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PREFACE

Annual Reports in Medicinal Chemistry continues to focus on providing timely and critical reviews of important topics in medicinal chemistry together with an emphasis on emerging topics in the biological sciences, which are expected to provide the basis for entirely new future therapies.

Volume 40 mostly retains the familiar format of previous volumes, this year with 29 chapters. Sections I–IV are disease-oriented and generally report on specific medicinal agents with updates from Volume 39. As in past volumes, annual updates have been limited only to the most active areas of research in favor of specifically focussed and mechanistically oriented chapters, where the objective is to provide the reader with the most important new results in a particular field.

Sections V and VI continue to emphasize important topics in medicinal chemistry, biology, and drug design as well as the critical interfaces among these disciplines. Included in Section V, Topics in Biology, is a chapter concerning alternative therapeutics indications for drug targets. Chapters in Section VI, Topics in Drug Design and Discovery include G-protein coupled reverse inverse agonists, Metabonomics, Prediction of blood-brain barrier permeation and pharmacogenetics.

Volume 40 concludes with an exciting chapter on the important topic of Pharmaceutical Innovation and last but not least is our regular chapter ''To Market, To Market'' covering NCE and NBE introductions worldwide in 2004. In addition to the chapter reviews, a comprehensive set of indices has been included to enable the reader to easily locate topics in Volumes 1–40 of this series.

Volume 40 *of Annual Reports in Medicinal Chemistry* was assembled with the superb editorial assistance of Hannah Young and I would like to thank her for her hard work and enduring support. Volume 40 completes my 7th and last year as Editor-in-Chief of Annual Reports in Medicinal Chemistry. During this period, it has been my pleasure to work with 12 enthusiastic and highly professional section editors and I thank them sincerely for their dedication. I would also like to thank all of the authors who have contributed during my tenure as Editor-in-Chief. Their insights and creative input to each chapter have contributed to the success of this series. I hope that you the reader will enjoy and profit from reading this volume.

Annette M. Doherty
Sandwich, UK
June 2005

CORRIGENDUM

We would like to correct some errors that occurred in Volumes 37 and 39 with our apologies to the authors and readers.

Annual Reports in Medicinal Chemistry Vol. 37

''Recent Advances in Pulmonary Hypertension Therapy'' by Russell A. Bialecki. The author refers to a paper written by Per A. Whiss (39 – P.A. Whiss and R. Larsson, Hemostasis, 28, 260 (1998).) and a paper written by his colleagues (38 – M. Grenegard, M.C. Gustafsson, R.G. Anderson and T. Bengtsson, Br. J. Pharmacol., 118, 2120 (1996).) at the Division of Pharmacology. The author incorectly defines these papers as ''confounding reports''. However, the results presented in these papers show the opposite, namely that GEA 3175 inhibits adenosine 5'-diphosphate-induced (39) but not thrombin-induced (38) aggregation of platelets from healthy humans.

Annual Reports in Medicinal Chemistry Vol. 39

''To Market, To Market – 2003'' by Shridhar Hedge and Jeffery Carter. Tadalafil should have been attributed to GlaxoSmithKline as the originator. The drug was indeed introduced by Lilly/ICOS as stated.

Tadalafil (Male sexual dysfunction) (94–98)

Country of Origin:	US
Originator:	GlaxoSmithKline
First Introduction:	UK, Germany
Introduced by:	Lilly/ICOS
Trade Name:	Cialis
CAS Registry No.:	171596-29-5
Molecular Weight:	389.41

Neuronal Nicotinic Acetylcholine Receptor Modulators: Recent Advances and Therapeutic Potential

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Contents

1. INTRODUCTION

The concept of nicotinic acetylcholine receptors (nAChRs) as targets with therapeutic potential is now well established, and has been the subject of several recent reviews [1–4]. Numerous publications continue to provide evidence for a role of nAChRs in the etiology and potential treatment of neurological diseases. This review will focus on recent developments supporting nAChR ligands as therapeutics in diverse diseases. Such developments include progression of compounds into the clinic, characterization in behavioral models and the discovery of new ligands with distinctive pharmacology, structure or therapeutic potential.

2. CLINICAL AND PRECLINICAL DEVELOPMENT COMPOUNDS

2.1. Cognition, dementia and schizophrenia

Impairment of various aspects of cognitive function is associated with a number of neurological and neuropsychiatric disorders, including schizophrenia, Alzheimer's

