathway, allowing for the identification of additional drugs of interest with dissimilar structure and mechanism of action. For a well-understood pathway, the gene expression profile could potentially pinpoint the specific step in the pathway inhibited by a drug.

This approach to characterizing kinase inhibitors was used by Gray et al. (24), in a study of gene expression changes induced in yeast cells by purine analogs that target human cyclin-dependent kinase 2 (CDK2) and *Saccharomyces cerevisiae* cyclin-dependent kinase cdc28p. Virtually the entire complement of yeast genes (6200 genes) was monitored for changes in expression in response to two CDK active site inhibitors with dissimilar structures and a related purine analog with poor CDK inhibition. More than 194 genes showed significant changes in expression, with both CDK active site inhibitors eliciting similar changes in expression for 63 of these genes. The majority of the affected genes were up-regulated in expression. The drug with poor CDK inhibition gave rise to changes in expression of only two genes, consistent with its inability to block cdc28p or other protein kinases.

A number of cell cycle–related genes were found to be affected by the two CDK inhibitors, including several cyclin and histone genes, consistent with previous data suggesting that these genes are regulated by cdc28p. Another interesting class of genes affected by the CDK inhibitors contained genes involved in phosphate metabolism. This is significant because, in addition to cdc28p, the CDK inhibitors also inhibit pho85p, a closely related kinase that is involved in the regulation of phosphate levels. This illustrates the potential for gene expression profiling to reveal multiple drug–target interactions within the cell. Other genes of interest that were up-regulated included genes encoding various heat-shock proteins and proteins involved in drug extrusion pump systems. Induction of heat-shock

genes suggests the cells exhibited a stress response to the CDK inhibitors. However, the induction of these stress response genes may not necessarily reflect a direct response to cdc28p inhibition, but rather may represent a response to the cascade of events stemming from the perturbation of the cell cycle. The change in expression of genes for drug pump–associated proteins suggests possible mechanisms of resistance and could explain differences in growth response to the drugs. It is important to note that not all changes in gene expression are likely to be informative. It's expected that many of the genes affected in this type of experiment will merely represent generic responses to gross cellular perturbations and will be affected by a large number of drugs, revealing very little information about an individual drug's unique properties.

The genes affected concordantly by both CDK inhibitors are not the only genes of interest. The majority of the affected genes by either inhibitor were unique to each drug, suggesting that the two CDK inhibitors interacted with more unique proteins than common proteins. Two basic questions arise from this analysis: how does one identify the affected genes specific to cdc28p inhibition (or any specific target perturbation) and how does one identify the pathways associated with the other affected genes? Both questions can be addressed through the use of genomics, as discussed in the next section.

The potential of gene expression profiling for drug classification is also illustrated by a study of adrenergic receptor–interactive drugs (25). Twenty drugs known to be agonists, antagonists, or both for *a*- and *β*-adrenergic receptors were used as a test set for the analysis of gene expression profile responses. Normal human aortic smooth muscle cells, which express both *a*- and *β*-adrenergic receptors, were used as the model cells for the treatments. Of the 6000 human genes analyzed for expression, a subset of 75 genes was selected for cluster analysis after filtering out genes with low or no response to drug and genes with the lowest variance. Cluster analysis of the drugs based on patterns of induced gene expression changes cleanly separated the agonists from the antagonists. Although the number of drugs analyzed was low, there was also some indication of drug clustering based on the specific receptor with which the drugs interact.

As the use of gene expression profiling of drug response expands, we can envision the development of reference libraries in which gene expression patterns are equated to classes of drugs, structures, and mechanisms of action. As new drugs are investigated, they could be mapped to drugs in the library to elucidate mechanisms, identify more specific drugs, or obtain leads with specific cellular effects.

3.4 Simulating Effects of Drugs through Cenomics

By coupling yeast genetics with microarray analysis, one can identify the transcriptionally regulated processes associated with each gene. This approach has been successfully used in studying the mitogen-activated protein kinase signaling pathway, allowing identification of new associated regulatory circuits (26, 27). The mutation of a target protein is also a way to block its activity and simulate the inhibition of the target by a drug. In the study of the CDK inhibitors by Gray et al. (24) described in the previous section, the gene expression profile for a yeast cdc28 mutant was compared with gene expression profiles of wild-type yeast cells treated with the cdc28p inhibitors. The changes in gene expression patterns resulting from cdc28 mutation had significant overlap with those found for the two CDK inhibitors. This analysis therefore helped to validate the responses observed, indicating that the CDK inhibitors were in fact targeting cdc28p in the cell. Not surprisingly, there were several differences in gene expression changes induced by the CDK inhibitors and the cdc28 mutation, consistent with the prediction that the CDK inhibitors would be less specific than cdc28 mutation. However, the difference could also be caused in part by the use of a temperature-sensitive conditional mutant of cdc28, which was required because loss of cdc28p is lethal. The change in temperature therefore adds another variable with which to contend. Whereas each method of disrupting the target seems to have its own unique effects, each unique effect can theoretically be determined and isolated within the gene ex-

pression data, thus allowing complex biological processes to be unraveled and understood.

A more global approach to characterizing gene expression patterns in yeast mutants was undertaken by Hughes et al. *(28)*. Almost *300* specific mutants were analyzed for the expression of virtually all yeast genes. This database provides a resource for analyzing regulatory pathways associated with the target genes and identification of genes associated with cellular processes. The database also provides a reference library for mapping gene mutants and drugs to the pathways they perturb. As with drug-induced gene expression patterns or fingerprints, mutant gene response patterns can be used to match mutants with similar fingerprints. For example, mutants known to affect sterol synthesis cluster together based on their gene expression profile. The expressed genes involved in sterol synthesis also cluster based on their expression across the ~300 mutants. The clustering of a mutant for a gene of unknown function with mutants of genes with known function suggests by association a function for the unknown gene. This was demonstrated with a mutant for a gene that clustered with mutants of genes involved in sterol biosynthesis. Further investigation into this gene provided evidence of its role in sterol biosynthesis.

Identifying similarities between a gene expression fingerprint for a drug and fingerprints for mutants can potentially reveal the pathway in which the drug interacts. Hughes et al. (28) found a significant similarity in gene expression fingerprints between cells treated with lovastatin, a hydroxy-methylglutaryl CoA reductase inhibitor, and a mutant in one of the yeast hydroxy-methylglutaryl CoA reductase genes. This concept was tested further by comparing gene expression fingerprints of drugs with unknown targets to known mutants. The fingerprint for the topical anesthetic dyclonine was identified as having significant similarity to that of mutants affecting sterol biosynthesis, and in particular, a sterol isomerase mutant. Additional biochemical analysis confirmed the sterol isomerase as the likely target for dyclonine in yeast. However, this information alone did not shed light on the mechanism for this anesthetic in humans. Further analysis showed that the human protein with the greatest sequence identity to the yeast sterol isomerase was a neurosteroid interactive receptor involved in regulating potassium channels. If this receptor were the target for dyclonine in humans, then a possible mechanism for dyclonine would involve disrupting signal transduction by perturbing potassium transport. This prediction requires experimental verification, a requirement we can expect from the results of most gene expression profiling studies.

Drug–target interactions have also been explored by Marton et al. *(29)* using expression profiling and yeast mutants. Cells with mutations in putative drug target proteins were used to confirm drug interactions and downstream effects on gene expression. The gene expression fingerprints found in "targetless" cells pointed to other pathway interactions for the drugs tested. The ability to define target-specific effects of drugs and their cross-reactions with other cellular components and pathways paves the way for future drug design with improved targeting and specificity.

Although it is considerably more difficult to generate specific mutations in mammalian cells than in yeast, several technologies are available that can allow mammalian cell systems to follow in the steps of the yeast system for gene expression profiling and drug analysis in mutants. The first is the production of knock-out mutations in engineered mice. A considerable number of knock-out mice have already been created, and these can be explored for alterations in gene expression profiles in a variety of cell types (30). A more amenable strategy involves the use of antisense technology to block the expression of specific RNAs and proteins. Antisense oligonucleotides can be used as therapeutic agents as well as tools for exploring targets *(31)*. Cho et al. (32) employed gene expression profiling to identify both the specific and non-specific interactions of antisense oligonucleotides. A reference library of human gene expression profiles for antisense oligonucleotides specific to potential targets could conceivably be used in the same way as the yeast mutants for identifying relevant pathways and drug interactions in the cell.

3.5 Molecular Toxicology

The use of microarrays, gene expression profiling, and the strategies described above are ideally suited for use in toxicology, an application referred to as toxicogenomics. The same gene expression data used in studying the targeting of drugs also applies to toxicologic analysis of drugs where the broader effects of the drugs are emphasized. Waring et al. (33) applied gene expression profiling to the characterization of 15 known and diverse hepatotoxins in a rat model. This study clearly demonstrated that diverse drugs or toxins could have distinct gene expression fingerprints while sharing some similarities based on mechanism of toxicity. Many of the genes affected by each toxin encoded proteins relevant to the known toxicity mechanisms and for proteins specifically involved in the metabolism of each toxin. These studies point to the potential use for gene expression profiling in predicting and characterizing toxic responses.

3.6 Bioinformatics Meets Chemoinformatics

The discussions above touch on only the surface of the potential applications of gene expression profiling as applied to drug development. The power of microarray technology and gene expression profiling will likely be further magnified by their interface with other information-intensive tools, such as used in chemoinformatics. For example, quantitative structure-activity relationship (QSAR) models relate the molecular descriptors of drugs to various biological or biochemical activities, ranging from the inhibition activity for a particular target to more in-depth drug response activities characterized in the NCI 60 cell lines. As we have already discussed, measurement of a single biological indicator of response, such as cell growth inhibition, may not reveal all the complex interactions a drug has in the cell. However, the gene or protein expression profiles before and after exposure to a drug hold the key to these interactions and to true functional definitions of drug activity. Thus, technologies that can reveal these interactions promise to be the future focus for designing and optimizing drugs. One approach that might fulfill this promise would be to combine the fields of bioinformatics with chemoinformatics, applying QSAR modeling to the gene expression activities affected by drugs in a model system. Bioinformatics has the potential to relate a group of genes affected by a drug to each specific cellular component affected by the drug, while relating this data to the therapeutic effectiveness. QSAR has the potential to reveal which substituents or residue substitutions in a drug analog series dictate the varied interactions with the cellular components. These data could form the basis for designing analogs with the highest therapeutically relevant activity and the greatest specificity.

4 CONCLUDING REMARKS

DNA microarray technology is proving to be a powerful genomics-based tool in a broad range of biomedical fields. In the field of drug development, this technology can provide insight into the full complement of cellular targets with which a drug might interact and assist in defining mechanisms of action. In the context of patient treatment, gene expression profiles can provide information that will assist in predicting the cellular response to a drug. Further, gene expression profiling is on the threshold of enabling the identification of disease genes and new therapeutic targets (34). Microarray technology is rapidly evolving, and innovations, such as multiplexing with nanocrystals embedded in microbeads (35), and fiber-optic biosensor arrays (36) are being developed that will further increase the speed, sensitivity, and power of these approaches. Nevertheless, several engineering challenges still exist. For example, improved detection methods and new surface chemistries are in demand (37), and there is a critical need for the development and promulgation of standards in the fabrication and use of microarrays. There is also an enormous demand for the development of bioinformatics tools that will allow us to probe deeper into the vast datasets generated by this technology to discover patterns with biological significance. Finally, many biological issues remain to be resolved as this technology moves into mainstream biomedical research, such as determining the appropriate normal "control"

tissue for comparison with disease tissue. Thus, microarray technology is still in the early phase of development and application in many biomedical research fields. However, the potential power of microarray technology is already clear, and it is virtually certain that the technology will play a critical role in drug discovery and development in the near future.

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CHAPTER TWELVE

SNPs: Single Nucleotide Polymorphisms and Pharmacogenomics—Individually Designed Drug Therapy

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Contents

1 INTRODUCTION

1.1 History

1.1.1 Watson and Crick to the Human Genome Project. It seems to have all started at the turn of the 20th century. Mendel's laws came back into favor within the scientific community, leading to discoveries in the cellular basis for heredity. But it wasn't until 1953 when Watson and Crick first described and built the elegant model of the structure of DNA that the field of genetics became not just a science, but an obsession. Over the last 50 years, scientists have been feverishly trying to unlock and decode the secrets of the human genome. Then, a monumental accomplishment was achieved when the first draft of the entire human genome sequence was published in February 2001 as a result of the Human Genome Project (1). This has sparked a renewed fervor for research in genomics that will certainly carry us through and beyond the 21st century, the post-genomic era.

1.1.2 Drug Response and Toxicity Variability. It is an incontrovertible fact that large interpatient variability exists in response to medications. Variation in response has existed as long as medications have been used for the prevention and treatment of disease. In many ways, the field of pharmacogenomics began serendipitously in the 1950s after seminal observations describing variability in response to medications. Examples included peripheral neuropathy from isoniazid among slow acetylators (2), prolonged apnea from succinylcholine caused by pseudocholinesterase deficiency

(3), and severe hypotension from debrisoquine among cytochrome P450 (CYP) 2D6 poor metabolizers (4). For the next 40 years, pharmacogenetic studies focused almost exclusively on the etiologies of altered variability in pharmacokinetic responses to medications.

As we entered the 1990s, pharmacogenomic studies began to include studies that examined pharmacodynamic variability in drug response. Now instead of examining only differences in drug metabolizing enzymes, scientists began to focus on genes that encode drug transporters, drug targets, and ion channels. The goal of this chapter is to provide a primer on pharrnacogenomics and describe how the human genome and molecular biology techniques are transforming medicine to create an era of personalized therapeutics.

1.2 Genetic/Genomic Definitions

1.2.1 Basics. The human genome is made up of DNA, which is organized into 23 chromosomes. DNA is a double helical structure that is composed of sugar, phosphate, and a nitrogenous base. The DNA strands are held together by hydrogen bonds. The four nitrogenous bases that make up DNA are adenine (A), guanine (G), cytosine (C), and thymine (T). The base pairing is consistent in that adenine always binds to thymine and cytosine always binds to guanine. A combination of three base pairs makes up a codon, and each codon specifies an amino acid that will be incorporated into a protein. The process of making a protein begins when an RNA polymerase attaches to a region of DNA known as the promoter region. This single-stranded chain now serves as a template to synthesize a single-stranded RNA molecule. Once RNA has been formed in the nucleus of the cell, it is transported to the ribosomes in the cytoplasm of the cell where translation will occur. However, before translation occurs, the RNA is processed and introns, non-coding regions of the DNA, are removed. The removal of non-coding regions is termed splicing. Exons, coding regions of DNA, constitute only 5% of the human genome. Once this has occurred, the RNA molecule is translated into amino acids and proteins. Because there are four nucleotides, a total of 64 different codons are possible. However, there are only 20 amino acids, hence several different codons may specify the same amino acid.

1.2.2 Pharmacogenetics or Pharmacogenomics?. Pharmacogenetics is literally a combination of pharmacology and genetics. It thus stands to reason that pharmacogenetics is the study of how an individual's genetic makeup may influence response to medications. The field of pharrnacogenetics has been around in one form or another for over 50 years. Historically, differences in drug response were observed and documented. Based on these phenotypic (i.e., the outward physical effect of a genotype) observations, scientists explored heredity and gene lines to pharmacogenetically explain these differences in response. Scientists further explored and concentrated efforts on the pharmacokinetics (i.e., absorption, distribution, metabolism, and excretion) of these medications in search of genetic differences in, for example, how fast or slow a particular drug is metabolized. Then came the Human Genome Project and birth of the term pharrnacogenomics.

There has been considerable debate about whether the terms pharmacogenetics and pharrnacogenomics mean the same thing or whether they truly are different sciences. Some state that pharmacogenomics is just the new *en* vogue terminology of late. Some state that pharmacogenetics looks at a single gene whereas pharmacogenomics looks at multiple interacting genes. Others state that there is a fundamental difference in the way the research problem or hypothesis is approached. Few would argue that pharmacogenomics has its roots in pharmacogenetics, but it is the recent technology in molecular biology along with the Human Genome Project that has shaped and defined the field of pharmacogenomics. Pharmacogenetics historically relied heavily on phenotypic observations to drive hypotheses about genetic differences (5). But, with today's technology, one can take a genome-wide approach to apriori hypothesize about differences in drug response and/or search for novel drug targets. This is the essence of pharmacogenomics. Furthermore, pharrnacogenetics typically concentrated its research in the area of pharmacokinetics thus

looking at drug-metabolizing enzyme polymorphisms (6). However, pharmacogenomics opens a genomic Pandora's box, allowing the ability to explore not only drug-metabolizing polymorphisms, but also drug target polymorphisms, drug transporter polymorphisms, and disease progression polymorphisms. If the goal of pharmacogenetics is to explain the inter-individual differences in drug response based on genetic information, then it is the promise of pharmacogenomics to truly individualize pharmacotherapy (5).

2 SINGLE NUCLEOTIDE POLYMORPHISMS

2.1 Definition

Single nucleotide polymorphisms (SNPs; pronounced "snips") are single base pair substitutions that occur in a sequence of DNA. It has been estimated that SNPs occur at a frequency of approximately 1 in 1000 base pairs. Because the human genome contains approximately 3 billion base pairs, there should be approximately 30,000,000 SNPs in the genome (7). However, the number of SNPs in coding regions (i.e., the regions that actually code for proteins) of the genome has been estimated at 500,000. Recently, a "SNP map" of the human genome was published containing 1.42 million SNPs (1). The researchers found an average density of one SNP every 1.9 kb, or approximately 1 in 1900 base pairs. They also estimated that of the 1.42 million SNPs identified, only 60,000 SNPs actually fell in coding regions. However, it is important to consider that, although a SNP may fall outside the coding region, it may be in linkage disequilibrium with a coding SNP, thus indirectly affecting a biologic or pharmacologic response. Irregardless, in order to be classified as a polymorphism, it must have a frequency of at least 1% in the population. However, to be of routine clinical use, the frequency of polymorphisms may need to be much higher.

2.2 Classification of SNPs

As alluded to above, there are several different types of SNPs. Coding SNPs are those polymorphisms that are located within the coding block of the gene, whereas noncoding SNPs occur outside of the coding block. Coding SNPs can further be classified as either synonymous or non-synonymous. A synonymous SNP is a polymorphism in which, although the codon has been changed, both the wild-type and the polymorphic variant code for the exact same amino acid. Thus, a non-synonymous SNP is one in which a different amino acid is coded and is therefore the most common SNP described in the literature. A non-synonymous SNP can further be classified as conservative or non-conservative. A conservative non-synonymous SNP confers an amino acid substitution of similar size and charge to that of the original amino acid, whereas a non-conservative non-synonymous SNP substitutes an amino acid that is very different in size and/or charge which may greatly impact protein folding. It thus stands to reason that a non-conservative nonsynonymous SNP may potentially cause the most obvious genetic variations.

It is becoming increasingly important given the dogma of pharmacogenomics to evaluate multiple SNPs. A haplotype is a collection of SNPs on a particular locus. The locus could be multiple genes, one entire gene or merely a segment of a gene. By examining haplotypes, the interaction between SNPs can be further addressed and elucidated. For example, SNPs can often travel together because of linkage disequilibrium, thus making it very important to study the haplotype. It therefore may be potentially misleading to only look at the individual SNPs without considering their interplay. This has indeed been proven to be the case in a seminal paper looking at β_2-adrenergic receptor polymorphisms is asthma (8). The details of this case are described in more detail in Section 4.3.6.2.

It is also very important to note that SNPs are not the only polymorphisms out there that affect drug response. Up until now, we have been discussing what happens when a single nucleotide base-pair is substituted. However, there are also polymorphisms causing insertion or deletion of a segment of DNA, splice site mutations resulting in exon skipping, microsatellite nucleotide repeats, gene duplication, point mutations resulting in early stop codons, and complete gene deletions. This makes for an incredibly complex variety of SNPs that are potentially responsible for in-

ter-individual response differences observed with certain medications.

3 TECHNOLOGIES USED IN PHARMACOGENOMICS

The cornerstone of genomic and pharmacogenomic studies is the ability to accurately identify genetic variations. The last few years have brought the development of many low-cost, high-throughput technologies that have permitted investigators to address genetic questions that were previously unapproachable. The molecular biology component of pharmacogenomics research essentially involves two fundamental activities: discovering new genetic variants (i.e., SNP discovery) and assaying for known mutations (i.e., genotyping). Selection of the most appropriate technology is based on which of these two activities are planned. While factors such as cost, accuracy, and throughput requirements are always important to consider, the most appropriate technology for a given project is driven by the state of knowledge concerning the gene, locus, or disease to be studied. For example, when there is an abundance of knowledge on the gene sequence and variation therein, the most appropriate technology will allow convenient robust assaying of known mutations (i.e., genotyping). When there is less information known on gene sequence and variation (polymorphism) within the gene, technologies that identify new genetic variation would be most appropriate.

3.1 SNP Discovery

A variety of techniques are available for SNP discovery. DNA sequencing remains the most direct method to determine the sequence of a target gene and remains the gold standard for detecting mutations. In this mode, an individual's sequence can be compared with many wild-type sequences to identify new polymorphism~Improvements in analytical software and platform have made modern automated DNA sequencers much more user friendly. Nonetheless, the use of DNA sequencing to identify population-wide variation is a costly, labor-intensive endeavor. Strategies to minimize the amount of sequencing allow for more efficient use of resources.

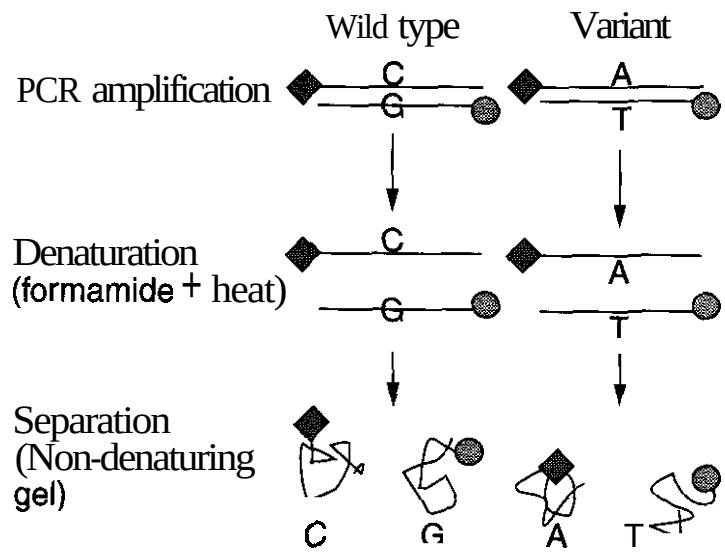

Figure 12.1. Single-strand conformation polymorphism analysis. Single-stranded DNAs are generated by denaturation of the PCR products and separated on a nondenaturing polyacrylamide gel. A fragment with a single-base modification generally forms a different conformer and migrates differently when compared with the wild-type DNA. Reproduced with permission from Ref. 9.

A commonly used strategy is to use polymerase chain reaction (PCR) techniques. Quite simply, one would PCR-amplify the genes of interest and scan the PCR products for the presence of variants. These gene scanning techniques differentiate PCR products which contain a variant sequence from those that do not. By only sequencing positive PCR products, gene scanning methods reduce the amount of sequencing required to discover new SNPs.