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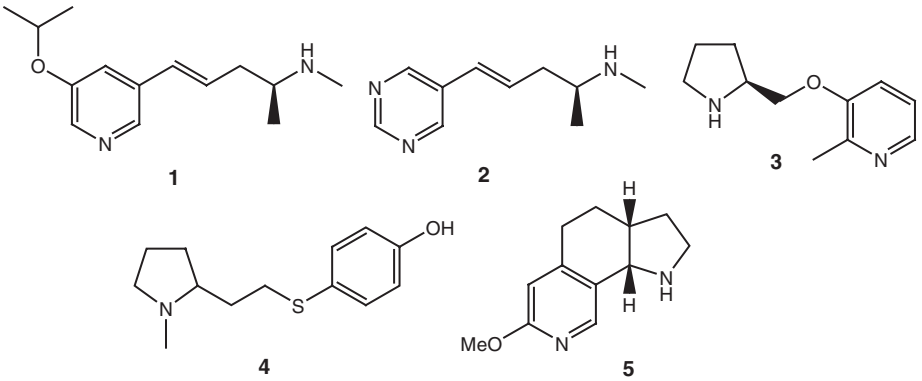
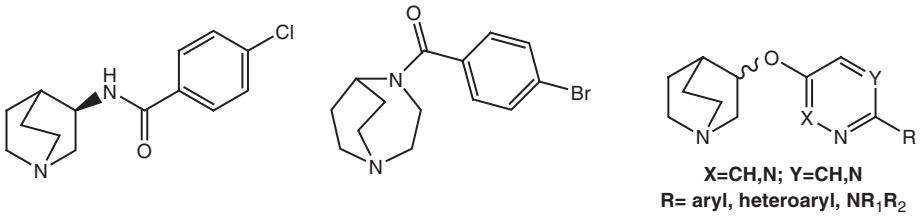
disease (AD), and attention deficit hyperactivity disorder. Currently, treatment options for neurodegenerative diseases are limited to AChE inhibitors to enhance cholinergic transmission and temporarily offset cognitive deficits. The role of nicotinic receptors in the etiology and treatment of cognitive disorders has been the subject of many recent papers and reviews [5–13]. The most compelling support for the concept of nicotinic ligands for the treatment of cognitive disorders in neurodegenerative diseases comes from the observation that a substantial loss of high affinity receptors accompanies disease progression [11,14]. Thus, a nicotinic drug that provides protection against neuronal degeneration and enhances cholinergic transmission may potentially be useful in both symptomatic improvement and delay of disease progression [8]. Cognitive deficits in schizophrenia include attentional disorders, slow information processing, working memory disorders and lack of flexibility of adaptive strategies [15,16]. A recent consensus meeting (MATRICS initiative) has identified cognitive impairment in schizophrenia as the underlying substratum for the negative symptoms, a hallmark of schizophrenia that contributes significantly to the lack of functionality of the patient [17]. Several recent papers have addressed the role of nAChRs in cognitive deficits in schizophrenia [1,7,9,11,12,18–22]. Presently, no treatment is approved to address these aspects of schizophrenia symptomatology.

The highly selective $\alpha_4\beta_2$ agonist ispronicline (TC-1734, **1**) has shown activity *in vivo* in several animal models indicative of cognitive enhancement (e.g., step through passive avoidance, object recognition, radial arm maze) [23]. In Phase I clinical studies, ispronicline in single oral doses up to 320 mg was well tolerated and possessed linear pharmacokinetics [23]. In preclinical studies, TC-1827 (**2**), a full agonist selective for the $\alpha_4\beta_2$ subtype, demonstrated potent activity in several species including mice, rats and non-human primates [24]. Cognitive improvement was observed in chemically-induced amnesia, as well as in aged and normal animals, as measured by performance in step through passive avoidance and object recognition models. It exhibited good pharmacokinetics, acceptable cardiovascular tolerability and lack of side effects associated with peripheral receptor stimulation in mice, rats and monkeys. The pyridyl ether ABT-089 (**3**) has been shown to be effective in preclinical models of impaired cognitive function, including aging, septal lesion, and scopolamine-induced deficits in the Morris water maze [25]. This compound recently completed Phase I clinical trials and was reported to have an excellent pharmacokinetic profile in humans, good cardiovascular and gastrointestinal tolerability, and positive signs of cognitive effect as measured by decreases in reaction time [25]. SIB-1553 (**4**) was shown to improve working memory performance in both aged and scopolamine-treated mice, with a cognitive enhancing effect equal to or greater than that of nicotine and with an improved margin of safety relative to nicotine [26]. SIB-1663 (**5**), a conformationally rigid analog of nicotine, activates $\alpha_3\beta_4$ and $\alpha_4\beta_4$ subtypes with little activity toward β_2-containing subtypes [27]. *In vivo*, animals treated with SIB-1663 showed improved performance in retention in the inhibitory avoidance paradigm.



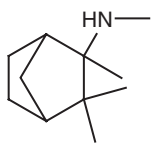
The therapeutic potential of α_7 receptor agonists to treat the cognitive and/or negative symptoms of schizophrenia is well supported in the literature [18,22,28]. The novel, α_7-selective nAChR agonist PNU-282987 (**6**) restored amphetamine-induced sensory gating deficits as determined by auditory evoked potentials in the hippocampal CA3 region [29]. The α_7 nAChR partial agonist SSR180711A (**7**) ($K_i = 50\,nM$, $E_{Max} = 38\%$, $EC_{50} = 0.8\,\mu M$,) has demonstrated efficacy in animal models predictive of cognitive deficits related to schizophrenia [30]. A series of 3-heteroaryloxy-quinuclidine agonists (**8**) with α_7 functional activity (EC_{50}'s in the 10 nM to 10 μM range) were reported to restore sensory gating in DBA/2-mice at concentrations of 10 to 40 μM [31].



2.2. Anxiety and depression

While a causal link between mood disorders and a dysfunction of the nicotinic cholinergic system has not been definitively established, compelling evidence exists suggesting a relationship [32–35]. A number of antidepressants in clinical use have been identified as antagonists at nicotinic receptors [32]. Nicotine and mecamylamine (**9**) have been shown to potentiate the effects of both imipramine and citalopram in the mouse tail-suspension test [36]. Mecamylamine also potentiates the effects of amitriptyline in the mouse forced swim test [37]. The novel 2, 7-diazaspiro[4.4]nonane TC-2216 (**10**) is a highly selective modulator for $\alpha_4\beta_2$

($K_i = 42\,nM$, no affinity for α_7, minimal interaction with $\alpha_3\beta_4$) which exhibited preclinical activity in the forced swim test, a behavioral model predictive of clinical antidepressant effects [38,39]. The pyridyl ether A-85380 (**11**) was also active in the forced swim test; it was suggested that nicotine and related agonists with antidepressant effects may be achieving their effect at least in part through interaction with the $\alpha_4\beta_2$ receptor [40]. The selective $\alpha_4\beta_2$ agonist A-186253 (**12**) has also demonstrated activity in the rat and mouse forced swim test models [40]. Evidence for a therapeutic application of nicotinic modulators in treatment of anxiety disorders is limited to a few studies reporting effects of nicotine or mecamylamine administration on measures of anxiety [34,41].



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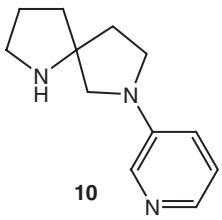

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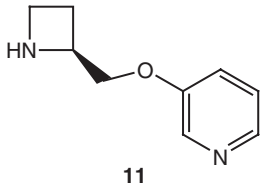

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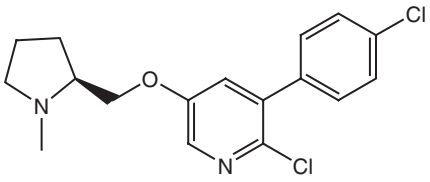

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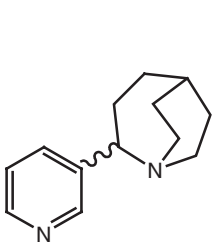
2.3. Neuroprotection

Recent reports and reviews citing nicotinic mechanisms in neuroprotection include *in vitro* and *in vivo* studies in brain regions implicated in neurodegenerative diseases such as cortical, hippocampal, and striatal structures [23,42–46]. Neuroprotection has been reported against a variety of insults including β-amyloid-mediated neuronal death, N-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP) toxicity, glutamate excitotoxicity, and growth factor, oxygen and glucose deprivation studies [12,42]. TC-1698 (**13**), a novel α_7 agonist, has been shown to provide neuroprotection against Aβ through an effect on the JAK2/PI-3K cascade [45]. Galanthamine and donepezil have been shown to protect rat cortical neurons against Aβ-enhanced glutamate toxicity, and the authors propose that these effects are mediated through activation of nicotinic receptors [43,46,47]. Ispronicline (**1**), in addition to its cognitive effects, exhibited neuroprotective properties *in vitro* in glutamate-induced toxicity in primary cortical neurons and in hippocampal slices following glucose/oxygen deprivation [23]. The α_7 antagonist MLA (**14**) has also been shown to partially protect against Aβ toxicity in primary neuron-enriched cultures [44].



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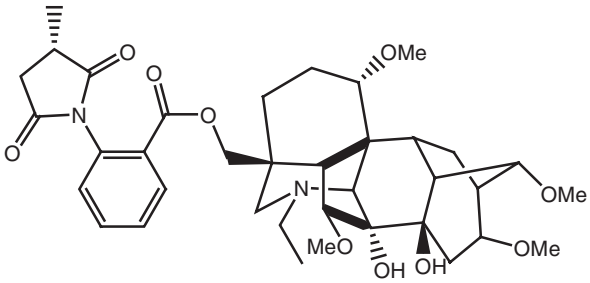

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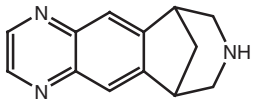
2.4. Addiction disorders

It is believed that activation of certain nicotinic receptor subtypes significantly contributes to the reinforcing effects of nicotine, cocaine and amphetamine through stimulated release of neurotransmitters [48–50]. Both antagonists and agonists at the $\alpha_4\beta_2$ subtype have been proposed as therapeutic agents for smoking cessation and drug addiction [51–53], and antagonists at the $\alpha_3\beta_4$ subtype appear to have anti-addictive properties [54,55]. Interestingly, the antidepressant/smoking cessation aid bupropion is not only a dopamine/noradrenaline uptake inhibitor, but also a noncompetitive antagonist at several nAChR subtypes. It has been proposed that this antagonism contributes to its clinical efficacy [33].

Varenicline (**15**), a partial agonist at the $\alpha_4\beta_2$ nAChR subtype, is reportedly in clinical development for smoking cessation [2,56]. SSR-591813 (**16**), a conformationally constrained pyridyl ether, is a novel ligand selective for the $\alpha_4\beta_2$ subtype ($K_i = 36$ nM; selectivity *vs.* other human receptor subtypes: 3 to 167-fold) [57]. SSR-591813 behaves as a partial agonist ($EC_{50} = 1.3\,\mu M$, 19% at 100 μM *vs. N, N*-dimethylphenylpiperazinium) at human $\alpha_4\beta_2$ nAChRs expressed in oocytes, and in dopamine release (brain microdialysis, 59% increase at 30 mg/kg i.p.; 2-fold less than that of nicotine). The compound shows activity in animal models of nicotine dependence at doses devoid of hypothermia and cardiovascular effects, reduces i.v. nicotine self-administration and antagonizes nicotine-induced behavioral sensitization in rats [57]. 18-methoxycoronaridine (**17**) is a noncompetitive antagonist of the $\alpha_3\beta_4$ subtype ($IC_{50} = 0.8\,\mu M$) [58]. In self-administration studies of methamphetamine and morphine in the rat, **17** and congeners reduced self-administration by up to 50% at 20 mg/kg [50,59]. The efficacy of analogs in this assay was directly proportional to their inhibitory potency at $\alpha_3\beta_4$.



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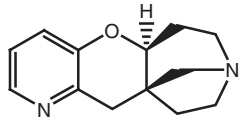

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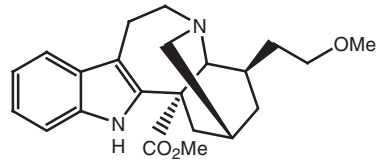

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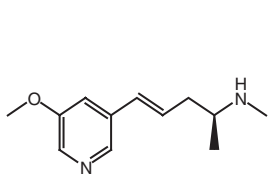
2.5. Analgesia

The potential for nicotinic agonists to produce analgesic effects is now well established and has been the subject of numerous reviews [1,60–64]. Thus far, inadequate therapeutic indices have prevented the successful development of analgesic nicotinics [63]. It has recently been proposed that development of agents targeted for specific pain states, such as neuropathic pain, may be more readily achieved than the development of broad spectrum analgesics [63].