One of the most widely used methods for discovering new variants is a conformation-based technique called single-strand conformation polymorphism (SSCP) analysis (Fig. 12.1). SSCP is based on the principle that a single-stranded DNA molecule has a specific sequence-dependent three-dimensional structure. Sequence variants can be observed by running single-stranded PCR products through a gel matrix. An amplicon with a single nucleotide substitution generally forms a different conformation and will migrate differently than wild-type DNA through a polyacrylamide gel during electrophoresis. By comparing the mobility of a series of test samples with that of a control sample, it is possible to identify those samples with a sequence variation.

Table 12.1 Members of the SNP Consortium

AP Biotech
AstraZenecca
Aventis
Bayer AG
Bristol-Myers Squibb
Hoffman-LaRoche
GlaxoSmithKline
Novartis
Pfizer
Searle

Other popular scanning methods for SNP discovery include chemical or enzymatic mismatch cleavage detection, denaturing gradient gel electrophoresis, and denaturing high performance liquid chromatography (dHPLC). The underlying principle of these methods is that the melting characteristics of double stranded DNA are largely defined by its sequence (9). Therefore, sequence variation will produce variable DNA denaturing and reannealing, such that during electrophoresis a single-base mismatch can produce conformational changes that result in differential migration of homoduplexes and heteroduplexes containing these base mismatches (10).

The Human Genome Project, the SNP Consortium, and other sequencing efforts have produced large amounts of human sequence data that are available in public databases. In fact, in the fourth quarter of 2001, the SNP Consortium, which was created by the Wellcome Trust and a group of pharmaceutical companies (Table 12.1), will complete a catalog of 1,000,000 SNPs for public usage (http://snp.cshl.org). The actual SNP identification and mapping will be performed at the five genomics institutes (Table 12.2). These sequence and SNP databases will provide a basis for rapid and efficient genome-wide SNP discovery using data assembled from sequences from libraries of cDNAs. For researchers, this represents an extremely valuable tool in which to do in *silico* searches to identify new candidate SNPs (11).

Table 12.2 Institutes Responsible for Identification, Mapping, and Analysis of SNPs

The Sanger Centre
Whitehead Institute for Biomedical Research
Washington University School of Medicine
Stanford Human Genome Center
Cold Spring Harbor Laboratory

3.2 Genotyping *Technologies*

There are many options available to investigators for SNP genotyping. These vary along a range of cost, throughput, robustness, and convenience. In comparing currently available and emerging technologies, a distinction should be made between the analytical biochemistries that underlie the different genotyping assays and the variety of platforms and modes of detection, or readout, of the genotyping results. For instance, it is sometimes suggested that DNA microarrays are a new powerful method of genotyping, when in reality DNA microarrays simply represent a means of spatially arranging biochemical reactions, whether hybridization alone or hybridization linked to an enzymatic reaction, so that the end result of the reaction can be efficiently quantified or scored (12).

Because genotyping assays require a high level of specificity, an additional distinction should be made between those biochemistries that rely primarily on differential hybridization for their specificity and those that derive specificity from the product of an enzymatic reaction. By combining one of the available allelic discrimination biochemistries with either a solution phase or solid (array) phase reaction platform and a common detection methodology [electrophoresis, fluorescence, fluorescence resonance energy transfer (FRET), etc.], a number of viable genotyping technologies have been developed.

3.2.1 Enzymatic-Based Techniques.

3.2.1.1 Polymerase Chain Reaction-Restriction Fragment Length Polymorphism. One of the simplest methods to genotype test samples is through restriction fragment length polymorphism (RFLP) analysis. With this method, after PCR, the PCR products are digested with an appropriate restriction enzyme and visualized by staining the gel after electrophoresis. If the test sample contains a genetic polymorphism that causes a gain or loss of the restriction site, then that sample will display a different migration pattern on the gel. Appropriate

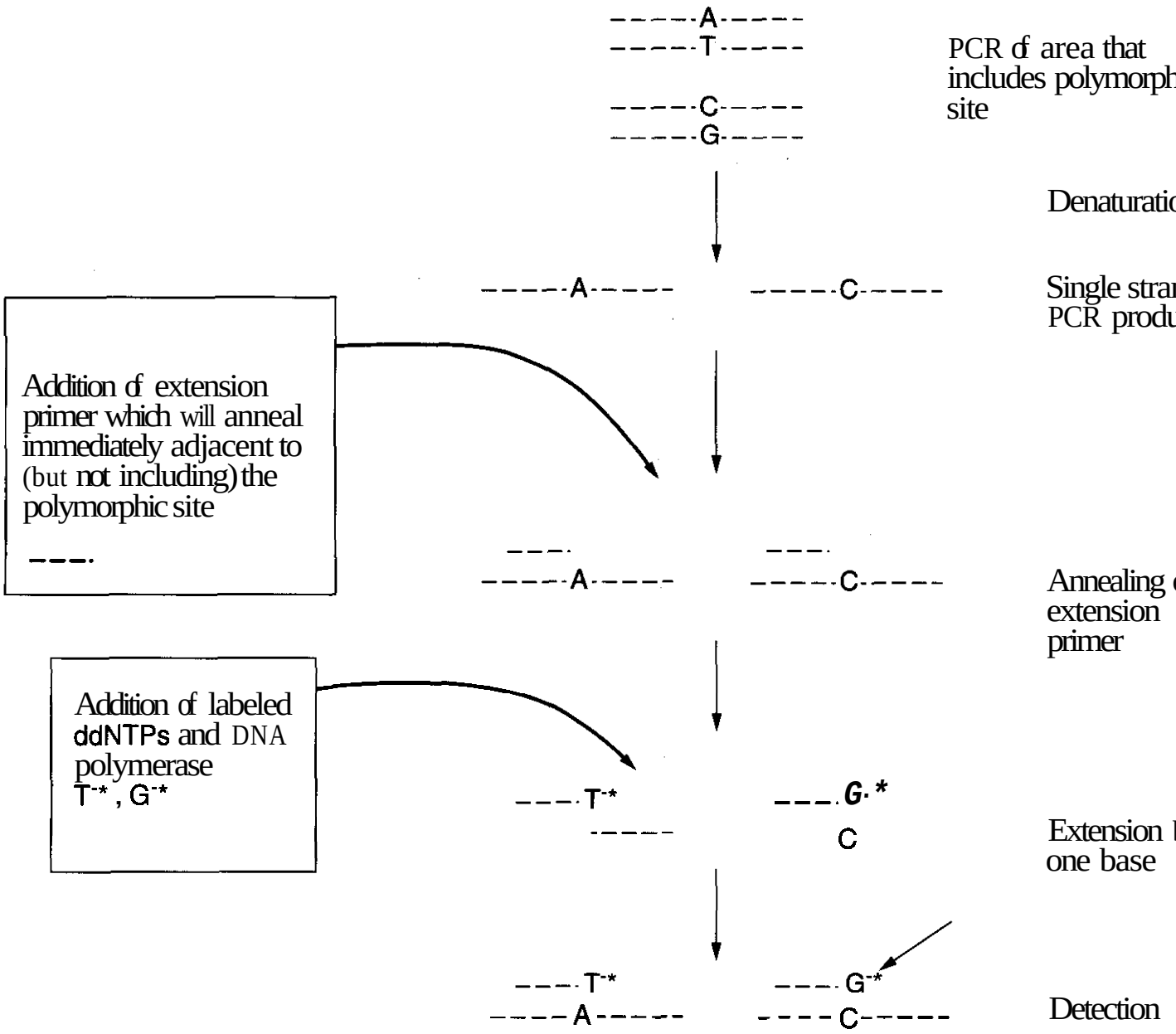

Figure 12.2. Single base primer extension. This patient is heterozygous for this A to C substitution. The labels from both ddNTPs can be detected in this sample. In the case of a homozygous genotype, only one of the labels would be detected. Detection might be through ELISA, fluorescence, FRET, or FP.

quality control measures require that positive and negative controls be present in each assay to confirm that the restriction enzyme is active.

The main advantages of RFLP analysis are that it is simple to develop and use. It also does not require expensive equipment, and it is effective for genotyping a small number of samples. The principle disadvantages are that it is time consuming, labor intensive, and not amenable for large numbers of samples or genotypes. Another drawback is that not all SNPs produce a usable restriction site.

3.2.1.2 Single Base Primer Extension. One of the most effective ways to detecting polymorphism is a technique known as single-base primer extension (Fig. **12.2).** In this simple assay, a region containing the polymorphism of interest is first amplified in a PCR reaction. The double-stranded PCR product is prepared into a single-stranded DNA fragment by heat or enzymatic digestion. A third primer is then used in the primer extension reaction. On the single-stranded PCR product, this third primer, called the primer extension primer, anneals immediately adjacent to, but only including, the site of the known mutation. After addition of DNA polymerase and labeled chain-terminating dideoxynucleotides corresponding to the wild-type and variant sequence, one of the two dideoxynucleotides will be added onto the primer at the site of the mutation. Unlike regular deoxynucleotides, which will extend indefinitely, dideoxynucle-

otides will only extend by one base. After completion of the primer extension reaction, detection of the reaction products may occur in any number of ways (13, 14).

An important distinguishing feature of primer extension is that assay specificity arises from the enzymatic specificity of DNA polymerase, not from the hybridization of the extension primer. Consequently, single base extension is rapidly gaining acceptance as the reaction biochemistry of choice for high-throughput genotyping of SNPs. It is well suited for high-throughput applications because the reactions can occur under similar reaction conditions, assay design and optimization are minimal, and it is portable to a variety of detection platforms (15). For these reasons, single-base primer extension is being licensed and adapted for use of a variety of different platforms and detection systems, including indirect colorimetric detection on ELISA-style microtiter plates (Orchid BioSciences' SNPStream 25K and SNPware 96 kits), DNA array product capture systems (SNPcode kits for use on Affymetrix's GeneChip and GeneFlex; Aper's APEX), fluorescent bead-based sorting devices (Luminex's LabMAP), capillary DNA sequencers (ABI's SnaPshot and Amersham Pharmacia Biotech's SNuPe), mass spectrometry (Sequenom), and fluorescence polarization (Perkin Elmer).

3.2.1.3 Pyrosequencing. Another enzymatic genotyping method is pyrosequencing (Pyrosequencing AB). In this method, primer extension is monitored by luminometric detection of pyrophosphate, which is released on the addition of deoxynucleotides. The pyrophosphate is converted to ATP, which in turn stimulates luciferase to produce light which can be detected. As the deoxynucleotides are added, the complementary DNA strand is built up and the nucleotide sequence can be determined from the signal strength. Using pyrosequencing, the sequence of short 30–50 bp stretches of DNA can be determined (15, 16). This method available in a 96-well format and might be useful for small low-throughput applications, but the high cost may be an issue when high genotyping throughput is necessary.

3.2.2 Hybridization-Based Techniques

3.2.2.1 Allele-Specific Amplification. The allele-specific amplification (ASA) assay is based on the finding that PCR amplification will not occur if a primer has a mismatch at the 3' end. Therefore, to detect the presence of a polymorphism, two different forward primers can be designed for use with a common reverse primer such that the 3' end of each forward primer matches the expected nucleotide at the polymorphic site for both the normal and variant sequences. A sample can be tested for the variant by running two PCR reactions; one with the primer set containing the normal sequence and the second reaction containing the primer set containing the variant sequence. By monitoring for the formation of allele-specific PCR products, the identity of the test sample can be known. The results of the assay can be detected through electrophoresis. The addition of fluorescent dyes to primers allows for easy multiplexing of reactions. The principal drawback of ASA based methods is the lack of specificity because of the difficulty in optimizing reaction conditions so that only perfectly matched oligonucleotides will be amplified.

3.2.2.2 DNA Array Genotyping. Allele-specific hybridization biochemistry underlies many of the chip-based genotyping systems. DNA chip-based genotyping systems allow for the simultaneous analysis of many polymorphic loci on one genetic sample. Thousands or even hundreds of thousands of allele specific oligonucleotides are created and attached in an order pattern onto a solid glass or silicon surface creating an array of surface bound oligonucleotide probes. Each bound oligonucleotide functions as an allele-specific probe. After PCR amplification of the areas of interest with fluorescently labeled nucleotides, the sample is then hybridized to the chip. Perfectly matched probes hybridize more efficiently to the bound oligonucleotides and therefore give a stronger signal (10). As with other hybridization-based reactions, the drawback of this system is the difficulty in achieving the appropriate stringency of hybridization conditions so that only perfectly matched oligonucleotides will be retained and provide a positive signal.

Moreover, chips must be custom made and are therefore expensive and very inflexible. Adding a new SNP to the analysis means that the entire chip must be redesigned. Universal tag array systems are being used as a reaction sorting system and can lower the cost and increase the flexibility of chip-based genotyping systems. Many of the more specific enzyme-based biochemistries such as single base primer extension and oligonucleotide ligation assay (OLA) are being developed for array-based genotyping (17, 18).

3.2.2.3 Homogeneous Solution Hybridization with Fluorescence Resonance Energy Transfer. The Taqman (Applied Biosystems, Inc.) assay uses allele-specific hybridization to distinguish between alleles during PCR with fluorescence resonance energy transfer (FRET) detection. FRET detection is based on the observation that the fluorescent emissions from one fluorescent dye can be absorbed by another fluorescent dye when the two are in close physical proximity. This assay uses two sets of probes: a pair of PCR primers and a set of allele-specific Taqman probes. The Taqman probes differ at the polymorphic site, such that one probe is specific for the wild-type allele, whereas the other is specific for variant allele. Each allele-specific probe is also labeled with both a 5' fluorescent reporter dye and a 3' quencher dye. Both probes use the same 3' quencher dye [6-carboxy-*N,N,N',N'*-tetrachlorofluorescein (TAMRA)], whereas the wild-type allele and variant allele probes are also labeled with 6-carboxy-4,7,2',7'-tetrachlorofluorescein (TET) or 6-carboxyfluorescein (FAM), respectively. While the probes are intact, there is no fluorescent signal because of the close proximity of the quencher dye to the reporter dyes (19).

During the PCR amplification, one or both of the allele-specific probes anneal to the polymorphic region. During the extension phase, the 5' reporter dye is cleaved and released by the 5' nuclease activity of the *Taq* polymerase. Cleavage releases the 5' reporter dye from the probe, allowing it to emit its characteristic fluorescence (9).

Because post-PCR processing or manipulation is not required, the Taqman assay is simple to perform once the assay has been optimized. Unfortunately, the assay, which is based on hybridization, requires a highly specific assay condition to deliver robust genotypes. Also, because four oligonucleotide probes are required (two of which must be fluorescently labeled), these assays can be expensive to run.

3.2.3 Combined Enzymatic/Hybridization *Techniques*

3.2.3.1 Invasive Cleavage Assays. The Invader assay (Third Wave Technologies, Inc.) is a FRET-based enzymatic/hybridization combination genotyping method that offers the potential to genotype without prior PCR amplification. This assay uses two hybridization oligonucleotides (a wild-type and a variant-specific oligonucleotide) plus an Invader probe. The two hybridization probes partially overlap a known polymorphic site and compete for hybridization. When one probe successfully hybridizes, it forces the other probe into an overlapping position, which will be recognized and cleaved by the enzyme flap endonuclease **(20,** 21). The cleaved fragment acts as an "invader" probe in a second reaction, where it directs the cleavage of an end-labeled FRET probe-template construct (9). While avoiding PCR and genotyping directly from genomic DNA are hypothetical advantages of the Invader assay, this requires a large amount of genomic DNA, which can be in short supply. This advantage is completely lost if investigators must perform the assay on DNA fragments previously amplified by PCR (22).

3.2.3.2 OLA. The OLA uses an enzymatic reaction to increase the specificity of a hybridization-based approach. Three very specific oligonucleotide probes are used in OLA: one specific for the wild-type allele, one specific for the variant allele, and a common probe that carries a fluorescent label. PCR is used to create amplicons containing the polymorphic site. When the PCR products are incubated with all three probes, the 5' region of the common probe anneals just downstream of the polymorphic site. The 3' end of either of the allele specific probes anneals adjacent to the 5' end of the common probe. In the presence of thermostable DNA ligase, the two probes will join only if there is a perfect match. The results of the assay can be observed either by gel

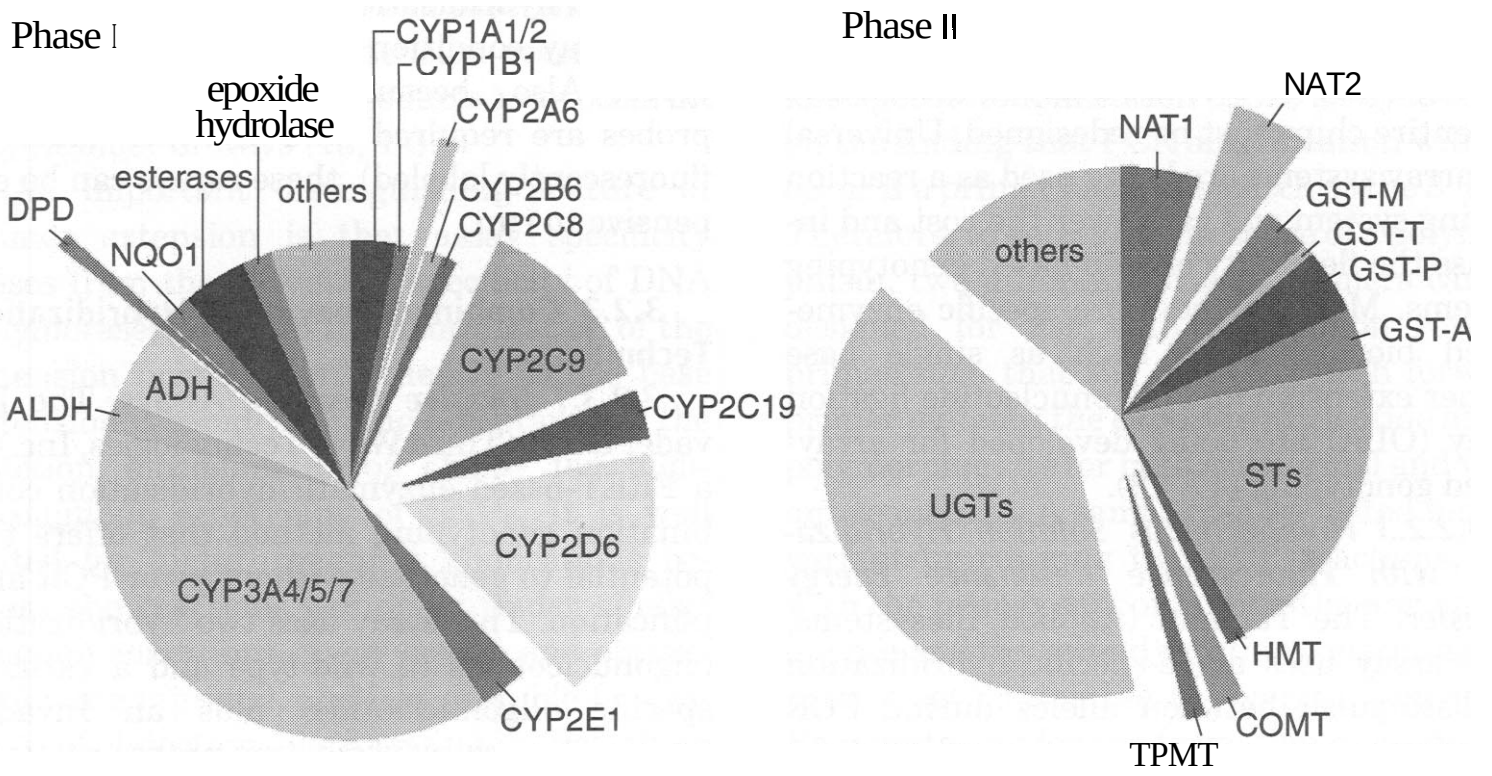

Figure 12.3. Drug-metabolizing enzymes. Those drug-metabolizing enzyme polymorphisms that have already been associated with altered drug effects are separated from the pie chart. For each of the corresponding phases, the size of the pie slice approximates that drug-metabolizing enzyme's contribution to the overall metabolism of drugs. ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; CYP, cytochrome P450; DPD, dihydropyrimidine dehydrogenase; NQO1, NADPH: quinine oxidoreductase or DT diaphorase; COMT, catechol *O*-methyltransferase; GST, glutathione S-transferase; HMT, histamine methyltransferase; NAT, *N*-acetyltransferase; STs, sulfotransferases; TPMT, thiopurine methyltransferase; UGTs, uridine 5'-triphosphate glucuronosyltransferases. Reproduced with permission from Ref. 6.

electrophoresis of the ligation products or by capture of the products on a microarray built with a set of oligonucleotides that are complementary to a tag sequence on one of the ligation probes (17). The need for three specific probes increases the costs of this genotyping assay.

4 PHARMACOGENOMICS

4.1 Drug Metabolizing Enzymes

More than 40 years ago, the deficiency of glucose-6-phosphate dehydrogenase (G6PD) and of the arylamine N-acetyltransferase type 2 (NAT2) were the first examples that revealed that hereditary variants of drug-metabolizing enzymes could be responsible for side effects and interindividual variability in response to drugs (23, 24). Since then, significant progress has been made in the field of pharmacogenetics. Much of that work involves investigation into the clinical relevance of genetic variability in the enzymes responsible for the metabolism of both endogenous and exogenous substrates (Fig. 12.3).

These drug-metabolizing enzymes, such as the cytochrome P450s (CYP), are responsible for the metabolic elimination of most of the drugs currently used in medicine. Genetically determined variability in the function of these enzymes can have a profound effect on drug safety and efficacy.

There are many molecular mechanisms of variability or inactivation of drug metabolizing enzymes. These include splice site mutations resulting in exon skipping (CYP2C19), microsatellite nucleotide repeats (CYP2D6), gene duplication (CYP2D6), point mutations resulting in early stop codons (CYP2D6), amino acid substitutions that alter protein stability or catalytic activity (e.g., TPMT, NAT2, CYP2D6, CYP2C19, and CYP2C9), or complete gene deletions (CYP2D6).

Table 12.3 Selected Substrates of Polymorphic Drug Metabolizing Enzymes

CYP2D6	CYP2C9	CYP2C19	CYP3A4/5/7
Propranolol	S-Warfarin	Omeprazole	Clarithromycin
Metoprolol	Glipizide	Lansoprazole	Erythromycin
Timolol	Glimepiride	Carisoprodol	Midazolam
Amitriptyline	Tolbutamide	Diazepam	Triazolam
Nortriptyline	Phenytoin	Pantoprazole	Cyclosporine
Imipramine	Celecoxib	Citalopram	Tacrolimus
Desipramine	Ibuprofen	Clomipramine	Indinavir
Paroxetine	Losartan	Hexobarbital	Nelfinavir
Fluoxetine	Irbesartan		Ritonavir
Venlafaxine	Torsemide		Saquinavir
Codeine			Cisapride
Dextromethorphan			Astemizole
Tolterodine			Estradiol
Propafenone			Hydrocortisone
Mexiletine			Progesterone
			Testosterone
			Sildenafil
			Trazodone
			Vincristine
			Zaleplon
			Zolpidem
			Amlodipine
			Diltiazem
			Nifedipine
			Verapamil

4.1.1 Polymorphisms in the Cytochrome P450 System. Cytochrome P450 (CYP) enzymes, a very large gene family comprised of numerous isoforms, oxidatively metabolize xenobiotics, including many drugs. Specializing in the removal of lipophilic foreign chemicals, these enzymes rank among the most abundant proteins in the liver. Table 12.3 lists selected medications that are metabolized by CYP enzymes.