The metanicotine TC-2696 (**18**), a selective $\alpha_4\beta_2$ agonist, is in clinical development for treatment of pain [65]. In preclinical models of pain, TC-2696 showed potency comparable to morphine with no development of tolerance to analgesic effects. Most notably, the analgesic effects were not associated with the nausea, vomiting or cardiovascular effects often seen with potent nicotinic agonists lacking adequate subtype selectivity. ABT-894, a second-generation nAChR agonist follow-on to ABT-594, is reportedly in clinical development for treatment of neuropathic pain [64]. No structure or pharmacological data have been disclosed. The 3,6-diazabicyclo[3.2.0]heptane (**19)** has been reported to be an effective analgesic in preclinical models of pain [66,67]. It has a binding affinity of 0.1 nM at the $\alpha_4\beta_2$ subtype, and was reported to possess good selectivity relative to ganglionic activation. In the ligation model for mechanical allodynia, an analgesic effect was seen ($ED_{50} = 1\,\mu M/kg$). A series of azabicyclic compounds, exemplified by the 3-azabicyclo[3.3.0]oct-6-ene (**20)**, were reported to show efficacy in preclinical models of pain (mouse hotplate and rat formalin, no data provided) [68].

Homoepiboxidine (**21**) has been prepared and characterized [69]. Like epiboxidine, homoepiboxidine is an agonist at $\alpha_4\beta_2$ and neuromuscular receptors, but is less active at ganglionic α_3 subunit-containing receptors. In providing analgesia, it was as efficacious as epibatidine in the hot-plate test, but 10-fold less potent. While it did have a longer duration of action than epibatidine, the functional selectivity proved inadequate to effectively separate analgesic properties from toxicity. The epibatidine analog (−)-2-fluoro-3-phenyl-deschloroepibatidine (**22**, $K_i = 0.26$ nM at $\alpha_4\beta_2$) has been found to be a potent nAChR antagonist in tail-flick and hot-plate tests (reversal of nicotine antinociception with $AD_{50} = 0.7$ mg/kg in hot-plate, 0.08 mg/kg in tail flick), as was 3′-aminoepibatidine (**23**, $K_i = 0.01$ nM at $\alpha_4\beta_2$; $AD_{50} = 30$ ng/kg in tail flick, 0.6 mg/kg in hot-plate) [70,71].



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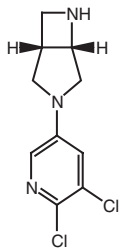

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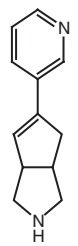

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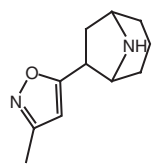

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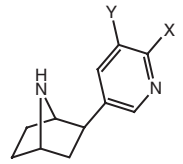


22 X=F, Y=Ph
23 X=Cl, Y=NH_2

2.6. Inflammation

Many recent reports suggest that α_7 modulation has therapeutic potential for treatment of inflammatory diseases. Nicotinic receptors, specifically the α_7 subtype, appear to be involved in the inflammatory process [72–75]. *In vivo* treatment with nicotine has been reported to modulate an inflammatory pathway through the α_7-stimulated suppression of high mobility group box 1 (HMGB1) secretion, thereby improving survival in models of sepsis [73].

3. NEW THERAPEUTIC INDICATIONS

Recent reports have continued to expand the number of indications for which nAChR ligands may prove to have a therapeutic effect. A relationship between nAChRs and angiogenesis [76,77] and a role for central and peripheral nAChRs in lower urinary tract dysfunction have been suggested [78–80]. A genetic defect in the $\alpha_4\beta_2$ receptor subtype has been associated with a form of epilepsy; thus, selective agonists may have potential as anticonvulsants [1,81,82]. Agonists at the α_7 subtype have been proposed for treatment of glaucoma, macular degeneration and diabetic retinopathy through a neuroprotective and antiangiogenic nicotinic mechanism [83], while antagonists may be beneficial in suppression of certain cancers [84].

4. NEW LIGAND CHARACTERIZATION

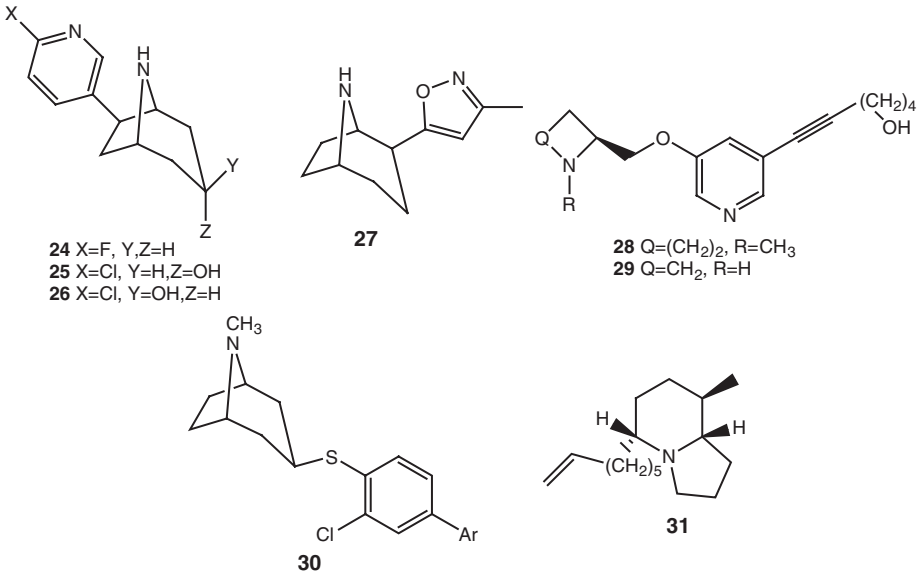
Numerous compounds appearing in recent journal and patent literature have not yet been characterized *in vivo*. Many of these compounds have been cited in previous review articles [85–88]. The ligands presented here are either new or additional data has recently been reported.

4.1. Heteromeric nAChR subtype selective ligands

The ring-expanded analog norchlorofluorohomoepibatidine (**24**, NCFHEB) shows significant subtype selectivity among several nAChRs. In contrast to most reported ligands, (+)−NCFHEB displayed 59-fold selectivity for $\alpha_3\beta_4$ *vs.* $\alpha_4\beta_2$ subtypes [89]. The related hydroxytropane (**25)** has binding affinities 160- to 500-fold less than those of epibatidine at the standard heteromeric nicotinic receptors, and is an agonist at $\alpha_3\beta_4$ ($K_i = 88$ nM, $EC_{50} = 2.1$ μM, 100-fold less potent than epibatidine) [90]. Surprisingly, the β-hydroxy epimer (**26)** is an antagonist at the same receptor ($K_i = 1023$ nM, $IC_{50} = 6.2$ uM) [91]. Several isoxazolyl-8-azabicyclo[3.2.1]octanes have been reported; binding affinity is generally poor at $\alpha_4\beta_2$ ($K_i = 194$-$26{,}000$ nM), with the exception of the 2-isoxazolyl β-isomer (**27)** ($K_i = 3$ nM) [92].

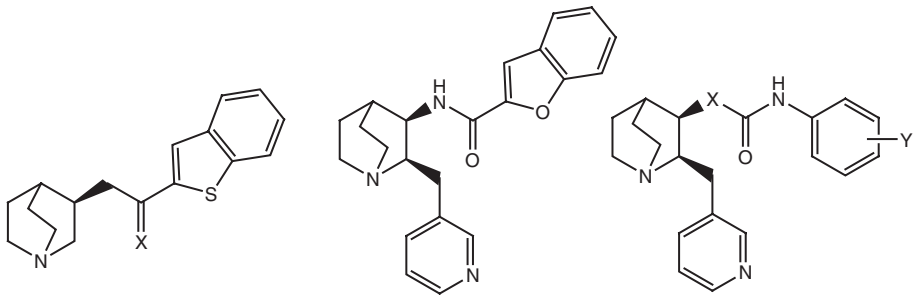
A finding was recently communicated in which selectivity across the nicotinic receptor subtypes was dramatically improved for a series of pyridyl ethers by

introducing an alkyne-containing appendage in the 5′-position [93]. The pyrrolidinylmethyl ether (**28**) had a $K_i = 0.8$ nM at $\alpha_4\beta_2$ and $K_i = 40,200$ nM at $\alpha_3\beta_4$, while the corresponding azetidinylmethyl ether (**29**) had an $\alpha_4\beta_2$ K_i of 0.09 nM and an $\alpha_3\beta_4$ K_i of 4,840 nM. A series of biarylthiotropanes (**30**) has been reported with selective agonist activity toward the β_4 subtype ($K_i = 15$–28 nM) [94,95]. Attempts to improve the bioavailability and increase affinity were disappointing, as the molecule was intolerant to modification. Indolizidine (−)-235B (**31**) has been reported as a potent open-channel blocker of $\alpha_4\beta_2$ nAChRs ($IC_{50} = 0.07\,\mu M$), and is selective *vs.* $\alpha_3\beta_2$ (40-fold) and $\alpha_3\beta_4$ (51-fold) [96].



4.2. α_7 nAChR subtype selective ligands

Compounds related to the well known 1-azabicyclo[2.2.2]oct-3-yl amides, but lacking the 3-amino group, have been reported as high affinity α_7-selective ligands [97]. The (+)-ketone (**32**) is a potent partial agonist (~30% at 10 μM) while (+)-**33** is a weak partial agonist (~20% at 100 μM). A novel series of 2-(pyridin-3-ylmethyl)-3-quinuclidinyl ligands has been disclosed recently [98]. The series, represented by amide **34**, carbamate **35** and urea **36**, demonstrated high affinity binding and selectivity for the α_7 nAChR (K_i values of 0.3 nM, 6 nM and 6 nM, respectively [99]. Introduction of the pyridylmethyl substituent was reported to improve the selectivity of the carbamates relative to muscarinic receptors and augment α_7 affinity in comparison to the known 3-quinuclidinyl carbamates. The 2,3-*cis* isomers gave higher affinity binding than the corresponding *trans* isomers. Amide **34** was also identified as a potent, full agonist at α_7 ($EC_{50} = 33$ nM, $I_{Max} = 1.0$ relative to ACh) with relatively low residual inhibition (desensitization).

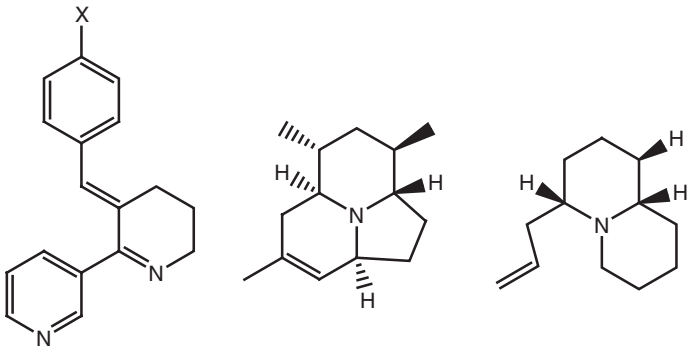


32 X=O
33 X=H_2

34

35 X=O, Y=4-Br
36 X=NH, Y=3-F

Structure-activity relationship data has been published for a series of benzylideneanabaseine derivatives [100]. While the unsubstituted benzylideneanabaseine (**37**) had an EC_{50} of 45 μM, the 4-hydroxy analog (**38**) had an EC_{50} of 2.3 μM. Substitution with a methylthio group or trifluoromethyl group abolished activity. The alkaloids (+)-205B (**39**) and (−)-1-*epi*-2071 (**40**) have been reported as selective inhibitors for α_7 receptors (**39**: $IC_{50} = 2.5\,\mu M$; 5.4 fold *vs.* $\alpha_4\beta_2$; **40**: $IC_{50} = 0.6\,\mu M$; 8.7-fold *vs.* $\alpha_4\beta_2$) [96].