When CYP mutations result in null alleles (inactivation), a complete lack of active enzyme and a severely compromised ability to metabolize drugs results. Drugs may reach toxic plasma concentrations if given in regular doses to these "poor metabolizers." For example, mutations in the gene encoding cytochrome P450 CYP2C9, which metabolizes warfarin, affects patients' response to the drug and their dose requirements (25).

4.1.1.1 CYP2D6. CYP2D6, also known as debrisoquine/sparteine hydroxylase, is highly polymorphic and is inactive in about 8% of Caucasian-Americans and 2–5% of African Americans (26). It is involved in the metabolism of approximately 30–40 commonly used drugs. Millions of patients with compromised metabolism are thus at risk of adverse drug reactions when prescribed drugs that are CYP2D6 substrates. Many such drugs are used for treating psychiatric (such as antidepressants and antipsychotics) and cardiovascular diseases (such as β-blockers and antiarrhythmics), where the therapeutic window can be narrow and side effects common.

More than 70 variant alleles of the CYP2D6 locus have been described, of which at least 15 encode non-functional gene products. These alleles as well as several functional allelic variants of CYP2D6 have been described that occur at variable frequencies in racially diverse populations (27). Most of the null alleles have interrupted open reading frames because of splice-site mutations, single base deletions, nonsense mutations, or deletion of the entire gene. Alleles encoding non-functional full-length proteins have also been described. Based on genetic diagnosis, it is now possible

to identify individuals with poor metabolizer (PM) phenotype as carriers of two null-alleles with over 99% certainty (28–30).

Whereas using a molecular diagnostic to identify CYP2D6 PMs has become much easier, it has remained much more difficult to predict the metabolic capacity of extensive metabolizers (EM; i.e., individuals carrying one or more functional gene copies) (30). Even in extensive metabolizers, CYP2D6 activity is known to vary greatly. For example, the CYP2D6 activity represented by the metabolic ratio (MR) values of debrisoquin and desipramine have been reported to show more than a 70-fold variation among extensive metabolizers in Korean and white populations (31, 32).

In poor metabolizers, the genes often contain inactivating mutations, which result in a complete lack of active enzyme and a severely compromised ability to metabolize drugs. Thus, poor metabolizers of CYP2D6 are potentially at risk for increased plasma concentrations of drugs given at conventional doses. For example, the metabolism of the antidepressant venlafaxine is controlled by genetic polymorphism. Poor metabolizers of CYP2D6 have significantly reduced venlafaxine clearance and an increased risk of cardiovascular toxicity (33).

Conversely, ultrarapid metabolizers (UM) often do not reach therapeutic concentrations when given standard doses. In some cases, these individuals inherit up to 13 copies of the CYP2D6 gene, arranged in tandem (34). This amplification polymorphism results in affected people metabolizing drugs that are CYP2D6 substrates so quickly that a therapeutic effect cannot be obtained at conventional doses. For example, it has been estimated that, whereas a daily dose of 10–20 mg nortriptyline would be sufficient for a patient who is a CYP2D6 poor metabolizer, an UM inheriting multiple copies of the gene could require as much as 500 mg/day (35). Ultrarapid metabolizers are found in 1–10% of Caucasians and 2–3% of African Americans. Among Ethiopian and Saudi Arabian populations, there is a very high frequency (20–30%) of the UM phenotype (36).

In addition to detoxifying and eliminating drugs and metabolites, CYP2D6 is required for activation of pro-drugs. For example, codeine must be converted to its active metabolite, morphine, by CYP2D6, rendering the 2–8% of the population who are homozygous for non-functional CYP2D6 alleles resistant to the analgesic effects of this commonly used medication. Thus, this common polymorphism explains at least some of the interindividual variability in pain relief from standard doses of codeine (6).

4.1.1.2 CYP2C9. CYP2C9 is one of the most abundant cytochromes P450 in the human liver and has been shown to metabolize a large number of drugs, including s-warfarin, losartan, glipizide, tetrahydrocannabinol, phenytoin, torsemide, celecoxib, and various nonsteroidal anti-inflammatory drugs (37–40).

There have been six different CYP2C9 alleles described. The two most common variant alleles (*CYP2C9*2* and *CYP2C9*3*) differ from the wild-type allele (*CYP2C9*1*) by a single point mutation: *CYP2C9*2* is characterized by an Arg144Cys amino acid substitution, whereas *CYP2C9*3* has an Ile359Leu substitution. *CYP2C9*2* and *3 have been reported to occur at a frequency of 8%and 6%, respectively, in the Caucasian population. Both of these variants are much less common in African-American (1%and 0.5%, respectively)and Asian populations (0%and 2–3%, respectively) (41). Both allelic variants are associated with reduced catalytic activity compared with the wild-type. They are reported to show approximately 12% (*2) and less than 5% (*3) of wild-type enzyme activity (42).

The potential clinical importance of these variants was recently demonstrated. Patients with one or more of these more common CYP2C9 variant alleles (*CYP2C9*2* or *CYP2C9*3*) require a significantly lower warfarin dose to maintain the desired level of anticoagulation, and these variant alleles were also associated with a greater likelihood of bleeding complications (25). Patients carrying at least one of these variants have also been shown to require 30% less phenytoin to achieve therapeutic phenytoin concentrations (42).

Much less is known about the less common CYP2C9 variants. *CYP2C9*4* results in an Ile359Thr substitution. It has been reported to be extremely rare (43). *CYP2C9*5* is reported to lead to an Asp360Glu substitution.

The *CYP2C9*5* variant has only been observed in African Americans, such that approximately 3% of this population carries the *CYP2C9*5* allele. In *vitro* intrinsic clearances for *CYP2C9*5*, calculated as the ratio of V_{max}/K_m, ranged from 8 to 18% of *CYP2C9*1* values in the initial report (44). The *CYP2C9*6* variant results in a frameshift mutation. To date, it has only been observed in one Caucasian patient (R. S. Kidd, personal communication).

4.1.1.3 CYP2C19. CYP2C19, also known as mephenytoin hydroxylase, was first described in 1993 (45). Since then, eight alleles have been identified. Each of the alleles other than *CYP2C19*1* have been associated with almost complete absence of gene expression. Most of the alleles (*CYP2C19*2*, *3, *4, *5, *6, *7, and *8) occur infrequently (approximately 3–5% in total) in random Caucasian and African-American populations. In all racial groups studied, *CYP2C19*2* is the allele most commonly associated with an inactive gene product. Within Asian populations, the higher frequency of *CYP2C19*2* and *CYP2C19*3* alleles accounts for the higher prevalence in this racial group (approximately 35% PM) (46,471.

CYP2C19 metabolizes many clinically important drugs (Table 12.3). Subjects with the CYP2C19 PM phenotype have an area under the curve (AUC) of omeprazole that is more than sixfold higher than efficient metabolizers (EM), and the drug has a severely prolonged half-life in PM individuals (48). A similar relationship is seen for other proton pump inhibitors (49). To reach similar plasma levels, PMs of CYP2C19 would take about 1–2 mg of omeprazole instead of the recommended dose of 20 mg (50).

Regarding proton pump inhibitors, the effect of CYP2C19 PM status is not limited to pharmacokinetic alterations. The difference in the pharmacokinetics has been shown to influence the outcome of H. Pylori eradication therapy. Furuta et al. showed that in patients with confirmed H. Pylori infection treated with omeprazole or lansoprazole plus clarithromycin and amoxicillin, CYP2C19 PMs had an eradication rate of 97.8% compared with a rate of 72.7% ($P < 0.001$) for CYP2C19 EMs (51).

In addition to the proton pump inhibitors, CYP2C19 genotype has also been shown to be associated with reduced elimination of diazepam (52), proguanil (53), imipramine (54), citaloprarn (55), carisoprodol (56), and hexobarbital (57).

4.1.1.4 CYP3A4/5/7. The CYP3A family consists of CYP3A4, CYP3A5, and CYP3A7. The CYP3A members are the most abundant CYPs in the human liver and small intestine. Substantial interindividual differences in CYP3A expression, exceeding 30-fold in some populations, contribute greatly to variation in oral bioavailability and systemic clearance of CYP3A substrates (58). One factor contributing to this large variability in 3A expression is the presence or absence of CYP3A5. CYP3A5 was previously detected in livers and small intestines of only some adult individuals, but the basis for this "polymorphic" expression was unknown (59, 60).

Recently, two important SNPs in CYP3A5 (*CYP3A5*3* and **6)* were described. Relative to the wild-type (*CYP3A5*1*), these mutations have been shown to cause alternative splicing and protein truncation, which results in the absence of CYP3A5. Only carriers of at least one *CYP3A5*1* allele have been shown to express large amounts of CYP3A5. The ethnic distribution of the *CYP3A5*1* allele indicates that relatively high levels of CYP3A5 are expressed by an estimated 30% of Caucasians, 30% of Japanese, 30% of Mexicans, 40% of Chinese, and more than 50% of African Americans, Southeast Asians, Pacific Islanders, and Southwestern American Indians (58).

For most Caucasians and African Americans who carry the *CYP3A5*1* allele, CYP3A5 accounts for at least 50% of the total CYP3A content. Because most CYP3A4 substrates are also substrates for CYP3A5, this CYP3A5 polymorphism influences overall CYP3A activity in humans (61). Thus, the presence or absence of CYP3A5 should contribute substantially to the total metabolic clearance of the many CYP3A substrates. Indeed, those heterozygous or homozygous for *CYP3A5*1* should have the highest clearance and lowest oral bioavailability of CYP3A substrates. Moreover, these people might be more likely to encounter a lack of efficacy from standard doses (58).

Important variation in other clinically relevant CYP enzymes such as CYP1A2,

CYP2A6, and CYP2E1 has been demonstrated and reviewed in detail elsewhere (50).

4.1.2 Polymorphisms in Other Important Drug Metabolizing Enzymes

4.1.2.1 **Dihydropyrimidine Dehydrogenase.** Dihydropyrimidine dehydrogenase (DPD) is the initial and rate-limiting enzyme in the catabolism of the chemotherapeutic agent 5-fluorouracil (5-FU). Diasio et al. conducted a familial study that suggested an autosomal recessive pattern of inheritance of deficiency of DPD. While it seems that a complete absence of DPD activity is extremely rare, even partial enzyme inactivity might result in severe toxicity from 5-FU (62). Prospectively evaluating the gene encoding DPD is a typical example of what could be a useful pharmacogenomic approach to preventing toxicity from a very effective drug that has a high level of toxicity (63).

4.1.2.2 ***N-Acetyltransferase-2.*** The *N*-acetyltransferase-2 (NAT2) polymorphism is one of the most common polymorphisms known in human populations. While more than 50% of Caucasians are NAT2 slow acetylator phenotype, there is a tremendous amount of interethnic variation in the frequency of the slow acetylator polymorphism (64). For instance, the slow acetylator phenotype is much more frequent in Egyptians but is much less frequent in Asians (65).

This polymorphism (NAT2) was discovered almost 50 years ago after differences were observed to isoniazid toxicity in tuberculosis patients (66). Subsequently, the differences in isoniazid toxicity were attributed to genetic variability in NAT2, a cytosolic phase II conjugation enzyme primarily responsible for deactivation of isoniazid (67). Indeed, the polymorphism was termed the "isoniazid acetylation polymorphism" for many years until the importance of the polymorphism in the metabolism and disposition of other drugs and chemical carcinogens was fully appreciated (65).

Since these first observations, a wealth of clinical evidence has shown that the disposition of a variety of drugs (including sulfonamides, dapsone, hydralazine, procainarnide, and caffeine) possessing primary aromatic amino or hydrazine functional groups is affected by the same genetic defect (68). In addition to metabolizing drugs, NAT2 is also known to catalyze both N-acetylation (usually deactivation) and *O*-acetylation (usually activation) of aromatic and heterocyclic amine carcinogens. Epidemiological studies suggest that the NAT2 acetylation polymorphisms modify risk of developing urinary bladder, colorectal, breast, head and neck, lung, and possibly prostate cancers. Associations between slow NAT2 acetylator genotypes and urinary bladder cancer and between rapid NAT2 acetylator genotypes and colorectal cancer are the most consistently reported (65). The importance of the NAT2 polymorphisms in clinical pharmacology and toxicology has been extensively reviewed (68, 69).

Grant et al. first demonstrated that the classical isoniazid slow acetylator phenotype is due, at least in part, to reduction of the expression of NAT2 protein (70). No polymorphism in the 5' or 3' regions of the gene have been reported. Eleven SNPs have been identified in the NAT2 coding region. Five of these are capable of producing the slow acetylator phenotype. The four most common of these are *NAT2*5*, *NAT2*6*, *NAT2*7*, and *NAT2*14*. *NAT2*4* is associated with the rapid acetylator phenotype and is considered the wild-type allele because of its absence of any of these substitutions. However, *NAT2*4* is not the most common allele in many ethnic groups, including Caucasians and Africans (71, 68).

4.1.2.3 **Thiopurine Methyltransferase.** Azathioprine, thioguanine, and 6-mercaptopurine are thiopurine drugs that are used to treat acute lymphoblastic leukemia, autoimmune disorders, inflammatory bowel disease, and organ transplant recipients. These drugs are metabolized by the genetically polymorphic enzyme thiopurine methyltransferase (TPMT). Thiopurines are very useful drugs, but they have a relatively narrow therapeutic index, with life-threatening myelosuppression as a major toxicity (72).

Population studies have found that approximately 11% of Caucasians are heterozygous and 0.3% homozygous for TPMT deficiency (73). For the TPMT polymorphism, all patients who inherit two non-functional TPMT alleles will develop dose-limiting hematopoi-

Table 12.4 Selected Substrates, Inducers, and Inhibitors of P-Glycoprotein (138)

P-Glycoprotein Substrates	P-Glycoprotein Inhibitors	P-Glycoprotein Inducers
Amiodarone	Amiodarone	Dexarnethasone
Anthracyclines	Atorvastatin	Phenobarbital
Cisplatin	Clarithromycin	Phenytoin
Cyclosporine	Cyclosporine	Rifampin
Cytarabine	Diltiazem	St. John's Wort
Dactinomycin	Erythromycin	
Daunorubicin	Itraconazole	
Dexamethasone	Ketoconazole	
Digoxin	Quinidine	
Docetaxel	Quinine	
Doxorubicin	Ritonavir	
Etoposide	Tacrolimus	
Fexofenadine	Tamoxifen	
Fluorouracil	Verapamil	
Glucocorticoids		
Indinavir		
Loperamide		
Losartan		
Methotrexate		
Mitoxantrone		
Nelfinavir		
Paclitaxel		
Ritonavir		
Saquinavir		
Sirolimus		
Tacrolimus		
Topotecan		
Vinblastine		
Vincristine		
Vindesine		
Vinorelbine		

etic toxicity. Patients deficient in this drug-metabolizing enzyme can require up to a 15-fold reduction in mercaptopurine to prevent fatal hematotoxicity (74–77).

The full clinical and molecular implications of this variant have recently been reviewed (72, 78).

4.2 Polymorphisms in Drug Transporter Genes

P-glycoprotein (P-gp) is an ATP-dependent drug efflux pump. In humans, P-gp is encoded by the multi-drug resistance gene (MDR-1) that is located on the long arm of chromosome 7. Overexpression of P-gp in neoplastic cells is associated with the phenomenon of multi-drug resistance to chemotherapeutic agents by promoting efflux of chemotherapy (79). P-glycoprotein is also expressed in normal cells including the intestinal epithelium, renal proximal tubule, liver, adrenal cortex, placenta, testes, and blood-brain barrier. Recently, P-gp has been implicated as the causative factor in numerous pharmacokinetic interactions (80). For example, amiodarone and quinidine therapy increases serum digoxin concentrations through inhibition of P-gp in the intestine and renal tubule, which increases digoxin absorption and decreases total body clearance. Table 12.4 provides a partial list of substrates, inhibitors, or inducers of P-gp. Considerable substrate overlap and tissue location exist between P-gp and the CYP 3A4 isoenzyme.

Table 12.5 Selected Polymorphisms Associated With Altered Drug Response

Gene/Gene Product	Medication	Effect Associated with Polymorphism	Reference
ALOX5	Lipoxygenase inhibitors	Improvement in FEV,	98
Angiotensin 1 receptor	Losartan	Reduction in mean arterial pressure	94
β_1-Adrenergic receptor	Metoprolol	Reduction in blood pressure	104
β_2-Adrenergic receptor	Albuterol	Improvement in FEV,	106, 107
Bradykinin B2 receptor	ACE inhibitors	ACE inhibitor induced cough	139
Dopamine D2 receptor	Dopamine antagonists	Anxiolytic/antidepressive effects of neuroleptics	140
Estrogen receptor	Equine estrogen	Increased bone mineral density	141
Gsa	β-blockers	Reduction in blood pressure	142
Platelet Fc	Heparin	Heparin induced thrombocytopenia	143
Serotonin transporter	SSRIs	Antidepressant response	144, 145

Sixteen SNPs have been identified to date in the MDR-1 gene (81). Most of the polymorphisms are either synonymous or occur in intronic regions. However, one synonymous polymorphism in exon 26 (C3435T) has functional significance by influencing the expression of P-gp in the intestine. Individuals with the TT genotype have a twofold lower expression of P-gp compared with the CC genotype (82). Thus, one could predict that individuals with the TT genotype would have higher concentrations of drugs that are P-gp substrates and indeed this is the case. In one study, subjects with the TT genotype had a statistically significant 38% higher digoxin C_{max} concentration compared with subjects with the CC genotype (82). Therefore, patients with the TT genotype would also be expected to have higher concentrations of other P-gp substrates. Alternatively, CC homozygotes through increased expression of P-gp may experience subtherapeutic concentrations of P-gp substrates and experience therapeutic failures.

Genotype frequencies at exon 26 are highly dependent on the ethnicity of the population studied. One recent investigation examined 1280 subjects from 10 different ethnic groups (British Caucasians, Ghanaians, Kenyans, African Americans, Sudanese, Portuguese, Southwest Asians, Chinese, Filipinos, and Saudis) (83). The frequency of the T allele (associated with lower expression of P-gp) was significantly lower in the African populations compared with the Asian and Caucasian groups (16–27% versus 41–66%). Based on this finding, persons of African descent would be expected to have higher P-gp expression compared with Caucasians and Asians. Consequently, plasma concentrations of drugs that are P-gp substrates may be lower among African populations than those of other racial backgrounds despite equivalent doses. Supporting this is a study that revealed that African-American subjects had lower plasma cyclosporine concentrations compared with Caucasians given equivalent doses (84). African-American patients have a higher rejection rate after transplantation compared with Caucasians. Plausibly, the higher frequency of the C allele among African Americans may result in subtherapeutic concentrations of P-gp substrates tacrolimus and cyclosporine and therefore increased risk of rejection. Likewise, the HIV-protease inhibitors may be less effective among patients with the CC genotype and may have diminished penetration into the central nervous system. Clearly, further study is needed to determine the association between the C3435T polymorphism with treatment outcomes among patients receiving P-gp substrates.

4.3 Polymorphisms in Drug Target Genes and Clinical Efficacy

The biomedical literature contains a multitude of publications that attempt to correlate genetic variation underlying differential response to medications. It is obviously not feasible to review all of the examples from the literature, however, Table 12.5 summarizes several of these polymorphisms that are associated with altered drug response. Our goal is to provide several illustrative examples of how

polymorphisms in drug targets are being used to establish a personalized medicine platform.

4.3.1 ACE Insertion/Deletion Polymorphism. One of the most studied polymorphisms occurs in the gene encoding angiotensin-converting enzyme. This polymorphism occurring in intron 16 is not a SNP. Rather it is an insertion/deletion (I/D) of a 287-base pair product. Numerous studies have demonstrated that the D allele is associated with higher concentrations of the hormone, angiotensin II (85). The functional consequences of these findings are apparent and have been supported by several examples from the literature demonstrating that the D allele is associated with increased risk of hypertension, myocardial infarction, and ventricular arrhythmias (86–88). Presence of the D allele is also associated with a poorer prognosis among patients with heart failure (89). Among 328 patients with heart failure followed at a cardiomyopathy clinic, after 2 years, the percentage of patients with transplant-free survival was 78% for the II genotype, 65% ID, and 60% DD ($P = 0.044$). Interestingly, these investigators also examined the impact of the ACE I/D polymorphism with β-blocker therapy in this cohort of patients. In the 208 patients who were not receiving β-blocker therapy, the 2-year transplant-free survival was 81% II, 61% ID, and 48% DD. However, a provocative finding was that β-blocker therapy obviated the influence of the D allele with poor prognosis. Among patients receiving β-blockers, the 2-year transplant-free survival was 70% for II genotype, 71% for ID, and 77% DD (P = NS).

Table 12.6 reviews several studies examining the impact of the insertion/deletion polymorphism with clinical response to ACE inhibitor therapy. Review of these studies reveals numerous conflicting results. Several possibilities exist for these equivocal findings including heterogeneous patient populations, sample size, endpoints, and study duration. However, perhaps the largest factor resides in the limitation of examining a single SNP. Medications act with numerous transporters and receptors to illicit a therapeutic response. Consequently, it is more plausible that variability in drug efficacy will be caused by polymorphisms in multiple genes involved in the drug response pathway. Thus, the findings with the ACE I/D polymorphism provide a rationale for future studies to eschew the simplicity of examining a single SNP and instead incorporate a more genomic approach.

One such effort examined 45 polymorphisms in genes encoding angiotensinogen, ACE, and the AT1 receptor (90). A total of 91 patients who had received an ACE inhibitor for the treatment of hypertension were retrospectively studied, and 10 polymorphisms associated with a good response were identified. These 10 polymorphisms, termed "genetic signatures," were then prospectively evaluated in 102 patients with hypertension who received ACE inhibitors. The response rate to ACE inhibitor therapy was 73% among the patients with the "genetic signatures" compared with 42% response rate in the cohort without the "genetic signatures." Thus, while focusing on a single SNP is easy, it is unlikely to yield sufficient predictive accuracy to be useful clinically. Future pharrnacogenomic studies will need to examine the impact of several SNPs and haplotypes to correlate these "genetic signatures" with drug variability.