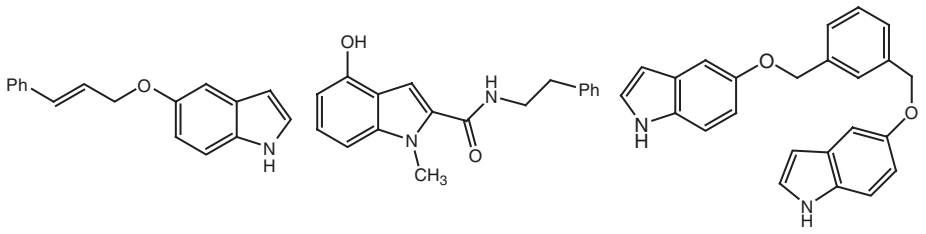
37 X=H
38 X=OH

39

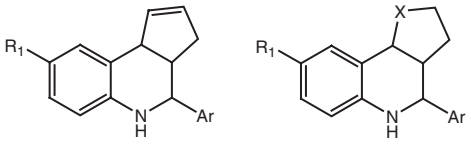
40

4.3. Allosteric modulators

The concept of modulation of nicotinic receptors through an allosteric site has gained popularity in the recent literature [101–105]. Several reviews have cited the therapeutic potential of such modulation [106,107]. Positive allosteric modulation of the α_7 receptor is considered particularly desirable since it should avoid the desensitization often associated with stimulation by full agonists. Several ligands reported to positively modulate the α_7 receptor have been based on substituted indole ethers or amides (**41**–**43**) [108–110]. Most recently, a novel series of compounds was reported based on tetra- and hexahydroquinoline scaffolds (**44**, **45**) [111].



41 42 43



44 45

R1= SO_2NH_2, $NHSO_2Ar$
Ar= Ph, Naphthyl, $PhNO_2$
X=O,S,CH_2

5. CONCLUSION

Intense research over the last several years has led to a number of positive developments in the field of nicotinic receptor modulation. Promising compounds are advancing through clinical trials. Many new and existing compounds have been characterized in animal models, with results supporting the potential of nicotinic ligands as therapeutics in the treatment of a variety of disease states. The increasing number of novel, diverse ligands with subtype specificity available for pharmacological study has allowed further elucidation of the roles of nicotinic receptors in normal and pathological states. Finally, as a result of the heightened understanding of receptor pharmacology, many new therapeutic targets with potential clinical application have been identified.

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Recent Advances in Selective Serotonergic Agents

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Contents

1. INTRODUCTION

Arguably, serotonin (5-HT) is one of the most studied of the neurotransmitters. The identification of 5-HT as a vasoconstricting agent over 50 years ago [1] and the discovery that more than one subtype of 5-HT receptor exists [2] marked the beginning of a monumental effort that has extended our knowledge, not only of 5-HT receptors, but of G-protein coupled receptors (GPCR's) in general. There are presently fourteen known 5-HT receptor subtypes, some of which exist as multiple splice variants. They are located both centrally and peripherally, influence a number of physiological functions, and are implicated in many disease states [3].

Numerous reports describe ligands that bind to multiple 5-HT receptor subtypes with high affinity or agents that interact with the 5-HT uptake site. The recent literature on these pursuits is extensive and merits review in its own right. The goal of this chapter is to summarize recent advances in selective 5-HT receptor modulators [4,5].

2. 5-HT_1 RECEPTOR FAMILY

5-HT_1 receptors make up the largest class of serotonin receptor subtypes. They are seven transmembrane receptors that are negatively coupled to adenylyl cyclase via

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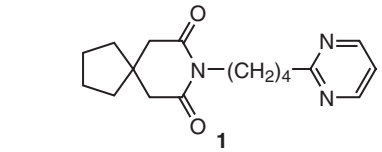
the G-proteins Go and Gi [6]. 5-HT_1 receptors are grouped into five major subtypes (5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E} and 5-HT_{1F}), based on conservation of structure, pharmacology and common signaling mechanisms [7]. Some 5-HT_1 receptor subtypes have received a great deal of attention with respect to drug development while others are less characterized and their potential as drug targets remains to be fully explored. Several reviews on 5-HT_1 receptors have appeared in the recent literature [8].

2.1. 5-HT_{1A} receptor ligands

Research efforts in the 5-HT_{1A} arena have delivered a number of clinical candidates. The partial agonist buspirone (Buspar®, **1**) is prescribed for the treatment of anxiety. Preclinical evidence suggests that full agonists may be useful as antiischemic agents [9]. A detailed list of the numerous recent additions to the 5-HT_{1A} agonist field is beyond the scope of this chapter. However, many of these new series and molecules are discussed in a 2003 review article [10]. Recent reports describe series that lack the carbonyl moiety of the stereotypical "spiroimide" scaffold inherent in compounds like buspirone. Indoleamine **2a** bound to 5-HT_{1A} receptors with a Ki value of 0.09 nM and >1000-fold selectivity over other biogenic amine receptors [11]. A related analog, **2b**, was shown to be a full agonist *in vitro* that displayed oral activity in a rat ultrasonic vocalization model (ID_{50} 1.5 mg/kg p.o.). A great deal of structural variation is tolerated within this general class of "carbonyl-lacking" long-chain arylpiperazine agonists. Potent affinity was seen with pyrimidopurine derivatives such as **3** (Ki 11 nM), which displayed anxiolytic-like activity in a behavioral conflict drinking test and antidepressant activity in a forced swimming model [12]. SAR within a series of nonselective dioxopyrrolopyrazines has led to the identification of 5-HT_{1A} agonists with improved 5-HT_{1A}/α_1 selectivity [13]. Compound **4** (CP-2503) demonstrated good 5-HT_{1A} affinity (Ki 4.1 nM) and >1000-fold selectivity vs. α_1, although only marginal selectivity vs. 5-HT_{2A} and 5-HT_3 were realized (3- and 2-fold, respectively). Compound **4** displayed full agonist activity *in vitro* and *in vivo* and anxiolytic-like effects in a light/dark box model.



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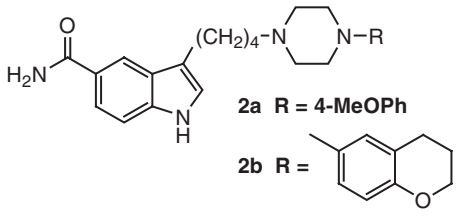
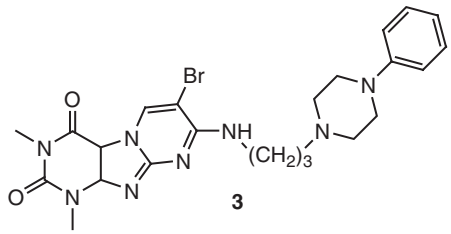
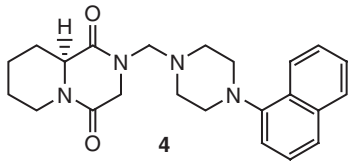


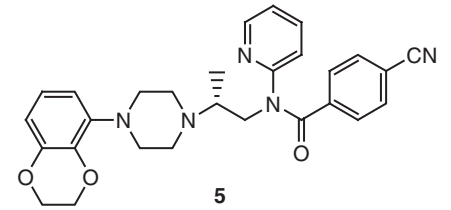
2a R = 4-MeOPh

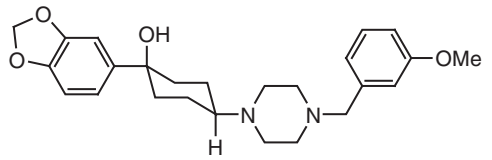
2b R =





Mounting evidence suggests that 5-HT_{1A} antagonists may reverse the cognitive deficits seen in Alzheimer's Disease [14]. Identifying 5-HT_{1A} full antagonists has been difficult, owing to the fact that weak partial agonists may appear as antagonists in assays mediated by post-synaptic 5-HT_{1A} receptors. Lecozotan (**5**), demonstrated potent affinity (Ki 1.7 nM), >100-fold selectivity, antagonist activity in two *in vitro* functional models, and activity in a rat fixed ratio responding model that is indicative of a full antagonist [15]. It is reported to be in Phase II clinical trials [16]. SAR in a series of aryl cyclohexanols has identified **6**, a selective 5-HT_{1A} antagonist (IC_{50} 2.2 nM) which demonstrated antagonist activity in both *in vivo* microdialysis and electrophysiology assays [17]. Computational models of 5-HT_{1A} antagonist pharmacophores have begun to appear in the literature [18,19].

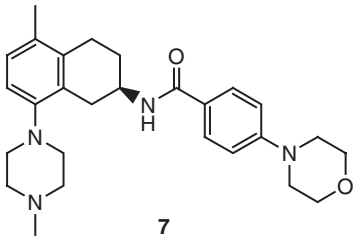




2.2. 5-HT_{1B} receptor ligands

Once thought to be a rodent-specific protein, the 5-HT_{1B} receptor has received heightened attention. Stimulation of the 5-HT_{1B} receptor is thought to underlie the peripheral vasoconstriction liabilities seen with many mixed 5-HT_{1B}/5-HT_{1D}

agonist triptan agents currently employed for the treatment of migraine [20]. 5-HT_{1B} agonists may have potential in the treatment of excessive aggressive behavior [21], and selective antagonists and inverse agonists could possess cognitive enhancing properties [22,23]. The identification of selective 5-HT_{1B} ligands, however, continues to be problematic. AR-A000002 (**7**), a selective 5-HT_{1B} antagonist, bound with high affinity [24] to native and recombinant guinea pig 5-HT_{1B} receptors (Ki 0.24 and 0.47 nM, respectively). It showed a 10-fold selectivity over 5-HT_{1D} and demonstrated antagonist activity *in vitro*. *In vivo*, compound **7** enhanced 5-HT release in guinea pig cortex [25] and displayed efficacy in animal models of anxiety and depression [26].



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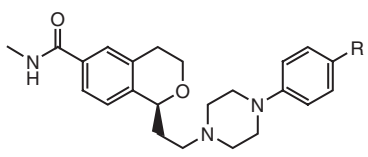
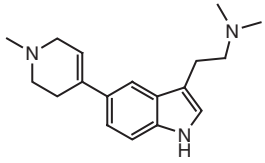
2.3. 5-HT_{1D} receptor ligands

The past five years has witnessed the introduction of a host of mixed 5-HT_{1B}/5-HT_{1D} into the marketplace as antimigraine agents which possess superior pharmacokinetic profiles and reduced cardiovascular side effects compared to first generation triptans [27]. However, efforts continue in the search for selective 5-HT_{1D} agonists, which may provide effective antimigraine therapy while eliminating the vasoconstricture liabilities in peripheral arteries thought to originate from 5-HT_{1B} agonist activity. Selective 5-HT_{1D} agonists have now entered clinical trials, but the results are equivocal. Two structurally-related analogs, PNU-109291 (**8**) and PNU-142633 (**9**), failed to demonstrate efficacy in clinical trials despite efficacy in animal models of migraine and excellent oral bioavailability [28–30]. ALX-0646 (**10**) is currently in Phase I clinical trials, where it is reported to have demonstrated minimal cardiovascular liability [31]. Preclinically, **10** displayed affinity for the 5-HT_{1D} receptor (Ki 8 nM) with 76-fold selectivity over 5-HT_{1B} [32] and fully blocked neurogenic dural inflammation. SAR studies around the basic structure of ALX-0646 have yielded potent, selective 5-HT_{1D} agonists [33,34]. Compound **11** had a Ki value for human 5-HT_{1D} receptors of 2.5 nM, and demonstrated good oral bioavailability in the rat (F 51%).