4.3.2 Angiotensin Type 1 Receptor. Angiotensin II produces its deleterious effects by interacting with the angiotensin type 1 receptor. A well-described polymorphism exists in the gene encoding the angiotensin type 1 receptor with either an adenine (A) or cytosine (C) at position 1166 of the 3' untranslated region of the gene. Case-control studies have shown an association between the C allele and increased risk of hypertension, aortic stiffness, and a worse prognosis among patients with idiopathic heart failure (91–93). Thus, angiotensin receptor blockers used in the management of hypertension and heart failure may be particularly effective among patients with the C allele. One study of healthy volunteers demonstrated that subjects carrying at least one C allele experienced a larger decrease in mean arterial pressure and an increase in glomerular filtration rate compared with subjects with the AA genotype (94). Although this finding warrants further study, it suggests that the C allele may be an important predictor of response to angiotensin receptor blockers. Because this polymorphism occurs in an un-

Table 12.6 Impact of Angiotensin-Converting Enzyme Polymorphisms and Response to ACE Inhibitor Therapy

Study Population	Results
27 Healthy volunteers	Captopril significantly reduced mean arterial pressure in the II and ID genotypes; no significant change in the DD group. Renal vascular resistance was significantly reduced in the II and ID genotypes with no significant change in the DD group (148).
27 Healthy volunteers	No correlation between ACE genotype and reduction in mean arterial pressure after single dose of enalapril (149).
530 Patients with diabetes	Largest reduction in albumin excretion rate occurred in lisinopril treated patients with the II genotype (150).
212 Non-diabetic patients with proteinuria	Largest reduction in proteinuria occurred in ramipril treated patients with the DD genotype (151).
104 Patients with hypertension	Statistically significantly greater reductions in systolic and diastolic blood pressure after 6 months of fosinopril therapy among patients with the DD genotype compared with II or ID genotypes (152).
57 Patients with hypertension	Absolute and percent reduction in diastolic blood pressure tended to decline more after 6 weeks of imidapril therapy among hypertensive patients with the II genotype compared with ID and DD genotypes (153).

translated region of the gene, it is likely that this polymorphism is in linkage with another marker.

4.3.3 ACE I/D and AGTR1 Interaction. In routine clinical practice, risk factors for disease tend to be either additive or synergistic. In general, a patient with hypertension, diabetes, and hyperlipidemia is at greater risk of suffering an acute coronary event compared with a patient with only one of these risk factors. Thus, interactions among polymorphism~may also be germane to pharmacogenomic studies. To this end, several studies have examined the impact of both the ACE DD and AGTRl CC genotype. One study demonstrated an increased risk of myocardial infarction among patients with the DD and CC genotypes for the ACE and AGTRl genes, respectively (95). However, a recent case-control study failed to demonstrate an association between these two polymorphisms with the risk of myocardial infarction (96). Another study revealed that the risk of ventricular arrhythmias among patients with systolic heart failure was significantly increased in patients with the DD ACE genotype and the CC genotype for the AT1 gene (97). Thus, once again, these results suggest that this group of patients may derive particular benefit from more aggressive management of their disease.

4.3.4 5-Lipoxygenase Polymorphisms. Leukotrienes mediate airway inflammation and play an integral role in the pathophysiology of asthma. Zileuton (Zyflo) inhibits the enzyme 5-lipoxygenase, reducing the formation of leukotrienes and thus improves the symptoms of asthma. A polymorphism exists in the gene encoding the 5-lipoxygenase (ALOX5) promoter region. This polymorphism contains three to six tandem repeats of GGGCGG. A recent study examined the impact of the tandem repeat polymorphism in the ALOX5 promoter region with response to an investigational 5-lipoxygnase inhibitor (98). The clinical outcome in the study was percent *change* in forced expiratory volume in 1 second (FEV_1) from baseline. The allele frequency for the wild-type ALOX5 promoter polymorphism was 0.77. Patients who were homozygous or heterozygous for the wild-type allele had an average *change* in FEV_1 from baseline of 18% and 23%, respectively. Patients with no wild-type alleles had a 1% decrease in FEV_1 from active treatment.

4.3.5 β_1-Adrenergic Receptor Polymorphism~. The β_1-adrenergic receptor (AR) is a G-protein–coupled receptor expressed in a number of cell types including the heart and kidneys. The gene that codes for the β_1-AR is intronless and is located on chromosome 10q21. There are two common SNPs within the β_1-AR gene at codon 49 and codon 389 (99).

Codon 49 is located in the extracellular tail of the amino terminus end of the receptor, a potentially important region for receptor binding, regulation, and expression (100). A non-synonymous SNP produces a glycine (Gly) for serine (Ser) substitution at codon 49 (Ser49Gly). Although there are no data *in viuo* associating this polymorphism with drug response, a recent site-directed *in uitro* mutagenesis study suggests that agonist-promoted down-regulation of the receptor is amplified with the Gly49 variant (101).

Codon 389 is located in the intracellular tail of the carboxy terminus end of the receptor, a potentially important region for G-protein coupling (100). A non-synonymous SNP produces a Gly for arginine (Arg) substitution at codon 389 (Arg389Gly). This polymorphism has been shown to vary by race, with African Americans possessing an allele frequency of 42% for the Gly389 variant while Caucasians possess a frequency of only 27% (102). Furthermore, *in uitro* mutagenesis studies have shown a functional difference with this polymorphism (103). In this study, those cells carrying the Arg389 variant had a nearly twofold greater resting activity rate, as measured by adenylyl cyclase levels, and an almost fourfold greater activity when stimulated with β-agonist, thus suggesting the Gly389 is a less active or perhaps less reactive receptor form. This theory has been recently put to the test in a prospective study of patients with hypertension, and indeed, this polymorphism may be an important determinant of the antihypertensive response to β-blocker therapy (104). Further studies are needed to evaluate whether this polymorphism confers the racial

differences observed in response to β-blocker therapy in both hypertensive and heart failure patients.

4.3.6 β_2-Adrenergic Receptor Polymorphisms

4.3.6.1 β_2-Adrenergic* Receptor *SNPs. The β_2-adrenergic receptor is a G-protein–coupled receptor that interacts with endogenous catecholamines and various pharmacologic agents. The mainstay of therapy for acute bronchoconstriction is the administration of β_2-AR agonists such as albuterol. A non-synonymous SNP in the β_2-AR gene produces a Gly for Arg substitution at codon 16 (Arg16Gly). In *vitro* studies indicate that the Gly16 form of the receptor undergoes enhanced agonist-mediated downregulation compared with the Arg allele (105). These findings have been supported by several clinical studies. One study demonstrated that subjects with the Arg16 homozygous genotype were 5.3 times more likely to have a positive bronchodilator response to a single dose of albuterol compared with Gly16 homozygotes (106). Another study enrolled 16 patients with asthma and measured FEV_1 response to an 8 mg oral dose of albuterol (107). The study population was divided into two groups: Arg16 homozygotes and Gly16 homozygotes. Patients who were homozygous for Arg16 had a fourfold greater FEV, response compared with Gly16 homozygotes despite nearly identical plasma albuterol concentrations. Moreover, the codon 16 genotype may also influence long-term response to albuterol. One study of 107 patients with mild-to-moderate asthma demonstrated that Arg16 homozygous patients receiving regularly scheduled albuterol had nearly double the number of asthma exacerbations per year compared with placebo. Furthermore, the rate of asthma exacerbations was significantly greater among Arg16 homozygotes during treatment with albuterol compared with heterozygotes and Gly16 homozygotes (108). A separate study found that Arg16 homozygotes receiving regularly scheduled albuterol experienced a decrease in morning peak expiratory flow rate (PEFR). In contrast, Gly16 homozygous patients who received regularly scheduled albuterol did not experience a decline in PEFR (109). These results suggest that the Arg16 homozygous genotype is associated with deleterious effects from regularly scheduled albuterol therapy and that patients with this genotype should only receive albuterol for breakthrough symptoms.

4.3.6.2 β_2-Adrenergic* Receptor *Haplotypes. In all, 13 polymorphisms occur in the β_2-AR gene. Thus, if these polymorphisms occurred completely randomly, one would expect a total of 2^{13} variations (haplotypes) in the β_2-AR gene. However, only 12 haplotypes occur, and 5 haplotypes describe 88% of the population (8). Thus, the polymorphisms in the β_2-AR are in strong linkage disequilibrium. A seminal paper has investigated the impact of haplotypes, rather than individual SNPs in predicting response to albuterol among patients with asthma. Importantly, there was no association between an individual SNP and response to albuterol. However, haplotype pair was significantly related to improvements in FEV_1 from albuterol (8). Therefore, examination of multiple SNPs in a receptor that is physiologically linked to drug response resulted in the best prediction of therapeutic efficacy. Focusing on multiple genes and/or multiple SNPs to determine disease associations or drug response is analogous to the multiple factors that a clinician must consider when dosing *a* medication. For example, when prescribing digoxin, the likelihood of prescribing the appropriate dose' is increased when a clinician considers multiple factors such as patient age, body size and weight, renal function, and concomitant drug therapy.

4.4 Single Gene Pharmacogenetic Studies in Genes influencing Disease Progression

Once again, it would not be feasible to review all the examples in the biomedical literature of polymorphisms influencing disease severity or progression. However, Table 12.7 summarizes many of these polymorphisms that influence disease severity and associated drug response. We will discuss a few of these disease states in detail.

4.4.1 Acute Coronary Syndromes. Several studies have examined the impact of SNPs on the natural history of patients undergoing percutaneous coronary intervention (PCI) for the treatment of acute coronary syndromes.

Table 12.7 Selected Polymorphisms Influencing Disease Severity and Associated Drug Response

Gene	Altered Disease Severity	Impact of Polymorphism on Drug Response	Reference
ACE	D allele ↑ risk of death or need for heart transplant	β-Blockers abolished poorer prognosis of patients with DD genotype	89
APOE	E4 allele ↑ in Alzheimer's disease	Presence of E4 allele associated with poor response to tacrine	117
APOE	Smokers with an E4 allele had a threefold ↑ risk of CHD event	Unknown	152
β_1	Heart failure patients with the Ser49Ser genotype had a worse 5-year prognosis compared with patients with a Gly variant	Unknown	153
β_2	Reduced survival rate among heart failure patients with an Ile164 allele	Unknown	154
	Reduced exercise capacity among heart failure patients with an Ile164 allele	Unknown	155
Calcitonin	Association between heterozogosity and ↓ fracture risk among postmenopausal women	Unknown	156
Cystathionine beta synthase	Risk of coronary artery disease	Response to homocysteine lowering from folic acid	157
Endothelin A	↑ frequency of TT homozygotes in idiopathic dilated cardiomyopathy	Unknown	158
Factor V	Risk of venous thrombosis	↑ Risk of venous thrombosis from oral contraceptives	159
Factor VII	Patients with the ArgArg genotype have a threefold ↑ risk of complications after PCI	Unknown	110
	Odds ratio of MI among patients with ArgGln genotype = 0.47 (0.27–0.81) compared with ArgArg genotype	Unknown	160
GP IIIa	Association between Pl^{A2} allele and acute coronary thrombosis	Unknown	112
G-protein	Increased BMI among TT homozygous primiparous women	Unknown	161
HERG, KvLQT1, MiRP1	Long QT syndrome	↑ Risk of drug induced Torsade de Pointes	162, 163
P-selectin	Risk of myocardial infarction	Unknown	164
Prothrombin	Risk of venous thrombosis	↑ Risk of venous thrombosis with oral contraceptives	165

CHD, coronary heart disease; PCI, percutaneous coronary intervention; MI, myocardial infarction; GP, glycoprotein; BMI, body mass index.

One of the major complications from PCI is restenosis of the target coronary artery, which occurs in as many as 40% of patients. Restenosis results in substantial morbidity and increases need for repeat coronary interventions. One study examined a polymorphism in the gene that encodes Factor VII. Factor VII has an integral role in the process of clot formation among patients with acute coronary syndromes. After plaque rupture, tissue factor released from the lipid core complexes with Factor VII, leading to the activation of Factor X, and ultimately Factor IIa (thrombin). This group of investigators studied 666 consecutive patients undergoing PCI (110). A polymorphism (Arg353Gln) in exon 8 of the Factor VII gene was studied to see if it influenced the rate of death, myocardial infarction, or urgent target vessel revascularization within 30 days of PCI. An earlier study showed the Gln allele was associated with a 20–30% lower Factor VII concentration (111). Thus, one would expect the Gln allele to be associated with reduced cardiovascular complications. Indeed, the incidence rate for the composite endpoint was 7.7% among Arg353Arg homozygotes compared with 2.5% among patients carrying at least one Gln allele (RR of 0.32; 95% CI, 0.08–0.90). Although statistically significant, the results are limited by the small number of events (43) in this study. Ironically, Factor VII concentrations were similar between Arg homozygotes and Gln carriers who reached the primary endpoint. Thus, the putative mechanism for this reduced risk among Gln carriers remains to be elucidated.

A second study examined a polymorphism in the glycoprotein IIIa gene. This protein plays an integral role in the final common pathway of platelet activation and fibrinogen cross linkage, resulting in the formation of a platelet plug. The two common allelic isoforms are Pl^{A1} and Pl^{A2}. A retrospective study demonstrated an association between the Pl^{A2} polymorphism with a heightened risk of acute coronary thrombosis (112). These findings were confirmed in a prospective study that examined the association between the GP IIIa Pl^{A1} and Pl^{A2} isoforms with angiographically confirmed restenosis. The study population consisted of 1150 patients undergoing PCI with stent placement who also had a follow-up cardiac catheterization at 6 months (113). Approximately 72% of the study population were Pl^{A1} homozygotes, 25% were heterozygotes, and 3% were Pl^{A2} homozygotes. At 6 months, 47% of patients with the Pl^{A2} allele had angiographically confirmed restenosis compared with 38% for Pl^{A1} homozygotes (OR 1.42; 95% CI, 1.09–1.84). The association between genotype and restenosis was strongest among Pl^{A2} homozygotes and women.

Although both studies demonstrate a statistically significant association between the studied polymorphism and complications after PCI, the results need to interpreted with consideration of the absolute risk reduction (ARR) and number needed to treat (NNT). A corollary to NNT, is the number needed for genetic effect (NNGE). The NNGE is calculated as 1/ARR. Thus, when examining the impact of the Arg353Arg genotype on increased complications after PCI, only 1 of 19 patients with this genotype would experience a complication after PCI (114). Likewise, in the study of the Pl^{A2} polymorphism, only 1 of 11 patients with the Pl^{A2} allele will have angiographically confirmed restenosis.

4.4.2 Alzheimer's Disease. One of the most well-studied disease association genes is the apolipoprotein E (APOE) gene located on chromosome 19; this is the gene related with the risk of developing Alzheimer's disease. Three allelic variants exist for APOE. The frequency for E3, E4, and E2 are 77%, 15%, and 8%, respectively. Several studies have determined an association between the presence of the E4 allele with late-onset (greater than 60 years of age) Alzheimer's disease (115, 116). Furthermore, a gene-dose response exists for the E4 allele and the risk of late-onset Alzheimer's. In one case-control study, the odds ratio for developing Alzheimer's disease was 3.9 for the E3/E4 genotype and 15.6 for the E4/E4 genotype. Interestingly, the allelic variant of APOE also influences treatment response with the cholinesterase inhibitor, tacrine (117). In one study, 83% of non-APOE4 carrying patients had improvements in cognition when given tacrine. In contrast, 60% of patients with an E4 allele were unchanged or declined after tacrine administration. Despite these data, single SNP studies of the APOE

gene would not justify withholding therapy because 40% of patients with the E4 allele had a positive response to tacrine (117). Future studies will need to incorporate a more genomic approach by examining other putative polymorphisms involved in response to pharmacologic therapy for Alzheimer's disease.

4.4.3 Hypercholesterolemia. Several studies have identified genetic polymorphisms influencing the clinical response to HMG-CoA reductase inhibitors (subsequently referred to as statins) (118–122). Rather than focusing on polymorphisms in drug targets, these studies examined polymorphisms in genes believed to influence the progression of atherosclerosis. All studies were randomized, double-blinded, and placebo-controlled, and are summarized in Table 12.8. Taken together, the results of these studies identify genetic subgroups of placebo-treated patients who have an increased risk of developing major coronary events. Furthermore, treatment with a statin abolished the deleterious effect of the genetic variant.

Three pharmacogenomic studies have been published from the Regression Growth Evaluation Statin Study (REGRESS) group. REGRESS examined the ability of pravastatin to retard progression of atherosclerosis among men with symptomatic coronary artery disease and hypercholesterolemia (123). In the first example, DNA was examined for a polymorphism in the gene encoding cholesteryl ester transfer protein (CETP), which is involved in the metabolism of high-density lipoprotein (HDL), a cardioprotective lipoprotein (122). The presence of the variation in CETP was referred to as B1 and its absence as B2. Response to pravastatin and placebo was examined by genotype with patients grouped as B1B1, B1B2, or B2B2. At baseline, patients with the B1B1 genotype had higher CETP and lower HDL concentrations than patients with the B2B2 genotype. Placebo-treated patients with the B1B1 genotype experienced the greatest progression of atherosclerosis, the B1B2 genotype had an intermediate progression, and the B2B2 group had the least disease progression. Pravastatin-treated patients with either the B1B1 or B1B2 genotype had significantly less atherosclerotic progression compared with patients receiving placebo. However, pravastatin-treated patients with the B2B2 genotype, (16% of the study population) derived no benefit from pravastatin treatment as measured by changes in mean luminal diameter of the coronary arteries. Although these results are provocative, a major limitation is that they do not examine hard clinical endpoints such as death and non-fatal myocardial infarction. Plaque composition, rather than size, is a better predictor of lesions susceptible to rupture resulting in an acute coronary event.

A second substudy from REGRESS examined a polymorphism in the promoter region of the stromelysin-1 gene [-1612 (5A/6A)] (120). Stromelysin-1 is involved in connective tissue remodeling and wound healing. The 6A allele results in reduced expression of stromelysin-1 and consequently increases connective tissue deposition and potentially increased atherosclerotic lesions. Patients were divided according to genotype (5A5A, 5A6A, and 6A6A). There were no differences in prognostic baseline characteristics, disease severity, or lipid values among the three genotypes. Furthermore, there was no association between the stromelysin-1 polymorphism and changes in lipid levels from pravastatin therapy. Placebo-treated patients with at least one 6A allele had the greatest number of clinical events (mostly restenosis). Pravastatin therapy reduced clinical events most effectively among 6A carriers.

A third pharmacogenomic substudy of REGRESS examined the impact of the −455 G/A polymorphism of the β-fibrinogen gene (119). Previous work revealed that carriers of the −455 A allele have higher concentrations of fibrinogen, a prothrombotic substance associated with increased risk of myocardial infarction and stroke (124). At baseline, patients with the −455 AA genotype had greater atherosclerotic disease severity compared with those with other genotypes. During follow-up, placebo-treated patients with the −455 AA genotype had the greatest disease progression. Once again, the effect of genotype on disease progression was abolished by pravastatin therapy.

The Cholesterol and Recurrent Events (CARE) trial enrolled patients with a history of myocardial infarction and hypercholesterol-

Table 12.8 Polymorphisms Influencing Clinical Response to Statins

Study (Statin)	N	FU (years)	Polymorphism Studied	Endpoint	Placebo Event Rate	Statin Event Rate	P
4S (simvastatin) (121)	966	5.5	Apolipoprotein E (E4, E3, E2)	Mortality	E4 Carriers; RR 1.9 (95%CI 1.1–3.1)	E4 Carriers; RR 0.33 (95%CI 0.16–0.7) Without E4; RR 0.66 (95% CI 0.35–1.24)	NR
REGRESS (pravastatin) (120)	496	2	Stromelysin-1(−1612 5A/6A)	Clinical events	5A5A = 12% 5A6A = 26% 6A6A = 14%	5A5A = 17% 6A6A = 9%	0.038
REGRESS (pravastatin) (122)	807	2	CETP (B1 or B2)	Average decrease in mean coronary diameter (mm)	B1B1 = 0.14 B1B2 = 0.10 B2B2 = 0.05	B1B1 = 0.05 B1B2 = 0.07 B2B2 = 0.09	0.01
REGRESS (pravastatin) (119)	682	2	p-Fibrinogen (−455 G/A)	Average decrease in mean segment diameter (mm)	GG = 0.09 GA = 0.10 AA = 0.24	GG = 0.07 GA = 0.05 AA = −0.06	0.024
CARE (pravastatin) (118)	767	5	GP IIIa (Pl^{A1},Pl^{A2})	CV death, non-fatal MI	Pl^{A1A2}; RR 1.32 (95% CI 0.99–1.76)	Pl^{A1A2}; RR 0.69 (P = 0.06)	NR

FU, follow-up; P, interaction between placebo and statin therapy; NR, not reported; RR, relative risk; CI, confidence interval; 4S, Scandinavian Simvastatin Survival Study; REGRESS, Regression Growth Evaluation Statin Study; CARE, Cholesterol and Recurrent Events; CETP, Cholesteryl ester transfer protein; GP, glycoprotein; CV, cardiovascular; MI, myocardial infarction.

emia (125). Overall, pravastatin therapy for 5 years reduced the risk of fatal coronary heart disease and non-fatal myocardial infarction. A substudy of CARE focused on whether polymorphism in the glycoprotein (GP) IIIa gene and the ACE I/D polymorphism were associated with the reduction in the primary endpoint of the study. The investigators found that the largest benefit of pravastatin treatment in reducing the primary endpoint occurred in patients with the GPIIIa $PI^{A1/A2}$ genotype who also carried at least one D allele of the ACE gene (118).

Finally, a pharmacogenomic study of the Scandinavian Simvastatin Survival Study (4S) investigated the impact of the apolipoprotein E4 allele with the prognosis and treatment response to either simvastatin or placebo (121). Among myocardial infarction survivors who received placebo and had at least one apolipoprotein E4 allele, the relative risk for all cause mortality was 1.9 (95% CI, 1.1–3.1). The detrimental impact of the E4 allele was not evident among patients who received simvastatin (RR 0.33; 95% CI, 0.16–0.69).

Taken together, the results of these five studies identify genetic subgroups of placebo-treated patients who have an increased risk of developing major coronary events. In all studies, treatment with a statin abolished the harmful effect associated with the genetic variant. These results support the observation that, in general, high-risk patients with ischemic heart disease derive the largest relative benefit from treatment. However, whether current recommendations regarding cholesterol-lowering therapy are applicable to patients in certain genetic subgroups is uncertain. At this time it is premature to withhold statin therapy from any patient meeting criteria for treatment based on national consensus guidelines. Future prospective genetic epidemiology studies may further delineate the role of genetic variants on the development and progression of coronary artery disease and response to treatment.

4.5 Clinical Relevance

Historically, pharmacogenetics targeted its pursuit on the reasons for toxicity and therefore drug safety. However, it is pharmacogenomics that promises to deliver individualized pharmacotherapy with greater efficacy (5) while still limiting toxicity. An example of the potential for improved efficacy is given in the example of clozapine. Clozapine is an atypical antipsychotic with superior efficacy in patients with treatment resistant schizophrenia. Despite being an effective anti-psychotic, clozapine therapy is limited by the serious adverse effects of tachycardia, orthostatic hypotension, and agranulocytosis. In fact, agranulocytosis is such a severe and worrisome adverse event that it has necessitated a national registry to track and monitor patients receiving clozapine therapy. In addition, large inter-individual response exists, with only 30–60% of patients responding to clozapine. A retrospective study examined 19 genetic polymorphism in 10 different genes with response to clozapine in 200 Caucasian schizophrenic patients (126). The results revealed that a combination of six polymorphisms resulted in a 76.7% prediction of treatment success with clozapine. The combination of polymorphisms had a sensitivity of 95% for identifying patients with a beneficial response to clozapine. It remains to be determined whether this combination of polymorphisms retains its high predictive value in a population of diverse ethnic backgrounds. However, this study should serve as a model for future prospective studies to incorporate a more genomic and/or haplotypic approach when attempting to correlate genetic variability with drug response.

5 RESEARCH AND DEVELOPMENT

The numbers are staggering. It costs the pharmaceutical industry approximately $880 million and 15 years to go from target identification through regulatory approval for a novel drug. One-half of this cost and time occur during phase II–III clinical trials. Contributing to the prodigious cost and time are the many inefficiencies of drug discovery, development, and clinical trials. Seventy-five percent of the costs of drug development are incurred from late-stage clinical trials. Incorporation of pharmacogenomic data may lead to a dramatic change in the way clinical trials are designed and conducted. Pharmacogenomics may re-

Table 12.9 Recent Drugs Removed/Restricted by the FDA

Alosteron
Astemizole
Bromfenac
Cerivastatin
Cisapride
Felbamate
Grapafloxacin
Mibefradil
Rapacuronium
Dexfenfluramine
Terfenadine
Troglitazone
Trovafloxacin

sult in more efficient trials that would be associated with a lower cost to bring a new chemical entity to market. Moreover, this could result in shorter time to drug approval, longer patent protection, and more importantly, increased time of market exclusivity. A recent report concluded that incorporation of genomic technologies currently available could result in savings of up to $300 million per novel drug and cut 2 years form the drug development process (www.bcg.com). This section will focus on specific examples of how pharmacogenomic data can be incorporated into clinical trials to identify responders and exclude nonresponders to reduce unnecessary adverse events.

5.1 Influence of Pharmacogenomics on Clinical Trials

In recent years, the complexity and cost of clinical trials has increased. It is not uncommon for phase III drug studies to involve thousands of patients with several years of follow-up. However, the results of these trials only provide information on the average treatment effect in a population, not for an individual patient. In addition, numerous drugs advance all the way to phase III trials only then failing to demonstrate any treatment benefit or having an unacceptable adverse event profile that prevents regulatory approval. Recent years have also seen several promising drugs approved by the FDA and then withdrawn from commerce or restricted secondary to serious adverse events (Table 12.9).

5.2 Use of Pharmacogenomic Data in the Clinical Trial Process

Assume that a company has a novel drug to treat chronic heart failure in phase II trials. Data from this study demonstrates that 35% of the study population receiving active drug achieves a therapeutic response compared with 15% of placebo-treated patients. Do these data warrant further study? If this hypothetical phase II trial had incorporated pharmacogenomic data, the potential exists that a genetic subgroup of responders could have been identified. Suppose that 60% of patients with the hypothetical AA genotype respond to this drug while patients with genotypes Aa and aa have a response rate similar to placebo. A homogeneous patient population (AA genotype) likely to respond to the medication could now be selected for a confirmatory phase III trial. The sample size needed for a phase III trial would be reduced because patients likely to respond to the medication have been enrolled. In fact, the majority of clinical trials that use pharmacogenomic information will require smaller sample sizes compared with trials in which no genotypic information is collected (127).

Alternatively, assume a company has developed an investigational drug to treat type 2 diabetes mellitus. Phase II studies demonstrate that 75% patients reduce their glycosylated hemoglobin (HbA_{1c}) levels by 1.5%. However, the drug is associated with a serious adverse event in 5% of patients. What impact will this serious adverse drug event have on the approval and use of this agent? If DNA samples had been collected from all study participants, a population of patients predisposed to developing this toxicity may have been identified. The susceptible patient population could then be excluded from phase III trials, and assuming that the agent is eventually approved, a bedside molecular diagnostic test could be used to prospectively identify patients predisposed to the serious adverse event. In this aforementioned example, everyone benefits, including the drug manufacturer, patients, and the health care system by avoiding the costs of a serious adverse drug event. Furthermore, pharmacogenomic data could also be used in post-marketing surveil-

lance, which would dramatically improve our current surveillance system (128).

Four pieces of information are important before planning a clinical trial using pharmacogenomic data (127). First, the allele frequency for the polymorphism of interest must be known. Most trials with a nominal outcome variable would require sample sizes of greater than 1000 patients for allele frequencies of less than 10%. For these rare SNPs, association with efficacy and or toxicity might only be discerned after the drug has been approved. However, studies of polymorphisms with an allele frequency of 30–50% would require fewer subjects compared with a trial that does not use pharmacogenomic information (127). Second, the gene action of the SNP must be understood. Does the SNP behave in a dominant, additive, or recessive fashion? A dominant action means that the genotype Aa displays the same phenotype as genotype AA. This example would require fewer study participants compared with alleles displaying additive or recessive action. Third, the investigator should have knowledge of genotype relative risk (GRR). This is potentially the most difficult of the factors to have *a priori* knowledge, and may require assumptions, as are typically done to estimate the sample size of most clinical trials. As with any trial, the smaller the genotype relative risk, the larger the number of subjects required. The GRR is likely to be small given the multitude of factors influencing drug response. Fourth, as the number of alleles tested increases, the sample size also must increase. For example, studying 10 loci would require a sample size 1.5 times larger than studying a single locus. Studies of 100 loci would require sample sizes twice as large compared with a single locus. Studying 100,000 loci necessitates a sample size three times larger compared with a single locus (127).

5.3 Examples from the Literature

A recent study prospectively genotyped all patients for the CYP2D6 gene and excluded poor metabolizers to enhance patient safety (129). The study was a randomized, double-blinded comparison of lamotrigine with desipramine in patients with unipolar depression. Desipramine is a substrate for CYP2D6 and poor metabolizers of this enzyme have serum desipramine concentrations markedly higher than extensive metabolizers (31). In all, 6.1% of subjects screened were excluded from the trial because they were identified as poor metabolizers. Clearly, using genotyping as an inclusion or exclusion criterion requires that the genotype(s) of interest can be determined in a rapid fashion. For trials that involve acute treatment, a point of care or "bedside" test would need to be developed to potentially exclude patients at an increased risk of adverse events.

Currently, 18 of the top 20 pharmaceutical companies are collecting DNA data in phase II and III trials. Among them is GlaxoSmithKline, which has added this component to studies in all major therapeutic areas of their drug development process (130). A survey from the SNP consortium estimates that within 5 years, 50% of clinical trials will involve genotyping. Unfortunately, in many cases, DNA collection is an optional part of the protocol. Reasons for this include lack of knowledge from the investigators or the perception that collection of genomic DNA from patients will lead to delays in Investigational Review Board (IRB) approval or patient recruitment.

It is understandable why the pharmaceutical industry may have some trepidation of using pharmacogenomics in the clinical trial process. The basic tenet of most of the pharmaceutical industry has been to develop drugs in a one-size-fits-all approach with the hope that they will become blockbusters, generating greater than $1 billion in annual sales. There has been some concern that pharmacogenomic data will reduce the market share of a drug by identifying the subgroups of patients who actually derive therapeutic benefit from a drug. Paradoxically though, integration of pharmacogenomics could actually increase market share for a drug. The goal for any new product is to capture 100% of the target market; however, for a drug, the target market is not composed of all patients with the particular disease that the drug is indicated to treat. Consider the following example. A pharmaceutical company develops a new drug to treat hypertension. What will be the market penetration for this new antihypertensive agent in the first year after regulatory approval? Hy-

pertension affects approximately 50 million Americans, but several classes of medications such as β-blockers, angiotensin-converting enzyme inhibitors, and angiotensin receptor blockers exist, with numerous drugs in each class. Consequently, it is difficult for any new drug in each of these classes to acquire a large market share. However, what if a new agent in one of these classes was approved with a molecular diagnostic to predict efficacy. A third or fourth in class drug approved with a diagnostic assay could provide a competitive niche demonstrating a high response rate in selected groups and a low adverse event rate in other groups. It is likely in this scenario that the market penetration as well as market share achievement will be greater if this drug is coupled with a molecular diagnostic test. Thus pharmacogenomic data could be an immensely useful marketing strategy for a drug company.

Several major unanswered questions exist as to how the FDA will react to new drug applications that contain pharmacogenomic data. Clearly, drugs approved for genetic subgroups will require molecular diagnostic testing and thus raise several compelling questions (131). First, how will this affect off-label prescribing? What impact will this have on liability? Second, what happens if a patient refuses to have a molecular diagnostic test performed? Are they now limiting themselves to new therapeutic agents? What happens to the low socioeconomic populations who have no insurance and can not afford the cost of the genetic test? Is this population now excluded from receiving potentially safe and effective pharmacotherapy? How will pharmacogenomics influence Orphan Drug status? In the United States, drugs developed for conditions affecting less than 200,000 individuals get 7 years of market exclusivity, unless an alternative medication is proven superior. Will this be the financial incentive that some companies require? Compelling questions indeed.

6 OBSTACLES FACING THE FIELD OF PHARMACOGENOMICS

6.1 Complexity and Cost

Current methods to sequence DNA are too costly and laborious to allow implementation into routine clinical practice. The cost to genotype one SNP can range anywhere from $1 to $50, and this prohibits phase II-III trials from examining several hundred putative SNPs per patient that influence drug toxicity or therapeutic benefit. An alternative approach would be to examine approximately 10–15 SNPs in candidate genes that are involved in the drug targets, drug transport, and metabolism. However, the candidate gene approach may not be plausible during initial drug discovery and development. Advances in high-throughput technology and increased competition should eventually reduce the cost of genotyping SNPs to $0.001/SNP, thus making it viable to evaluate 100,000–200,000 SNPs per patient. Another factor contributing to cost involves the initial investment in the equipment and technology required to fully integrate pharmacogenomics into all aspects of drug discovery and development. However, these upfront costs may result in significant cost savings by increasing efficiency of clinical trials with the identification and termination of drugs with little potential of eventual regulatory approval. The deluge of the data generated from pharmacogenomic studies also requires advances in bioinformatics and data mining strategies. The extent to which pharmacogenomics pervades clinical practice is largely dependent on the ability of bioinformatics to transform the prodigious amounts of data into knowledge. Consequently, the bioinformatics budgets of some pharmaceutical companies have increased 20–60%. A sure indication of the desperate need for this technology.

6.2 Will Pharmacogenomics Improve Medical Care?

It is still unclear if prospectively genotyping patients for many of the genetic variants described within this chapter improves medial care and whether it is cost effective. Genotyping patients for the presence of TPMT deficiency to prevent life-threatening hematological toxicity from azathioprine, mercaptopurine, or thioguanine provides an equivocal advantage compared with empirical dosing. However, in other examples, genotyping may not be an advantage over the current best medical care. For example, several studies

have demonstrated that individuals carrying mutant alleles for CYP2C9 have decreased metabolism or elimination of warfarin (25, 132–134). One retrospective trial also showed that these individuals have a higher risk for both minor and major bleeding (25). However, it remains to be elucidated whether prospectively genotyping patients receiving warfarin reduces bleeding complications when compared with the best available standard of care. This question can only be answered through a randomized controlled trial comparing a genomic approach with a traditional dosing approach.

Recently, the Statin Response Examined by Genetic HAP Markers Study (STRENGTH) completed enrollment of 600 patients with hyperlipidemia. Patients were randomized to one of four statin treatment regimens (cerivastatin, pravastatin, atorvastatin, or simvastatin) for a 16-week duration. The goal is to identify genetic markers that predict which of the four statins is most beneficial in reducing cholesterol levels. However, whether this approach is superior to management of patients in a specialized lipid clinic remains to be determined.

6.3 Paradigm Shift in Health Care

Transitioning from the one-size-fits-all approach to personalized medicine will create a paradigm shift for both health care providers and the pharmaceutical industry. There is some precedence for drugs specifically targeted for a subset of disease. Trastuzumab (Herceptin) is approved only for the 25–30% women whose breast cancer overexpresses the HER-2/neu protein. Trastuzumab is marketed along with a molecular diagnostic, the DAKO Hercep Test. This test is a semi-quantitative assay for testing breast tumor tissue that overexpresses the HER-2/neu protein. In its first year on the market, trastuzumab generated $188.4 million in sales. Thus, an agent that probably would not have received FDA approval is now an effective alternative for a specific subgroup of women with breast cancer.

Physicians will also need to adjust to the shift from the art of medicine to the science of medicine. Rather than selecting a drug based on experience, the selection may be based on the analysis from a computer program. Rather than making a diagnosis based on phenotypic symptoms, a disease may be diagnosed years before it manifests, based on an individual's genetic makeup.

6.4 Ethical Considerations

Pharmacogenomics has created a new lexicon that all health care providers must familiarize themselves, and thus precise language is fundamental when dealing with pharmacogenomics. It is imperative that pharmacogenomics be distinguished from genetic predisposition testing. All investigators in the field must convey this concept to the public, members of the health care team, and the insurance sector. In the majority of cases, identification of SNPs to predict drug response carries no prognostic information for diseases. Determination of SNPs to predict drug response is analogous to obtaining culture and sensitivity data to guide antimicrobial therapy. However, in other cases the potential for discrimination exists. One example involves the apolipoprotein E4 allele. Genetic variation in the apolipoprotein E4 allele may be examined to explain the variability among statin therapy (121). However, these results could have significant implications in predicting the risk of Alzheimer's disease later in life (115, 116). Clearly, an enormous potential for discrimination exists if an insurance carrier discovers the results of this diagnostic test. As an example, the New Jersey Genetic Privacy Act, passed in 1996, prevents employers and insurance carriers from discriminating based on genetic tests. Indeed, further state and federal legislation like New Jersey's would help to allay some of these ethical concerns and public fear of genetic testing.

Undeniably, genomics and pharmacogenomics opens up a whole host of legal, ethical, and societal issues that will have implications in patient confidentiality, discrimination, malpractice, and informed consent (135–137). However, as clinicians, scientists, and health care practitioners, we must always remember the unquestionable power of an individual's right to choose, and prospectively fight to ensure patients' rights and prevent genomic discrimination.

7 CONCLUSION

The publication of the draft of the Human Genome Project represents an acmatic scientific breakthrough. One of the first discernible benefits from the Human Genome Project will be advances in pharmacogenomics. Pharmacogenomics is likely to have a major role in the daily practice of medicine in the near future. In many ways, a proof of principle is required. Although trastuzumab is an effective agent, its use is based on differences in protein expression and not genetic variation. It is our belief that there will be a surge in the field after the first drug developed through functional genomics becomes FDA approved, or when the first assay to predict efficacy or toxicity of an existing drug is approved. Whatever our future holds, we must always remember the ultimate goal of pharmacogenomics: to develop or use truly individualized pharmacotherapy that will produce the most benefit and the least harm thus extending and enhancing human life.

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CHAPTER THIRTEEN

Plasmid DNA–Mediated Gene Therapy

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Contents

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1 INTRODUCTION

In the post-genome era, many defective genes responsible for various disease phenotypes have already been or will soon be discovered. Introduction of the normal genes effectively into the target cells and/or tissues to complement the defective genes is a daunting challenge to scientists of various disciplines. Gene therapy methodology was originally designed to correct inheritable disorders but is now being considered for treating acquired disorders, such as cancer and AIDS.

Gene therapy is broadly divided into two different categories, one that uses viruses and the one that doesn't. Replication-defective viruses with a part or the entirety of their coding sequences replaced by a therapeutic gene are used to transduce cells with very high efficiency. Retrovirus, adenovirus, adeno-associated virus, herpes simplex virus, papilloma virus, sendai virus, etc., have been used for various gene therapy approaches either clinically or experimentally. Although viral vectors have shown efficacy in transducing cells and tissues, they have several drawbacks that limit their widespread use. For example, adenoviral vector induces strong host immune responses, which induce toxicity and limit the duration of the transgene expression. Immunogenicity makes repeated dose impossible. Retroviral vector requires dividing cells for gene expression, and it may integrate randomly into the host genome, posing risk for mutation and neoplastic transformation. Viral vectors are also'difficult to produce in large scale. These concerns have prompted the development of non-viral vectors as a less toxic and more scalable alternative for gene therapy. However, the efficiency of non-viral vectors still needs improvement; so does the duration of the transgene expression. These aspects are the central concerns of the current non-viral vector development.

The carrier for the therapeutic gene in non-viral vectors is often a recombinant plasmid DNA (pDNA). Recombinant DNA is a synthetic DNA made by connecting different DNA fragments from different sources into one recombinant molecule, whereas plasmid DNA is a non-chromosomal, circular, and supercoiled bacterial DNA, which is produced in bacterial cultures by fermentation. A therapeutic or a marker gene can be introduced into pDNA by simple recombinant procedure. pDNA can be purified by simple physical and chemical methods. Advanced technologies exist such that multigram quantities of pDNA can be routinely produced from a single lot. Larger scale production can also be done on demand. Standard pDNA is 4–10 kilobase pairs in size (MW 1×10^6 to 3×10^6). Other than the bacterial sequences, pDNA for gene therapy contains a transgene, which is usually the expressible sequence of a genomic DNA, popularly known as complementary DNA (cDNA) that encodes a protein. Transcription of the transgene to make messenger RNA is controlled by another gene sequence called promoter, which locates upstream of the coding sequence of transgene. To ensure proper termination of transcription, a transcription termination gene sequence is often inserted downstream of the transgene. A polyadenylation sequence and sometimes an intron, i.e., a non-expressible gene sequence, is also added to ensure proper processing of the mRNA product of the transgene (Fig. 13.1). In this chapter we will emphasize the usefulness of a transgene in the form of plasmid DNA associated with non-viral vectors to decipher various biological functions and for development of gene therapy.

2 DIRECT DELIVERY WITH NAKED PLASMID DNA

2.1 History and Recent Therapeutic Uses

The simplest of all DNA delivery systems is the injection of naked pDNA to the organ or tissue of interest. Intramuscular injection of

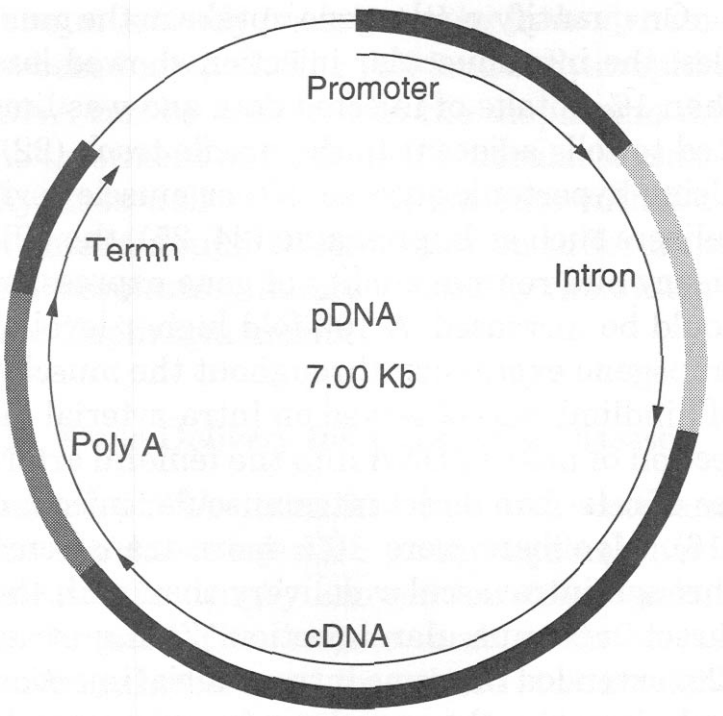

Figure 13.1. Schematic representation of a typical plasmid DNA. Promoter, promoter sequence; intron, intron sequence; cDNA, complementary DNA encoding a gene; poly A, poly-adenylation sequence; termn, termination sequence.

naked pDNA was the first instance where the skeletal muscle was transfected (1). Since then, a variety of tissues had been transfected with direct gene transfer. Naked pDNA had been injected into the interstitial space of liver (2–4), thyroid (5), heart muscles (6), brain (7), and urological organs (8). The naked DNA containing one or more anticancer genes were also injected in various tumors with mixed efficacy (9–11).

This technique of direct injection of pDNA into an intact organ had become a useful tool to analyze the gene expression and promoter function in the respective organ. By the use of a viral promoter, such as that from cytomegalovirus (CMV), a high level of expression was attained initially with pDNA vectors. However, the expression declined sharply to near background within 2–3 weeks. Herweijer et al. (3) have shown that, although the maximum decrease in the expression level occurred within 2 days from pDNA transfer, pDNA could be detected for at least 12 weeks after injection (0.2 copies per genome). Promoter activation associated with the immune responses raised against the expressed transgene product was believed to be the major cause of this early decline in gene-expression level. This fact is corroborated in immunosuppressed mice that showed extended level of transgene expressions.

To sustain therapeutic response initiated by the transfection of pDNA containing therapeutic gene, it is necessary to ensure a long-lasting gene expression eliciting little to no expression-related cytotoxicity. Towards this end, few gene transfer techniques can sustain transgene expression over an period of time. Mouse liver transferred with high-expressing human factor IX (hFIX) pDNA yielded therapeutic-level gene expression over 1.5 years, eliciting no expression-related or therapy-related toxicities (4). A declined transgene expression observed after liver regeneration further suggested that the maintenance of the plasmid had declined, rather than the transgene being integrated into the host genome. Hence, sustained gene expressions required a transcriptionally active vector DNA that could persist for long time in the organ. These results demonstrated that non-viral plasmid transfer could lead to extended life of transgene-expression level without genome integration.

Non-viral gene therapy had become a useful tool in generating various therapeutic effects in diverse animal models. One of these effects is to induce anticancer response in tumor models by introducing anticancer genes. In an effort to elicit anticancer effect in tumorogenic cells implanted in medullary thyroid cancer (MTC) rat-xenografts, NO synthase II (NOS II) gene, which catalyzes nitric oxide (NO) production, was incorporated in a plasmid under the control of CMV promoter and injected in the rat. The gene triggered a suicidal effect (apoptosis) on the tumor cells by the production of NO, which specifically activated the macrophages against the fast dividing tumor cells, leaving normal cells untouched (9). Most importantly, the activated NOS gene in a tumor cell induced the cytotoxicity in the neighboring tumor cells, i.e., an NO-mediated bystander antitumor effect was also observed (10). The fact that nitric oxide mediated tumorogenic effect doesn't require transfection of all neoplastic cells promised a capable suicide gene therapy approach for human cancer.

Similar bystander effect could be obtained by other apoptosis-inducing molecules such as Fas ligand (FasL). FasL and its receptor Fas are membrane proteins, which on mutual interaction initiate an apoptotic signal in Fas-bearing cells. pDNA could be used to deliver these apoptosis-inducing gene to initiate killing of transfected and non-transfected surrounding cells. On direct injection of FasL-encoding pDNA vector into the inflamed thyroid (12), pathogenic lymphocytes were inhibited to enter into thyroid leaving the already-infiltrated T-cells dead. Thus, FasL expressing in thyroicytes might lead to potential remedial therapy for the experimental autoimmune thyroiditis (EAT).

Direct pDNA transfer technique had been used to examine the role of a physiologically related protein transduction signal pathway toward certain endogenous disease phenotype, such as investigating the function of the tissue kallikrein-kinin system (KKS) in the central control of blood pressure homeostasis (13). Kallikrein is a proteinase enzyme, which converts kininogen to vasodilative kinin peptides. The human tissue kallikrein gene, in the form of naked pDNA (CMV-cHK), was directly delivered by intracerebroventricular injection into hypertensive rats. The expression of human tissue kallikrein protein was identified in the cortex, cerebellum, brain stem, hippocampus, and hypothalamus of the treated rats. The expression level and its effect could lead to understanding the role of vasodilative KKS on the pathogenesis of hypertension.

Direct gene transfer to skeletal muscle was mainly used for the treatment of variety of diseases, e.g., muscular dystrophies, chronic ischemic limb syndromes, etc. Specifically engineered pDNA and the vector systems were developed that enabled regulated and tissue-specific transgene expression in skeletal muscle in *vivo* (14). Naked pDNA based gene transfer (1,15) has been used in different animal models, e.g., in correcting Duchenne Muscular Dystrophy (16), to supply sources of therapeutic protein systemically (17, 18), for genetic-vaccination against pathogens (19) and tumor cells (20, 21). However, clinical use of this technique is limited because of inefficient gene expression specifically in large animals.

On quantifying the gene uptake in the muscles, the intramuscular injection showed less than 1% uptake of injected dose and was limited to cells adjacent to the needle track (22). Using hypertonic sucrose (23) or muscle revitalizers such as bupivacaine (24, 25), the efficiency and reproducibility of gene expression could be increased. A 100-fold higher level of transgene expression throughout the muscles of hindlimb was observed on intra-arterial injection of naked pDNA into the femoral arteries of rats than direct intramuscular injection (16). Myofibers were 10% more transfected through intravascular delivery than with the direct intramuscular injection. Zhang et al. (26) extended the same intra-arterial injection technique to the non-human primates. It could be hypothesized that pDNA was extravasated by the intravascular pressure during the injection, most likely by convective flow across the endothelium (27, 28). Once the pDNA is extravascular, the muscle cells in *situ* with the help of a receptor mediated uptake process pick up the naked pDNA. This was evidenced by the fact that the naked pDNA was taken up by hepatocytes in *vivo* by a receptor-mediated process (28). Several DNA receptors had been discovered in human leukocytes (29), peritoneal macrophages (30), and in wide variety of tissues and tumor cells (31–35). At this point, the poorly understood mechanism of molecular recognition and characterization of the cell surface receptor(s) involved in the binding and internalization of DNA need a fresh look. Liu et al. (36) and Zhang et al. (37) reported that rapid injection of pDNA in a large volume (e.g., 5 μg of DNA/20 g mouse in 1.5–2.0 mL of saline solution) through the tail vein left the injected DNA in the inferior vena cava. The DNA flowed back to the tissues linked to this vascular system, primarily the liver. The hydrodynamic pressure forced DNA into the liver cells before it was mixed with blood. By this process, the liver showed the highest expression of gene; internal organs like lung, spleen, heart, and kidney were also efficiently transfected. Our lab had recently shown that briefly clamping the vena cava following tail vein injection of pDNA in a small volume efficiently transfected both liver (38) and diaphragm (39). The result is potentially important because dia-

phragm is barely transfected by hydrodynamics-based method. The full-length dystrophin cDNA can be delivered to the diaphragm for the treatment of Duchenne Muscular Dystrophy. It is well known that patients with Duchenne Muscular Dystrophy often suffer from fatal respiratory failure caused by the dystrophic diaphragm muscle.

2.2 Gene Delivery for Myocardial Diseases

Naked pDNA transfer into myocardium through direct injection or through coronary vasculature usually showed low transfection efficiencies. The results had nevertheless proven valuable in studies aimed at characterizing the role of promoters in cardiac tissue and for examining the influences of naturally occurring mechanical and hormonal stimuli of the myocardium on expression of transferred foreign genes (40).

Kitsis et al. (41) have demonstrated that the tissue-specific promoter chimeras injected into the heart could respond accurately to shift in thyroid hormone levels *in vivo*. Injection of pDNA with gene constructs driven by cellular promoters resulted in detectable levels of reporter gene activities. The cellular promoter was derived from the rat a-myosin heavy.chain (α-MHC) gene whose expression *in vivo* is restricted to cardiac muscle and is positively regulated by thyroid hormone. This method proved valuable to identify the regulatory portion of genes expressing specifically in cardiac muscles. Direct DNA injection had been extended to evaluate and characterize the activation properties of a cardiac-specific promoter/enhancer of the slow/cardiac troponin C (cTnC) gene that express in cardiac striated muscles (42). Myocardial direct DNA injection was also used to analyze the transcriptional regulation of brain creatine kinase (BCK) gene in the heart (43). pDNA constructs containing BCK promoter and CAT or luciferase reporter gene was delivered into the left lateral wall and apex of the ventricle on the heart. The study might provide insight into the embryonic gene expressing mechanism during cardiogenesis. Because the BCK gene, the major gene for cytoplasmic creatine kinase expressed in the embryonic heart, is down-regulated during cardiogenesis, it is reinstated in response to stimuli such as ischemia, hypertrophy, or heart failure in the adult.

The method of direct pDNA injection was used to explore the effect of specific pathophysiological state on cardiac gene expression, such as ischemia (44), myocardial infarction, reperfusion injury, hypertension (45, 46), etc. Ischemia is a disease state formed when tissues are starved for blood supply and nutrients because of deficient supply of blood through possibly narrowed or blocked arteries. Sporadic myocardial ischemia is commonly associated with coronary arterial diseases. To eliminate the ischemia related disease phenotype, a therapeutic gene is required, which could be selectively up-regulated by the signals related to the heightened period of ischemic activity and consequently down regulated when the activity subdues. In this context, Prentice et al. (44) introduced expression plasmids containing muscle-specific a-MHC promoters and hypoxia-responsive enhancer (HRE) elements linked to a reporter gene in cultured cells or into the rabbit myocardium and measured the regulation of these constructs by hypoxia or experimental ischemia. It was shown that the expression of reporter gene was induced by both hypoxia *in vitro* and by a short interval of ischemia *in vivo*.

There were different reports concerning the stability of plasmid-based transgenes in both skeletal and cardiac muscles. Lin et al. (47) have shown that the rat cardiac myocytes could express β-galactosidase gene under the control of the Rous sarcoma virus promoter by the injection of pDNA encoding the reporter gene directly into the left ventricular wall. β-galactosidase expressed in cardiac myocytes was detected in rat hearts for at least 4 weeks after injection of the β-galactosidase gene. In post-mitotic cardiac and skeletal muscle cells, the transgene expression of the pDNA declines with time probably because of the episomal localization of the DNA (16, 47). The reason that the striated muscles showed higher capacity of uptake and expressing pDNA following direct injection was not clear; the efficient gene transfer might be induced by cellu-

lar membrane rupturing and destabilization followed by inflammation caused by the injection needle (48).

2.3 Gene Therapy for Angiogenesis

Because ischemia is formed because of a deficient supply of blood to tissues, formation of new blood vessels is curative against this disease. One of the mechanisms that involve the formation of new blood vessels is angiogenesis. Angiogenesis is the process of new blood vessel development for the vascularization of various organs, for wound healing (49) and to allow cancer development and proliferation (50).

Therapeutic angiogenesis involves restocking angiogenic growth factors by administering recombinant proteins or endothelial growth factor gene. The recombinant proteins have severe limitation on its usage because it is expensive and difficult for large-scale production. On the other hand, gene therapy provides a systemic and long-term effect with modification in the effective dosage of the therapeutic agent. To evade potential problem of pathological angiogenesis, transient gene expression is usually preferred for this kind of treatment. Tsurumi et al. (51) introduced naked pDNA encoding vascular endothelial growth factor (VEGF) by intramuscular (IM) injection into ischemic hind limb muscles of a rabbit model and observed that the vessels and blood-capillaries were increased in rabbit muscles injected with VEGF compared with controls. An enhanced vascularity-induced perfusion followed by increased blood flow in the ischemic limbs was also observed. In clinical trial, Simovic et al. (52) introduced naked pDNA encoding human VEGF gene by direct intramuscular injection to chronic ischemic limbs of patients to treat peripheral neuropathy caused by critical limb ischemia. The patients showed decreased neuropathic disability in the treated limbs, indicating that a long-term therapy might improve the integrity in tissues of ischemic limb and consequent retrieval of limb.

2.4 Gene Therapy for Autoimmune Diseases

Autoimmune disease is a pathogenic condition in which one's immune system mistakenly targets and attacks person's own cells, tissues, and organs. Inflammation is a prevalent symptom for this disease caused by the excessive presence of large number of immune cells and molecules in the target site of the body. Against autoimmune diseases or other inflammatory conditions, the delivery of cytokines or cytokine inhibitors through gene therapy is proved very effective. Interferon γ (IFN-γ), interleukin-1 (IL-1, a or β), IL-12, and tumor necrosis factor α (TNFα) are the most frequently addressed inflammatory cytokines in illness related to autoimmune/inflammatory diseases. Other than these cytokines, transforming growth factor β (TGF β) is also a key regulatory cytokine (53), because TGF β inhibits T- and B-cell responses, dysregulation of which lead to elevation of autoimmune disease conditions.

In animal models, pDNA constructs with the encoding anti-inflammatory cytokine genes for IL-10 (54), IL-4 (55), and TGF β1 (56) were injected into either tibialis anterior or rectus femoris muscles in nonobese diabetic (NOD) mouse against autoimmune diabetic disease. Although there was no marked decrease in severity of insulitis, the diabetes was reduced in NOD mice injected with IL10 compared with nontreated NOD mice. In another experiment, treatment of autoimmunity prone NOD mice with pCMV-TGF-β1 (57) resulted in considerable elevation of TGF-p 1 level in the plasma. The increased levels of TGF-β1 exerted various immunosuppressive effects such as there was suppression of delayed-type hypersensitivity (DTH) and prevention of insulitic and diabetic incidence in this kind of mice. TGF-β1, IL-4, and IFN-γ gene coding plasmid vectors were also injected IM to rodent models for treatingexperimental allergic encephalomyelitis (EAE) (57), systemic lupus erythematosus (SLE) (58), colitis (59), and streptococcal cell wall-induced arthritis (SCW-arthritis) (60).

3 IMPROVING PLASMID DNA-MEDIATED GENE TRANSFER BY ELECTROPORATION

Electroporation is a process of exposing cells to a controlled electric field for the purpose of cellular membrane permeabilization (61). The intense localized electric pulses destabilize the

membrane allowing molecules, which otherwise do not gain access inside the cell, to enter cells. A variety of genetic materials were inserted into the cells in vitro by electroporation (62–64). Electrochemotherapy, a cell electropermeabilization approach that facilitates the cellular entry of hydrophilic anticancer agents such as bleomycin, was used to obtain drastically improved antitumor effect (65) in malignant melanoma.

Recently, in vivo electroporation has emerged as a leading technology for developing non-viral gene therapies and nucleic acid vaccines (NAV). Naked pDNA injections accompanied by electroporation showed 10- to 1000-fold increases in gene expression compared with the same injections without electroporation (66–68), and the duration of a gene expression after electroporation in vivo was dependent on target tissue and plasmid constructs (66). A broader variety of cells showed reporter gene expression (66–69).

One of the advantages of electroporation is that specially designed electrode directed against a given tissue could provide a specific gene expression for the treated tissue. A high level of controlled gene expression was obtained by using a tissue-specific promoter directed against a given tissue with different pulsing parameters (66).

The electroporation-mediated in *vivo* gene transfer had been successfully used for hepatic parenchyma (70, 71), hepatocellular carcinoma (72), skin (73, 74), skeletal muscle (69, 75,761, mouse testes (77), melanoma (65), human primary myoblast (78), glomeruli (79), brain (80), human primary hematopoietic stem cells (81), human esophageal tumor (82), and rat skeletal muscle for correcting anemia of renal failure (83).

Electroporation requires a high electric field strength, which eventually restricts its use because it induces some tissue damage. Efficient and safe electro-transfer for delivering exogenous material into tissues must be developed before the clinical potential of gene therapy can be realized. Our laboratory had recently developed a novel design of a syringe electrode, with which electric field was directly delivered to the cells where pDNA had been iniected. and the iniected DNA could be confined to the high field region. High transfection efficiency was observed with comparatively lower field strength, which incurred minimal tissue damage (Liu and Huang, unpublished observations).

A technique called microelectroporation was used locally to introduce a transgene into chick embryo and express the gene in spatial and temporal manner (84, 85). By the microelectroporation technique, DNA molecules were efficiently introduced into the optic vesicle (84), sensory placodes, surface ectoderm (86), neuroepithelium of the CNS, and into the somites and limb mesenchyme (87).

4 IMPROVING PLASMID DNA TRANSFER MEDIATED BY GENE GUN

Gene gun is a physical way of administering genes in vitro or in vivo. pDNA makes an electrostatic complex with gold or tungsten microparticles. DNA-coated metal particles are placed in the interior of Teflon-coated tubing, and the DNA is readily expelled by a flow of regulated, highly compressed helium gas. The delivery is highly localized to the tissue part. For regulating the speed and hence impact-pressure, the projectiles can be targeted to different tissue depths and areas. The process involves easy and speedy preparation of the delivery vehicle while keeping DNA intact. This technique sometimes allows DNA to gain direct access to nucleus bypassing endosome/lysosome wherein they would have possibly enzymatically degraded. Because of the benefit of accessibility, this technique is especially suitable for gene transfer to skin (88) and for superficial wounds (49, 89). There are several examples in various animal models that have shown a high level of transgene expression in the epidermis and dermis of the skin (90, 91).

By the gene gun technique, mouse skin was transfected with IL-6 and hemagglutinin-encoded DNA to elicit protective immune responses against equine influenza virus (92). Several different large animals such as rhesus monkeys (93), pigs (94), and horses (95) were also immunized against virus by transfecting gene encoding the viral antigen.

Other than skin, detectable transgene activities were also noticed in various organs including liver (96, 97), lungs (98), pancreas

(99), kidney (100), muscle (101), and cornea (102). Gene gun was also used to treat against tumor growth (103). pDNA containing IL-12 gene delivered by gene gun to the epidermal cells over an implanted intradermal tumor gave detectable levels of the gene product at the treatment site. This eventually led to complete tumor regression within 7 days (104). A particle bombardment of TGF β1 encoded plasmid to rat tissue enhanced the tensile strength of the tissue by almost twofold compared with the control (90). Porcine partial-thickness wounds, when transfected with a vector expressing epidermal growth factor (EGF), showed an increased rate of reepithelialization that duly shortened the healing time by 20%, (105).

5 LIPID-BASED VECTORS

The lipid-based DNA delivery system was first used on the premise that naked DNA on injection *in vivo* might be degraded by endogenous DNAase. The lipidic delivery vehicle would provide protection to the DNA during circulation through the blood stream, and with necessary chemical modification, this vehicle would confer targeting potential to the DNA towards specific cellular sites.

Lipids are a class of molecule that have a tendency to self-assemble in aqueous or organic media. Depending on the molecular architecture and/or the presence of co-solute, they assume structures such as micelle, emulsion, or liposome in aqueous media and reverse micelle in organic media. Among them, liposomes have shown wide diversity in biological relevance. They constitute a bilayered structure, which can encapsulate water-soluble molecules in its aqueous hydrophilic core or water-insoluble molecules in its hydrophobic bilayer. Liposome-based drug delivery systems have shown promise in clinical use, and several products have already been approved by FDA. Fraley et al. (106) have shown for the first time that DNA could also be delivered to cellular targets by encapsulating DNA in liposomal aqueous core. They used a composite liposomal system containing anionic and nonionic lipids but with a limited DNA encapsulation efficiency. To enhance gene transfection and targeting ability of the liposome to specific tissue site, Wang and Huang (107) used pH-sensitive immunoliposomes made by coating anionic liposomes with target specific antibody and encapsulating plasmid. These liposomes, on intraperitoneal injection, showed specific transfection to tumor cells in an ascites tumor model of nude mice. Very recently, the immunoliposomes were revisited for targeting brain. An exogenous gene was targeted to the brain through the blood-brain barrier by intravenous injection of pegylated immunoliposomes conjugated with antibody to rat transferrin receptor. The whole entity was targeted to the brain through the transcytosis of transferrin receptor (108).

Felgner et al. (109), for the first time, used cationic liposomes for gene transfection. The primary idea was to electrostatically condense DNA with more than one equivalent of cationic lipid and the negatively charged cells would take up the net positively charged entity when fed to cell. Since then, a plethora of literature developed in fine tuning the chemical structure of lipids by systematic structure-activity studies to elucidate the structure of the optimal cationic lipid for gene transfection. Herein, pDNA played a significant role. pDNA reconstructed with recombinant marker/reporter gene and suitable promoter is primarily used as a tool to screen for the efficient transfection-lipid from a library of cationic lipidic molecules. The electrostatic lipid-DNA complex formed by first generation cationic lipids is termed as "lipoplex." Cationic polymers also condense DNA with or without the presence of lipid; these polymers can deliver DNA to cells. This provides the basis for the second-generation gene delivery systems. Cationic polymers, cationic dendrimers, etc. condense DNA to make a complex called "polyplex." If liposomes, cationic or anionic, are used to encapsulate the pre-condensed cationic polymer-DNA complex and are used to deliver DNA to cells, then the whole entity is termed as "lipopolyplex." We will talk about these second-generation gene delivery systems later in this chapter.

5.1 First Generation of Cationic Lipids

Lipids with a glycerol backbone containing aliphatic carbon chain(s): *N*-[1-(2,3-dioley-

loxy)propyl]-*N,N,N*-trimethylammonium-chloride (DOTMA), *N*-(2-hydroxyethyl)-*N,N*-dimethyl-2,3-bis(tetradecyloxy)-1-propanaminium chloride (DMRIE), *N*-[1-(2,3-dioleyloxy)propyl]-*N*-hydroxyethyl-*N,N*-dimethyl-ammonium chloride (DORIE) (110), 2,3-dioleyloxy-*N*-[2(sperminecar-boxamido)ethyl]-*N,N*-dimethyl-1-propanaminium trifluoroacetate (DOSPA), *N*-(3-aminopropyl)-*N,N*-dimethyl-2,3-bis(dodecyloxy)-1-propanaminium bromide (GAP-DLRIE) (111), *N*-[1-(2,3-dioleoyloxy)propyl]-*N′,N′,N′*-trimethylammonium chloride (DOTAP) (112), and *N, N*-(2-hydroxyethyl)-*N*-methyl-2,3-bis(myristoyloxy)-1-propanaminium iodide (DMDHP) (113). These lipids are linked to glycerol moiety through ether or ester-bond and have either a single cationic charge with none, single, or multiple hydroxyl groups or multiple cationic charges by possessing spermidine derivatives (Fig. 13.2).

Lipids with long chain alkylamine/alkylamide moiety: dimethyldioctadecylammonium bromide (DDAB) (114), DOGS (115), lipopoly(L-lysine) (LPLL) (116), GS2888 (117), *O,O'*-ditetradecanolyl-*N*-(trimethylammonio acetyl)diethanolamine chloride (14DEA2) (118), diC14amidine (119), *N,N*-di-n-hexadecyl-N, N-dihydroxyethylammonium bromide (DHDEAB) (120), and TFX. These are basically non-glycerol–based lipids, with single or multiple charges. The charges arise because of the presence of ammonium or multiple amines or spermidines. Some of these lipids also possess multiple hydroxyl groups.

Lipids with cholesterol moiety: 3β-[*N*-(*N′, N′*-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol) (121) and other cholesterol derivatives (122), biglycosylated cholic acid derivative (123), and cholesterol with polyamines (124) and spermidines (125, 126).

Lipids with L-a-phosphatidylethanolamine derivatives with spermidines: 1,2-dipalmitoyl-phosphatidylethanolamidospermine(DPPES) (116) or L-a-phosphatidylcholine with phosphonate diester (127, 128).

Lipids with imidazole derivative: 1-[2-[9-(Z)-octadecenoyloxy]ethyl]]-2-[8](Z)-heptadecenyl]-3-[hydroxyethyl]imidazolinium chloride (DOTIM) (129) or non-glycerol–based lipids with long-chain alkylacyl carnitine ester (130).

There are many other lipids that have been synthesized and tested for gene transfection. Only a few of these lipids have shown consistent and relatively high levels of transfection in various cell lines and the in *vivo* system. Most of these first-generation formulations do not circulate long in the blood because the DNA/cationic lipid complex disintegrates in the presence of negatively charged blood proteins. They are also grossly non-targetable to cells. To overcome these problems, a number of new delivery formulations have been created and tested for gene transfection efficiency. We will discuss these second-generation cationic formulations later in this chapter.

5.2 Cellular Barriers for Transfection

To develop an efficient gene delivery system, it seems necessary to understand the extra- and intracellular processes involved in the overall transfection mechanism. This will lead to understanding the mechanism, which is necessary for developing novel lipid-based non-viral vectors. For this purpose, cationic liposomes and pDNA are used widely to understand the cellular mechanism involved in the transfection (Fig. 13.3).

5.2.1 Structure of Lipid/DNA Complex.

Positively charged cationic liposomes electrostatically interact with negatively charged nucleic acid sequences to form fused, aggregated complexes, which are capable of entering a cell. These aggregates have a very heterogeneous distribution with respect to particle size and net charge. The lipid-to-DNA mass and charge ratio, which defines the size and net surface charge of the complex, is an important factor to determine transfection efficiency. Hence, the structural features associated with this kind of complex need to be interpreted. Structural features revealed by electron microscopy include lipid-coated DNA strands, aggregates of liposome intercalating DNA, DNA entrapped between the lamellae of aggregated multi-lamellar structure, and tubular structure consisting of fused liposome around DNA (131–136). Polycations such as polylysine or multivalent cationic lipid condense DNA into small compact particles (137).

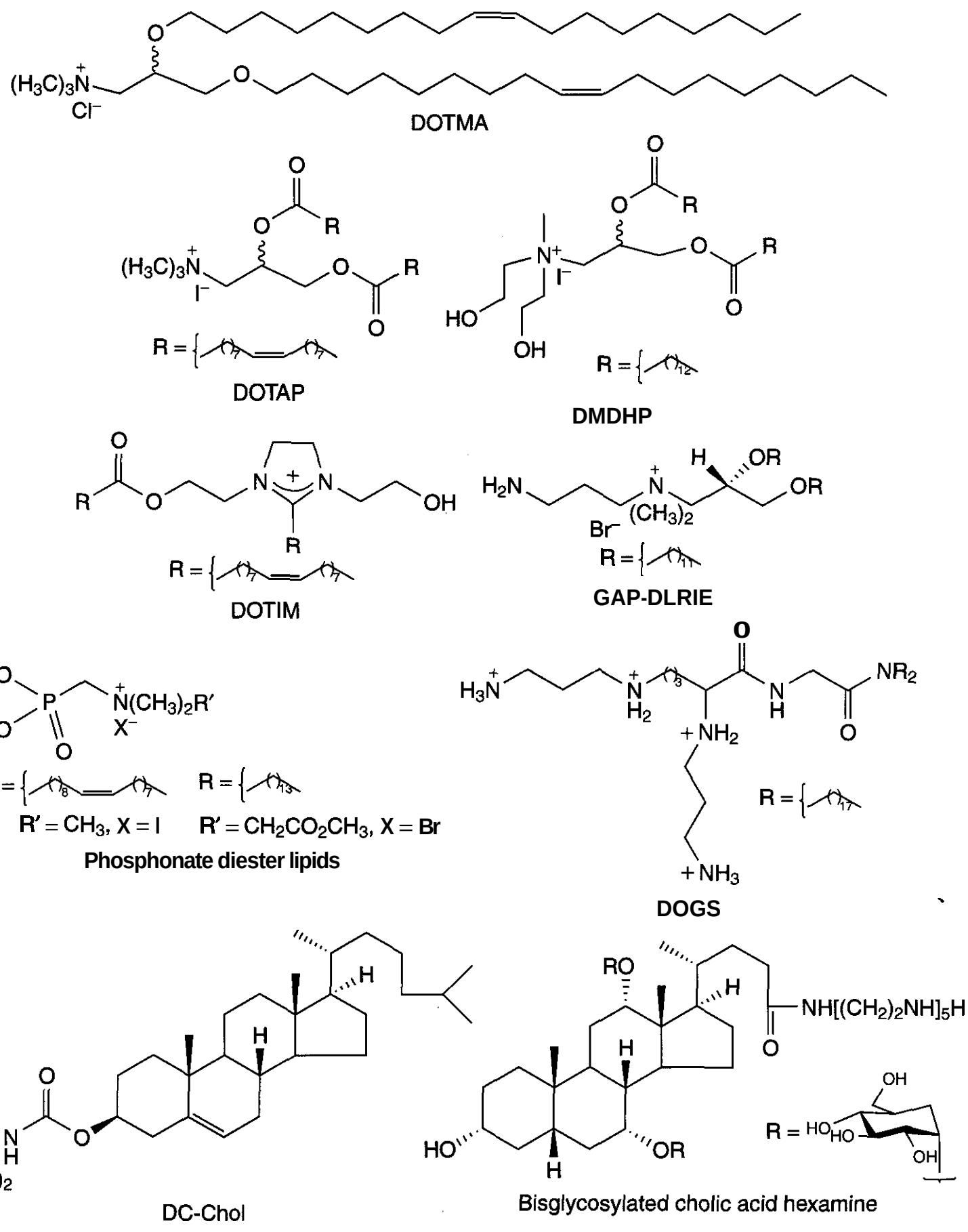

Figure 13.2. Structures of some representative cationic lipids.

Cells can efficiently uptake these particles compared with the particles obtained by condensation of DNA by monovalent cationic lipids (Sorgi et al., unpublished data).

5.2.2 Entry of DNA Into Cells. The presence of negatively charged proteins and glycophospholipid impart negative charges on the cell surfaces. It was hypothesized that membrane fusion between cationic liposome and negatively charged cell membranes is the primary means of cell entry **(109, 138).** The hypothesis was made based on the premise that cationic and anionic liposomes readily fuse **(139, 140).** It is conceptualized that the interaction of the cationic complex with the cell is

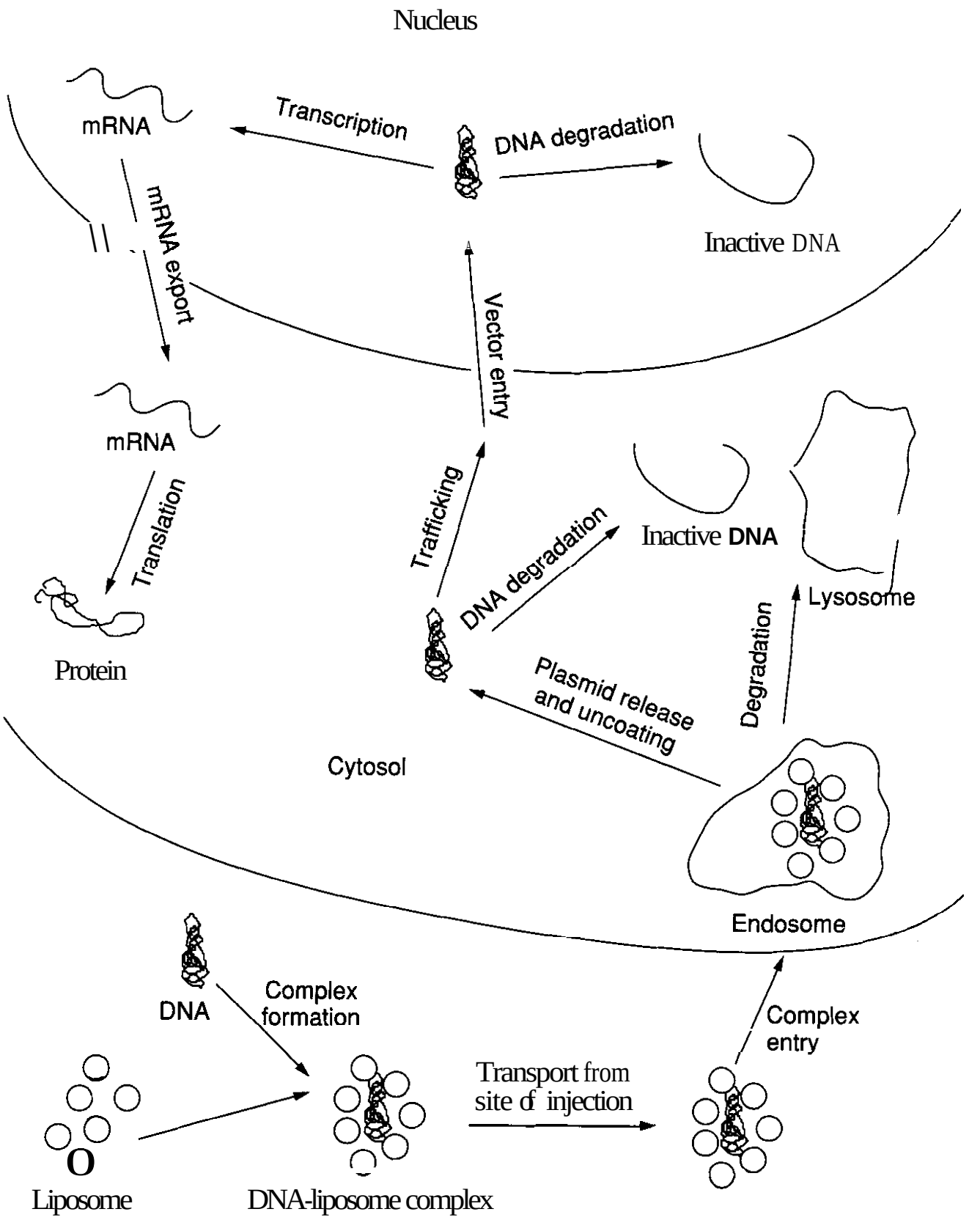

Figure 13.3. Schematic illustration of the processes involved in gene delivery and expression.

primarily electrostatic and does not involve any specific receptor for the cationic moiety. However, Mounkes et al. **(141)** showed that heparin sulfates, the highly anionic polysaccharides, serve as an important cell surface receptor for cationic lipid-DNA complexes. Other experiments also suggested that the mechanism of DNA-transfer to animal cells from cationic liposomes might not entail a simple fusion of liposomal and cellular membranes **(113).** The non-differentiated edged airway epithelial cells endocytose liposome-DNA complexes consequent to their relatively high negative charge and phagocytic activity compared with differentiated cells **(142).** Evidences now strongly suggest that slow endocytosis of intact lipid/DNA complex is the primary method (143–146) for cellular entry.

5.2.3 Fate of Complex Inside the Cell: Escape into Cytosol. After cellular entry, the lipid/DNA complex is engulfed into lower pH

compartment called "early endosomes" in the peri-membranous region (147–150). In an attempt to elucidate intracellular tracking of lipid/DNA complex, Zhou and Huang (117) showed that liposome composed of lipopolylysine (LPLL) and 1,2-dioeoyl-sn-glycero-3-phosphatidylethanolamine (DOPE), condense DNA to form electron dense particles recognized by thin-section transmission EM, and the majority of them reside in vesicular compartments. The endosomal contents usually pass into the lysosome, the cell organelle in the perinuclear region that houses a host of degredative enzymes. The hydrolytic enzymes degrade most if not all the lysosomal contents (111,151–153). It becomes necessary that the endosomal content must free itself at the endosome stage to keep the DNA intact. Disruption of endosomal vesicle is visualized in the liposomal system containing DOPE as a helper or co-lipid. DOPE with its tendency to promote significant polymorphic changes in the lipid phase stimulates membrane fusion or destabilization (117, 154, 155), which is followed by leakage of endosomal content into the cytoplasm. DOPE in aqueous media assumes inverted hexagonal phase II structure, which is frequently obtained in regions of membrane where it fuses with another membrane. Thus, one may assume that DOPE or liposome formulation containing DOPE might fuse with endosomal membrane and destabilize it to leak out the content from the endosome.

If the early endosomal release is not possible, another way to keep DNA intact in lysosome is to protect the DNA from lysosomal degradation. Cationic liposomes formulated with cholesterol believed to offer a useful role in keeping DNA intact (121,156,157). Straubinger et al. (158) have demonstrated that the lysosomal enzymes work at lower pH, i.e., pH < 6. It was also shown that cholesterol-containing liposomes, which possess greater stability and lower ion-permeability compared with DOPE-containing liposomes, provide an improved stability to the lipid-DNA complex in the cytosol (158–160). It is easily conceivable that if the endosomal content passes onto lysosome before being released from endosomes, the lipid/DNA complex could remain secured in the lysosome.

Cationic lipids can destabilize a cellular membrane because of its intrinsic detergent property. Therefore, destabilization of endosomal and/or lysosomal membrane may be a contribution from the cationic lipids itself. In the same context, it was shown that the cationic lipid/DOPE or cationic lipid/cholesterol liposome formulation exhibit surface anisotropies in terms of increased liposomal surface pH (161,162). The surface pH of the liposomal formulations exhibits at least two pH units higher than the pH of the solution at which they are made. Therefore, a liposomal solution made at physiological pH may in reality exhibit a surface pH ≥ 9, which is detrimental for both the stability of endosome and activity of lysosomal enzymes. Endosomal disruptions were also done with fusogenic peptides, which promote pH-dependent fusion of small liposomes when associated with lipid bilayer. When these peptides were co-delivered with lipid/DNA complex, they imparted formidable endosomal disruption by changing its usual random coil conformation into amphipathic a-helix conformation at lower pH, resulting in consequent cytoplasmic delivery of DNA (163).

5.2.4 Entry of DNA in Nucleus. The success of lipid-mediated gene transfer is severely limited by the inefficient transport of transfected DNA from the cytoplasm into the nucleus (146). In general, macromolecules enter the nucleus through nuclear pores. Molecular aggregates with size more than 55 Å in diameter or molecular weight greater than 40 kD use the nuclear pore complex (NPC) to access nucleus. NPC bind with molecular aggregates associated with nuclear localization signal (NLS) peptide and help it to translocate across the nuclear membrane (164–166). One of the typical signals is the NLS from SV40 large T antigen (PKRRRKV) (167), which had been conjugated to many different molecules to gain nuclear access (168, 169). NLS peptide-conjugated plasmid DNA was delivered efficiently inside the nucleus with an enhanced gene expression (170). Similarly, SV40 T antigen NLS co-delivered with DOTAP cationic liposome-mediated efficient gene transfer and expression in the cell (171). The genes delivered inside the nucleus require uncoating

from the lipidic shell before the transcription starts. It is generally assumed that pDNA is displaced from the complex by anionic macromolecules in the nucleus. In this regard, an alternative hypothesis for cytosolic release of DNA from lipid/DNA complex had been proposed. Xu and Szoka (160) have demonstrated by model studies that the pDNA was released from cationic liposome/DNA complexes by anionic liposomes exhibiting compositions mimicking the cytoplasmic face of the lipid monolayer of the cellular membrane. Membrane destabilization followed by flip-flop of the liposome/DNA complex by the anionic lipids by electrostatic interaction resulted in charge neutralized lipidic ion pairs followed by release of pDNA.

To evade the nuclear transport, an alternative approach was developed that uses T7-based cytoplasmic expression vectors. Here the inefficiently nuclear-transported DNA could be expressed in the cytoplasm itself (172–175).

6 *IN VIVO USE* OF CATIONIC LIPOSOME/DNA COMPLEX (LIPOPLEX)

6.1 Intravenous Injection

This is a widely used mode of gene delivery in animals. On injecting liposome/DNA complex (lipoplex) intravenously (IV), Stewart et al. (156) for the first time showed the residence of DNA primarily in heart and lungs even after 9 days with minimal toxicity. IV-injected lipoplex expressed transgene in almost all organs, includingthe lung, kidney, heart, spleen, liver, brain, etc., and the expression stayed for 9 weeks with apparently no treatment-related toxicity (176). Toxicity and antitumor response was evaluated in mice and pigs with high doses of lipoplex containing MHC gene incorporated pDNA (177).

Recently, more work has been done to increase the overall transfection efficiency with much higher targeting capability and reproducibility of the liposomal delivery system (159, 178, 179). Intravenously administered lipid/DNA complex avidly reacts with blood components. So, it is necessary to keep the complex intact in the blood until it reaches the organ of interest. Sakurai et al. (180) have shown that in the presence of erythrocytes, cationic lipid/cholesterol formulation didn't induce fusion between erythrocytes, whereas the cationic lipid/DOPE formulation possessing high fluidity in its structure induced fusion between the erythrocytes after a short incubation period. This offered an explanation for why cholesterol makes more superior formulation with cationic lipids for in vivo purposes (159).

A repeated systemic intravenous injection of cytokine gene (IFN β1) by lipoplexes gave a systemic expression of human interferon-β in mice, thereby increasing the possibility of cytokines used for therapeutic purposes in a systemic manner (181). Hwang et al. observed an enhanced and highly selective liver targeting by IV injecting cationic liposome/pDNA containing β-sitosterol β-D-glucoside (Sit-G) (182). IV injections showed gene expression in all major organs including the heart, lung, liver, spleen, and kidney, with the lung being most efficiently transfected (178, 183). For efficient targeting and gene expression in the lung, intravenous injection was favored over intratracheal instillation. Uyechi et al. (184) have shown by injecting fluorescently tagged lipoplexes through the vein that the entire lung lobe was homogenously fluorescent, whereas intratracheal administration resulted in regional distribution of lipoplex, concentrated around bronchiales and distal airways.

6.2 Direct injection

Direct injection to tissue is a common approach for cationic lipid-mediated gene therapy. Intratumoral injection of DNA-liposome complexes containing either E6 or E7 antisense plasmid resulted in significant growth inhibition of C3 tumors grown in a syngeneic mouse model (185). E6 and E7 are two oncogenes responsible for the maintenance of the malignant state of HPV-position tumors. Nabel et al. (186) have directly injected recombinant pDNA containing murine class I major histocompatibility (MHC) gene into localized arterial segment of various major organs and showed that the direct gene transfer by liposomal transfection did not lead to treatment related toxicity, autoimmunity, or gonadal localization of the transgene in mice. The toxicity

of gene delivery by DNA-liposomes was also analyzed in pigs and rabbits in vivo. There were no clinically significant immunopathology in major organs such as the brain, heart, lung, liver, kidney, spleen, and skeletal muscles. To induce local tumor immunity in patients with stage IV melanoma, Nabel et al. (187) injected pDNA encoding MHC class I antigen complexed with cationic lipid directly into the cutaneous tumor nodules. Treated lesions exhibited presence of T-cells, followed by an enhanced reactivity of tumor infiltrating lymphocytes. As a result, local inhibition of tumor followed by complete diminution of tumor was observed in some of the treated patients. Mohr et al. (188) have shown that direct liposome-pDNA complex injection to intrahepatic hepatocellular carcinoma produced by human HCC cells seemed far superior to systemic administration for gene therapy for localized intrahepatic tumors, because the direct administration to tumors left the surrounding normal hepatic cells untouched. In another typical example, in vivo, direct intratumor injection of plasmid containing the coding sequence for the human IL-2 gene complexed with cationic lipid formulation resulted in retention of intact pDNA in the tumor tissue and IL-2 secretion by cell cultures derived from the injected tumors. Formulation of this lipid with the cationic lipid inhibited DNA degradation and enhanced in vivo transfection efficiency over pDNA alone (189).

Airway administration of liposome complexes was used for the treatment of pulmonary diseases including cystic fibrosis. Cationic liposome/DNA complex showed no adverse effect towards airway epithelial integrity (190); therefore, the cationic lipid-based delivery system proved to be appropriate for use in human trials for cystic fibrosis (CF). A series of pre-clinical trials were done in CF patients with intranasal instillation to evaluate the risk factors associated with the treatment (191–193). Because there was no apparent toxicity associated with lipoplexes as was seen from these trials, progress had been made in delivering the complexes to the entire lung by aerosol in CF patients (192,194,195). By nebulization, the DNA-liposome complex was delivered into the airways of mutant mice to obtain human cystic fibrosis transmembrane conductance regulator (CFTR) cDNA expression in the respiratory tract (196). A study conducted on CF patients revealed that pCMV-CFTR/cationic liposome complex on administration to the nasal epithelium gave no evidence for excess nasal inflammation, or any adverse events related to active treatment. Gene transfer and expression assayed by PCR revealed the presence of transgene DNA in seven of the eight treated patients up to 28 days after treatment (192). Intranasal instillation technique was also used in the mouse model, to incorporate cationic lipid/DNA complexes (112, 197). The CFTR dysfunctional gene gives rise to multiple defects in airway epithelia such as altered Cl^- and Na+ permeability. Zhang et al. (198) have shown that the goblet cells were more efficiently targeted with lipoplexes than any other cells in the entire spectrum of lung airway epithelia. This was ascertained by the fact that an efficiently reduced mucous sulfation to levels seen in non-CF airways was observed with lipoplex/DNA despite low levels of CFTR gene expression in lung epithelial cells in human bronchial xenograft model of mice compared with non-CF airways of control mice. This kind of apparent complexities in CFTR function presented challenges in the design of different lipoplex formulations that were capable of generating the endogenous patterns of CFTR gene expression in specific lung epithelial cells.

6.3 Intraperitoneal Injection

Intraperitoneal (IP) injection of DNA/lipoplex was done to transfect cells in the peritoneum region. Nude mice bearing disseminated human ovarian tumors derived from p185-overexpressing SKOV-3 ovarian cancer cells were injected with E1A gene/lipoplex intraperitoneally (199). These tumors resemble stage III human ovarian cancer. The expression of E1A protein decreased the expression of p185 oncoprotein and hence increased the survival rate of mice (200). 70% of the treated group survived for 1.5 years from the last injection, but the untreated group barely survived more than 16 weeks. The treatment of complex containing 1/13 of the original lipid dose also worked as efficiently as the normal dose. There was no apparent toxicity or major organ

pathologic change. There was no trace of E1A DNA in the liver, lung, heart, spleen, brain, uterus, and ovaries of the treated mice even after 1.5 years (199).

7 SECOND GENERATION OF LIPIDIC DELIVERY SYSTEM (LIPOPOLYPLEX)

7.1 Polycation-Condensed DNA Entrapped in Cationic Liposome: LPD-I Formulation

One of the prerequisites for a bio-entity entering the cell and nucleus is to possess a small size. The delivery of DNA was efficient through cationic lipidic formulation–mediated gene delivery, but the nuclear transport from cytoplasm was quiet an inefficient process (146). Generally, lipoplexes formed by multiple charged cationic lipids made small (~20 nm), highly condensed DNA/lipidic complex. These complexes were found to be more transfecting than the lipoplex formed by mono-cationic lipids and DNA. Synthetic cationic polymers like poly-L-lysine (PLL) could also condense DNA into very small compacted particles. This compact complex was allowed to freely mix with cationic liposomal solution. The overall complex formed was termed LPD-I (200). A sucrose gradient ultracentrifugation of the heterogeneous mixture showed the existence of various populations associated with varied amount of lipid. Negative-stain EM studies of purified complexes showed electron dense structures ranging from elongated rod-shaped to ball-shaped particles. The purified fractions were severalfold more transfection efficient than unpurified ones. The fractions, which contained more lipids, were more efficient in gene transfection than those containing fewer lipids. The transfection efficiency of LPD-I was severalfold higher than the corresponding cationic liposome-DNA complex. It is hypothesized that the small compacted structure of DNA resulted in high cellular and nuclear uptake, the DNA is protected efficiently against the enzymatic degradation, and PLL may have mimicked the nuclear localization signal for nuclear delivery of DNA. An alternative cationic polymer, protamine sulfate, from salmon sperm was used as a substitution for PLL (201). Protamine sulfate is a small (MW ~4000 versus ~18,500 for PLL), highly positively charged peptide and very basic because of the presence of 21 arginine residues, which contains a nuclear localization signal (202). It is naturally occurring (whereas PLL is synthetic), a United States Pharmacopeia (USP) grade compound, FDA-approved, and finds its use as an antidote to heparin-induced anti-coagulation. With a routine history of human administration, the issues of toxicity and immunogenicity were minimal for protamine sulfate.

Both protamine sulfate and PLL were compared for their transfection efficiencies. With the same amount of DNA and cationic lipid, PLL reached its efficiency plateau at an amount one-half the amount of protamine sulfate, but the overall efficiency of protamine sulfate was two- to sevenfold higher than PLL in different cell lines. The extent of transfection efficiency for LPD-I was 7- to 45-fold higher than the levels shown by DNA/cationic lipid complex. Phosphate and free base form of protamine showed lower levels of transfection and the activity was comparable with lipoplex. It is noteworthy that arginine, which exists as a salt of sulfate, and the lysine as free base or as a phosphated salt, showed a variance in the relative transfection efficiencies even though the percentage of basic (arginine + lysine) residues remained relatively constant.

On intravenous injection, LPD-I made with DOTAP had shown very high gene expression in the heart, lung, liver, spleen, and kidney, with the highest expression found in the lung (178). The in *vivo* gene expression had steadily increased with increase in cationic lipid and/or protamine, but the protamine-DNA charge ratio was kept below 2:1 to avoid forming large aggregates. After LPD injection transgene expression was detected as early as 1 h, it peaked at 6 h and declined thereafter. The lung consistently showed the highest gene expression, which lasted for 2 days. Even after 4 days of LPD injection, gene expression could still be detected in the lung, spleen, and liver.

In a bio-distribution study with labeled DNA and liposome, it was revealed that DNA was rapidly removed from circulation by the liver after injection of protamine-DNA complexes and uptake by the lung was always less than 10% of injected dose at all time points. However, DNA formulated in LPD was

trapped in the lung to an extent of 40% within 5 min of LPD injection. Southern blot analysis revealed that the pDNA injected with LPD was detectable in the lung in much higher quantity than the control even 6 h after IV injection. Intraportal injection gave significantly lower gene expression than with intravenous injection.

7.2 Polycation Condensed DNA in Anionic Liposomes: LPD-II Formulation

Some disadvantages always accompany the cationic lipid-mediated gene transfection. Cytotoxic effect in cells/tissues is the primary concern, which requires early attention. The cationic lipids exhibit relatively large sizes, whereas complexation with DNA provide suboptimal DNA condensation and have limited efficiency and lack of tissue specificity. Anionic liposomes used in gene therapy have shown poor encapsulation efficiencies because of large size and excessive negative charge of uncondensed pDNA. The use of anionic liposomes was revisited during a recent approach, which also used the concept of condensing DNA by cationic polymers. A delivery vector was developed wherein polylysine-condensed pDNA was trapped in folate-targeted anionic liposomes through charge interaction. It had a structural similarity to LPD-I and it was named LPD-II (203). It differed from LPD-I in that anionic lipids instead of cationic lipids were used. This novel vector was more efficient and less cytotoxic compared with conventional cationic liposomal vectors.

Folate-targeted LPD-II particles were generated by mixing anionic liposomes composed of DOPE/cholesteryl hemisuccinate (CHEMS)/folate-polyethyleneglycol (PEG)-DOPE and the cationic DNA-polylysine (1:0.75, w/w) complexes. Structural analysis of LPD-II by negative-stain EM showed that the DNA-polylysine (which appears individually as rod shaped) and lipid complex seemed to be a highly electron dense, spherical core with a low-density coating. The mean diameter of these particles was 74 ± 14 nm, i.e., smaller than the empty liposomes.

KB cells expressing folate receptors were transfected with LPD-II particles containing luciferase reporter gene, and high transfection efficiency was observed. The activity could be inhibited by the presence of excess free folate. Control LPD-II particles generated with nontargeted liposomes was only active at low lipid/DNA ratios, suggesting that the transfection by LPD-II particles was only receptor dependent when the over all charge was negative. Compared with DC-Chol/DOPE/DNA liposome complexes, LPD-II showed ~20- to 30-fold more transfection activity. On replacing DOPE with DOPC in the original formulation, the transfection was severely reduced. This indicated that the fusogenic activity of DOPE was essential for the transfection activity of LPD-II particles.

The therapeutic applications of antisense oligonucleotides (ODN) are currently limited by their low physiological stability, inefficient cellular uptake, and the lack of tissue specificity. The use of various vectors renders phosphodiester (PO) ODN resistant to enzymatic digestion. KB cells, which overexpress folate binding protein, were also transfected with LPD-11–containing targeting ligand folate to deliver ODN against epidermal growth factor receptor (EGFR). This resulted in down-regulation of EGFR and growth inhibition of KB cells (178). The modified backbone ODN, i.e., phosphorothioate (PS) and monomethylphosphonate (MP), are more stable to enzymatic degradation compared with PO ODN, but they suffer from increased toxicity and decreased specificity. In one study, PO ODN against EGFR had shown growth inhibitory effect to KB cells compared with that of PS/PO ODN when delivered with LPD-II, indicating that LPD-II could also protect PO ODN from the attack by enzymes inside cells (204).

8 EMULSION-MEDIATED GENE TRANSFER

8.1 General Development

Emulsions are one of the most widely studied colloidal dispersion systems for the delivery of drugs (205,206). The oil-in-water emulsion is made of oil dispersed in an aqueous phase with a suitable emulsifier such as phospholipids and non-ionic or ionic surfactants. Castor oil or soybean oil is predominantly used as the core oil phase. Non-ionic surfactants such as Tween, Span, Brij, and pluronic copolymers are used as co-emulsifiers. The ionic co-emul-

sifiers are phospholipids or cationic lipids. A number of structure-activity studies had been done with different emulsion formulations, which were subsequently used for gene delivery *in uitro* and *in vivo*. The non-ionic surfactants such as Tween, Span, Brij, and pluronic copolymers were found to be excellent co-emulsifiers when used along with castor oil, DC-Chol, and DOPE (207). The *in vitro* transfection study on BL-6 cells showed that the Tween surfactant–containing formulations had more serum resistivity and exhibited higher transfection in serum-containing media than in the absence of serum. One of the Brij-containing formulations, i.e., one with 2-oxyethylene chains, showed the highest transfection efficiency in presence of serum. The toxicity of each formulation is minimal. In DOTAP, soybean oil, and pDNA emulsion complexes, it was observed that despite the change of ζ potential with the varying amount of DNA, the structure and the size of the emulsion complex remained mostly unchanged (208). The stability of this emulsion complex was shown to be high and inhibited DNAase-I digestion of pDNA. In serum-containing media, the emulsion showed much higher transfection efficiency compared with the lipofectamine/DNA transfection complex. Inclusion of polyethyleneglycol-PE in the emulsion complex created a high-level transfection that was observed even in the media containing 90% of serum. This result suggested that *in uiuo* transfection could be done with this emulsion complex. Another soybean oil-DOTAP emulsion was used to transfer genes to the epithelial cells of the mouse nasal cavity through intranasal instillation. The emulsion showed enhanced stability against heparin exchange and exhibited higher level of transfection compared with commercially available liposome/DNA complexes in nasal cavity mucosa (209).

8.2 Reconstituted Chylomicron Remnants for Gene Transfer

Chylomicrons are triglyceride-rich lipoproteins that are slowly modified during the circulation in blood. The core glyceride structure is hydrolyzed by the lipoprotein lipase. It is by the apolipoprotein-specific receptors that the hepatocytes in the lever consume these remnants (210,211). It had been shown that even reconstituted chylomicron remnants formed by purified lipids could be taken up by the hepatocytes following intravenous injections (212). In our laboratory, we modified the system and used it for delivery of DNA into liver cells (213). DNA is predominantly hydrophilic in nature and therefore cannot be included in the hydrophobic interior of the reconstituted chylomicron remnants (RCR). 3β-[*N*-(*N'*, N',*N'*-Trimethylaminoethane)carbamoyl] cholesterol (TC-Chol), a quaternary ammonium analog of DC-Chol, was used in various amounts to make complexes with DNA. The resultant hydrophobic complexes extracted from aqueous solution were emulsified with commercially available lipids (olive oil: L-a-phosphatidyl choline, L-a-lysophosphatidyl choline, cholesteryl oleate) by homogenization. DNA/TC-Chol complex was incorporated into the internal oil space of RCR and remained protected against DNAase I digestion. The RCR-containing DNA/TC-Chol complex showed transgene luciferase expression in the liver 100-fold higher than with naked DNA injection when injected intraportally. The expression was also seen in the spleen, lung, and heart, but was 25- to 800-fold lower than in the liver. The gene expressions obtained through tail vein injection were 100-fold lower than of the mice injected intraportally. This was likely because the RCR didn't contain any apolipoproteins on its surface, which could have otherwise facilitated the receptor-mediated uptake. Moreover, the colloidal stability might not be as good as the ones containing apolipoproteins. RCR didn't employ any protein or peptide that acts as an antigen. So, it could be injected repetitively by using a catheter method that had been established for multiple portal vein infusion (214). We have recently improved the formulation by adding pegylated lipid to the surface of RCR and showed that the circulation time of the particle was prolonged significantly (215).

9 GENE DELIVERY BY POLYMERIC SYSTEMS (POLYPLEX)

9.1 General Development and Uses

An alternative non-viral gene delivery system has been developed that uses polymers, either

cationic or neutral. Cationic polymers are predominantly used because they efficiently condense DNA to very small particles. These complexes are called "polyplexes." Wu et al. (216) used polylysine-asialo-orosomucoidconjugate to condense pDNA and target the pDNA to liver. pDNA condensed with protamine/polylysine-conjugate of iron transport protein, transferrin, was efficiently delivered to eukaryotic cells (217). pDNA condensed by CD3 antibody-polylysine conjugate showed receptor-mediated endocytosis to efficiently internalize pDNA into T-lymphocytes (218). Depending on the cell-binding ligand, specific targeting was obtained in different cell lines (219).

Several of the most effective polymeric delivery systems were polyamidoamine dendrimers (220) and polyethyleneimines (PEI) (221). Being non-biodegradable, these synthetic polymers posed a potential toxicity to cell; therefore, biodegradable polypeptides like PLL and protamine were used for condensation and delivery of gene, but they had limited efficacy in transfection (222, 223). They were usually used with cationic lipids to obtain enhanced transfection activity (178,201). Among the biodegradable polymers, chitosan (224) and β-cyclodextrin–based polymers (225) were also used for gene transfection.

Although the cationic polymers shared the same mechanism of DNA condensation, the transfection efficiency greatly varied between polymers. Even different molecular weights and isomeric forms of the same polymer showed different physicochemical characteristics, transfection efficiency, and toxicity (221, 226–228).

Ligand-conjugated polymers were used for *in vivo* targeting and expression of genes. Kircheis et al. (229) reported an enhanced level of transfection in subcutaneous neuro2a tumors on intratumoral injection of transferrin-PEI/pDNA compared with naked pDNA injection. On pegylating the Tf-PEI/pDNA complex, the whole entity became serum resistant without losing its targetability, and the complex could be efficiently targeted to neuro2a tumors in a mouse model after IV injection (230). Alvarez-Maya et al. (231) cross-linked neurotensin with PLL and made polyplexes with pDNA. On injecting polyplex into the substantia nigra of rat, a high level of transfection was observed in nigrostriatal dopamine neurons and was detectable up to 15 days.

EBV-based plasmid vector containing thymidine kinase (TK) gene coupled with polyamidoamine (PAMAM) dendrimer (EBV/polyplex) was used in suicide gene therapy of cancer (232). Huh7 hepatocellular carcinoma (HCC) tumors in SCID mice were injected intratumorally with TK genes containing EBV/dendrimer polyplex to show remarkable suppression of tumor growth, leading to prolongation of survival time. Gene transfer to the lung was obtained by intravenous injection of G9 PAMAM dendrimer-complexed pDNA into mice. This resulted in high levels of transgene expression in the alveoli at 12 and 24 h, followed by a second peak of expression 3–5 days after administration. However, the direct endobronchial administration of this polyplex primarily targeted bronchial epithelium (233). Topical *in vivo* delivery of pDNA to the hairless mice skin was done with PAMAM dendrimer polyplex. The polyplex was incorporated in or coated on the surface of poly(DL-lactide-co-glycolide) (PLGA) or collagen-based biodegradable membranes (234).

In an effort to transfer genes to rabbit carotid artery, Turunen et al. (235) used DNA/fractured dendrimer (generation 6) polyplex to obtain a high level of gene transfer (4.4%) compared with what was obtained by lipoplex. The arterial gene transfer was particularly useful because it could be used as a tool for treating various vascular diseases. In *vivo* gene transfer methods in this study employed a gene delivery reservoir (collar) around the carotid artery, which served as a reservoir for the gene delivery solution. This type of local gene transfer with cationic polymer-pDNA complexes provided a technically efficient way of treating arterial diseases during vascular surgeries. These dendrimer-based polyplexes showed a clear advantage over polylysine in that they can buffer the drop of endosomal pH inside the endosome leading to an increase in transfection efficiency (236, 237, 223).

Qin et al. (238) used starburst PAMAM dendrimers to transfer genes into a murine cardiac transplantation model. These starburst dendrimers are a special class of its kind, which are highly branched spherical polymers

with large number of amino groups on the surface. At the time of transplantation of whole heart in the recipient mice, the dendrimer/β-gal pDNA was directly injected into the graft tissue. X-gal staining revealed a highly efficient and wide spread transgene expression in both myocytes and the graft infiltrating cells with the peak lasting up to 14 days. For organ-transplantation, severe tissue rejection is a common immune response. Viral IL-10, a cytokine synthesis inhibitory factor, is able to regulate a variety of negative immune responses by suppressing the synthesis of IFN-y or inhibiting IL-1, IL-6, IL-8, IL-12, and TNF-a! (239–242). Direct injection of this dendrimer/viral IL-10 gene with a-MHC promoter polyplex to the cardiac tissue showed an increased survival of the cardiac allograft. As little as 0.31 μg of the injected pDNA led to an increased mean survival from 13.9 to 38.6 days.

9.2 Targeted Gene Delivery by Antibodies Conjugated with Polycations

Polyamines had shown efficient transfection to lung endothelium. An efficient targeted transfection vector to the lungs could be achieved by conjugating a targeting ligand against platelet endothelial cell adhesion molecule-l (PECAM-l) to polyamines. This ligand-polyamine complex was targeted efficiently to the pulmonary endothelial cells. A chemical vector was synthesized by covalent conjugation of polyethylenimine (PEI) and anti-PECAM antibody (Ab) (243). The cationic complex was shown to deliver DNA specifically to mouse lung endothelial cells. The highest gene expression was obtained at relatively low plus-to-minus charge ratios. The PEI conjugated with a control IgG did not enhance transfection of mouse lung endothelial cells. Intravenous injection of this anti-PECAM Ab-PEI /DNA showed an increased lung expression in mice compared with other modes of injection (243).

TNF-α in blood was found to be about five-fold less in the mice injected with PEI-anti-PECAM Ab/DNA than in the mice injected with PEI/DNA. The decrease in TNF-a was partially reversible by pretreating mice with Ab to PECAM. The immunosuppressant dexamethasone not only improved the persistence and level of gene expression in the lung but also shortened the refractory period for repeated dosing when injected along with anti-PECAM Ab/DNA. This supported a potential therapeutic role of dexamethasone in lung gene transfer with Ab-polymer conjugates.

Similarly, Ferkol et al. (244) targeted the polymeric immunoglobulin receptor (pIgR), which is expressed in lung and liver tissues, and transferred pDNA to these tissues. The targeting ligand, anti-secretory component (SC) Fab antibody, was covalently linked to poly-L-lysine. The polycation, after condensing pDNA, was delivered successfully to airway epithelium in *uiuo*. Tissues that do not express the pIgR, the spleen and heart, were not transfected. In addition, conjugate prepared with irrelevant Fab fragments did not produce detectable transgene activity. This complex specifically targeted pIgR-bearing tissues, but after repeated dosing, increased humoral immune response against anti-SC Fab antibody was observed (245).

10 NON-VIRAL VECTOR-RELATED CYTOTOXICITY

It is known from the 1980s that bacterial DNA stimulates the formation of cytotoxic IFN-a!, β, and IL-12 when the DNA is taken up by macrophages. It in turn leads to NK cell activation and production of pro-inflammatory cytokine IFN-y. This is accompanied by the proliferation of B-cells and therefore the reduction of apoptosis and release of IL-6 and IL-12 (246–249). These pro-inflammatory effects were found to be caused by some immuno-stimulatory sequences in prokaryotic DNA that contained unmethylated CpG dinucleotide motif flanked by two 5' purines and two 3' pyrimidines. Plasmid DNA, which is derived from bacterial DNA, induces these immune responses. The unmethylated CpG motif–containing sequence occurs four times more frequently in prokaryote DNA than in eukaryotic DNA. Moreover, the CpG motifs are usually 75% more methylated in mammalian DNA than in prokaryotic DNA (250,251). On methylation of the cytosine bases in plasmids, the immuno-stimulatory effect is decreased considerably (252). Immature den-

dritic cells tend to produce pro-inflammatory IFN-a, β, IL-6, Il-12, and TNF-a during exposure to CpG containing oligonucleotide or bacterial plasmid (253,254).

These immune-stirnulatory effects leading to inflammatory cytokine productions had a negative impact on the systemic gene delivery of caionic lipid/DNA complexes. On recognition of pDNA by splenic macrophages during circulation, there was every chance that the pDNA would elicit an immune response, which might lead to phagocytosis of the complex and hence decreased transgene expression. High levels of pro-inflammatory cytokine also led to inactivation of several promoters, resulting in a decrease in transgene expression (255–257). The death of animals by high dose lipoplex injection for obtaining high transgene expression (258) could be attributed both to the high concentration of lipid and pDNA mediated toxicity. In the case of local lipoplex administration in animals, minimal toxicity was observed. However, on intravenous injection or intratracheal instillation, high levels of IFN-γ and TNF-α were observed (259–261). Minimized CpCr triggered inflammation and immune responses followed by prolonged gene expression were obtained by pretreating cytokine-neutralizing antibodies during intravenous injection of lipoplex (259). Repeated dosing without any antibody treatment led to silenced transgene expression after 1 or 2 weeks (178, 259).

It is not clear how cytokine production decreases the transgene expression, but various efforts to minimize CpG-related immune responses and toxicity and to enhance transgene expression is worth mentioning. Hofmann et al. (262) have used PCR amplified fragments containing encoded therapeutic gene and regulatory elements for preparing LPD. On delivering, a similar level of gene expression comparable with pDNA lipoplex was obtained. However. much lower level of cytokine response was observed, which sustained the gene expression for a longer period than pDNA. PCR fragments contain fewer CpG motifs than the full-length pDNA; this led to reduced CpG-triggered adverse effects. Yew et al. (263) have shown that during mutating CpG or its flanking motifs in the plasmid, a decreased level of cytokine and increased transgene expression could be obtained. A limited interaction of plasmid with immune cells could also lead to decreased cytokine response. It could be achieved by sequentially injecting cationic liposomes and free pDNA. Song et al. (264) used this process for the purpose of efficiently transfecting the lung by prolonging the residency time and interaction of DNA with pulmonary endothelium. Tan et al. (255) used the same concept to show that the sequential injection led to the formation of a lower level of cytokines compared with lipoplex injection. It is evident from the above efforts that understanding the detailed mechanism of CpG-induced immune response is required for increasing the efficacy of pDNA-mediated gene delivery.

11 CONCLUSION

Since the first attempt of using plasmid DNA for gene delivery to cells, two decades have seen many attempts to enhance the efficacy of genetic vectors, to test various therapeutically important genes, and to understand the mechanism of the gene delivery, associated toxicity, and factors inhibiting gene expression. A number of gene therapy clinical trials have also been performed. Although no single vector is superior over other vectors, each in *vivo* gene transfer application will find its vector system for optimal performance. Understanding cellular barriers and possible means to overcome will undoubtly further improve the performance of a non-viral vector.

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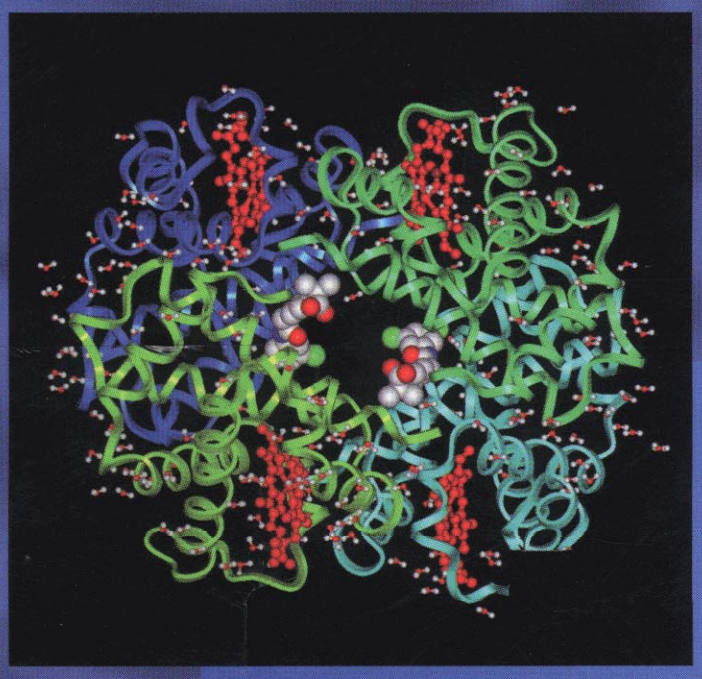

VOLUME 4

Autocoids, Diagnostics, and Drugs from New Biology

In addition to having cell numbers associated with this disease, the biology of the eosinophil also implicates this cell as a major contributor to the disease process. Eosinophils are phagocytic granulocytes that mature in bone marrow, migrate to the blood stream, and eventually localize at the site of injury. They contain highly toxic components that are released upon degranulation, leading to destruction of bronchial epithelium, mucosal edema, and bronchial hyperresponsiveness (430–432).

IL-5 is detectable in bronchial biopsies and bronchoalveolar lavage (BAL) fluid of asthmatics (433, 434). Additionally, inhalation of IL-5 by asthmatics has been shown to cause airway hyperresponsiveness (AHR) and an increase in sputum eosinophils (435).

IL-5 is produced primarily by activated CD4+ T-cells (436, 437), and in lower levels by eosinophils (438), mast cells (439, 440), basophils, B-cells, NK cells (441, 442), and endothelial cells (443). The expression of IL-5 is predominantly regulated at the transcriptional level (444), and can be induced by a variety of stimulants, usually through activation of the T-cell receptor and a second signaling pathway. IL-1α and PMA can induce IL-5 expression; histamine can also increase the production of IL-5 in activated T-cells (445).

Structurally, IL-5 is a disulfide-linked homodimeric glycoprotein between 45 and 60 kDa (446). Glycosylation does not seem to be a requisite for signaling; however, IL-5 must be in native dimeric form for bioactivity (447). Each monomer of IL-5 consists of four α-helices with an antiparallel β-sheet between opposing monomers. The monomers are maintained in dimeric form by cysteine bonds at residues 44 and 86 (448). Mutagenesis shows residues His38, Lys39, and His41 in the second helix; Glu89 and Arg91 in the β-strand; and Thr109, Glu110, TRP111, and Iso112 in the fourth helix as being important contributors to the IL-5 interaction with the hIL-5Rα-chain (449, 450). Glu13 on IL-5 has been identified as a contact point for the β-chain of the IL-5 receptor.

The IL-5R α-chain specifically binds IL-5 with low affinity (451). When associated with a signal-transducing β-unit that is also used by other hematopoietin receptors such as GM-CSF and IL-3, IL-5 binds with high affinity (452–454). Both subunits are necessary for signal transduction (455). The pathway for signal transduction includes activation of two Janus kinases (JAK1 and JAK2) and the signal transduction/activator STAT5 (456, 457).

9.4.1 IL-5 Knockout and Transgenic Mice. Animal models have helped define the significance of IL-5 and the eosinophil in the disease process. IL5 administered to mice results in an increase of eosinophils (458). Experiments with IL-5-deficient and IL-5-transgenic mice confirm a role for this cytokine in controlling eosinophilia (459, 460). In IL-5 knockout mice, no eosinophils are produced in response to parasite infection or sensitization with ovalbumin, and there is minimal development of lung inflammation or tissue damage. When IL-5 expression is reconstituted in these mice, pulmonary eosinophilia, tissue destruction, and airflow limitation can be observed after allergen challenge (459).

Transgenic mice that have constitutive expression of IL-5 with detectable levels of IL-5 in the serum and persistent eosinophilia have also been described (461). These mice are described as normal, which may suggest that activation and degranulation of eosinophils may be necessary for disease pathology.

9.4.2 IL-5 Modulators/Clinical Data. Modulation of IL-5 can occur by inhibiting its production and synthesis, or through direct binding to IL-5 receptor or ligand. Cytokines can regulate IL-5 levels by inhibiting production. For example, IFNγ and IL-10 have demonstrated they can inhibit IL-5 production *in vitro* (462), whereas IL-12 indirectly modulates IL-5 by biasing toward a Th1 subset population (463).

Small molecule antagonists such as CsA, FK506, and rapamycin all inhibit IL-5 production (464, 465). Glucocorticoids, in addition to decreasing bronchial hyperresponsiveness, can also downregulate IL-5 production (466–468). OM-01 suppresses IL-5 protein production, mRNA expression, and transcriptional activity in PBMCs with no effect on either IL-2 or IL-4 (469, 470). (The structure for OM-01 has not been disclosed.)