8 R = OMe
9 R = $CONH_2$



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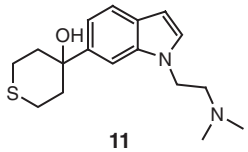


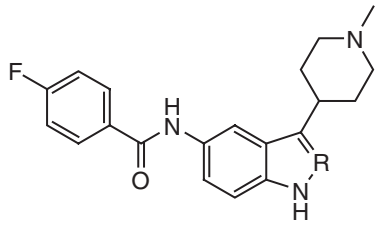
11

2.4. 5-HT$_{1E}$ receptor ligands

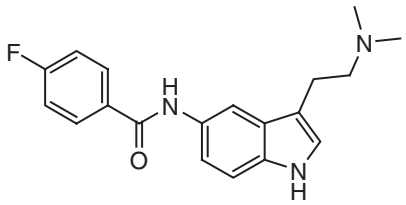
Progress in the 5-HT$_{1E}$ area has been hindered, in part, by the difficulty of identifying a suitable animal species which expresses this receptor. A recent report suggests that purported 5-HT$_{1E}$ receptors identified in rat and mouse may in fact be more closely related to the 5-HT$_{1F}$ receptor [35]. The report goes on to describe the identification and cloning of 5-HT$_{1E}$ receptors from guinea pig genomic DNA. To date, no selective 5-HT$_{1E}$ receptor ligands and no specific pharmacological functions have been identified for the 5-HT$_{1E}$ receptor. Using known tryptamine and ergoline derivatives, one group has performed a Comparative Molecular Field Analysis study and has proposed a model of structure-affinity requirements [36].

2.5. 5-HT$_{1F}$ receptor ligands

The past four years has witnessed an increase in efforts to identify selective 5-HT$_{1F}$ agonists as drug targets for the treatment of migraine [37]. Such selective ligands may be devoid of the cardiovascular liabilities inherent in currently used tryptans which possess high 5-HT$_{1B}$ affinity [20]. SAR studies around the discontinued clinical candidate LY334370 (**12**) have yielded a number of publications. Replacement of the typical indole moiety with bioisosteric groups such as azaindole [38] and furo[3,2b]pyridine [39] yielded derivatives with potent 5-HT$_{1F}$ affinity and reasonable selectivity for other 5-HT$_1$ receptors. Substituting indazole for indole gave compound **13**, which displayed a Ki value of 3.9 nM for 5-HT$_{1F}$ affinity and $>$200-fold selectivity for 5-HT$_{1A}$, 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors [40]. Compound **14** was the highlight of a fourth report [41]. This full agonist showed good potency (Ki 8.2 nM) with a 32-fold selectivity over 5-HT$_{1A}$ and $>$100-fold selectivity over a number of other biogenic amine receptors. It inhibited neurogenic dural inflammation (ID_{50} 4.3 ng/kg p.o.) and did not induce contractions in a rabbit saphenous vein preparation at concentrations up to 100 μM.



12 R = C-H
13 R = N



14

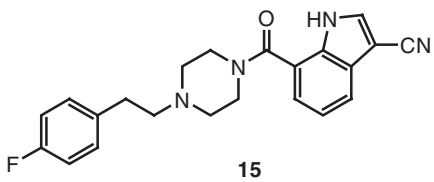
3. 5-HT$_2$ RECEPTOR FAMILY

The 5-HT$_2$ receptor family represents a significant component, both in terms of function and clinical use, of the serotonin receptor subtypes. As a subfamily, these

GPCR's are positively coupled through the Gq/11 family of G-proteins eliciting their second messenger effects predominantly through increases in activity of phospholiopase C (diacylglycerol pathway) and/or phospholipase A (arachidonic acid pathway). Three distinct subtypes of the 5-HT_2 receptors exist: 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C}. These subtypes share an overall amino acid homology of approximately 50% [42]. Although interest in 5-HT_{2A} antagonists for use in the antipsychotic field continues, the majority of effort in this area in the recent past has been focused almost exclusively on 5-HT_{2C} agonist ligands.

3.1. 5-HT_{2A} receptor ligands

Since the clinical demise of M100907, the most widely investigated selective 5-HT_{2A} antagonist (Ki 0.4 nM) targeted at schizophrenia [43], news of clinical efforts targeting selective ligands has been scarce. Recently a novel, selective antagonist for this receptor subtype, EMD-281014 (**15**), has been reported [44]. This potent ligand (Ki 0.35 nM) shows excellent selectivity over a wide range of related receptors. In rodent behavioral assays examining its *in vivo* potency in a number of anxiety paradigms, **15** had shown activity (i.v. dosed) only in preventing the symptoms of hyperarousal following severe stress. As in the past, combination therapies for schizoaffective disorders utilizing 5-HT_{2A} ligands have continued to be of interest. Selective 5-HT_{2A}/D_2 antagonists for use as potential antipsychotics have been reported [45], with **16** highlighted as a potent dual antagonist (Ki: 2.8 nM 5-HT_{2A}; 16 nM D_2) possessing oral activity in a rodent model of 5-HT_{2A} potency (ED_{50} 0.03 mg/kg p.o.). The researchers report that the ratio of 5-HT_{2A} antagonism to D_2 antagonism can be adjusted based on the core ring system and butyrophenone substitution.



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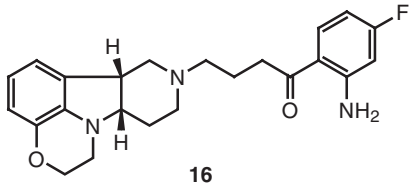


16

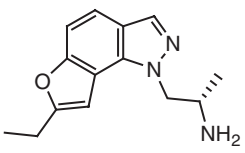
3.2. 5-HT_{2B} receptor ligands

Initially identified in rat stomach fundus [46], the 5-HT_{2B} receptor has been implicated in the treatment of migraines and gastric motility [47–49]. Additionally, 5-HT_{2B} receptor activation has been reported to produce hyperphagia [50], anxiolysis [51] and cell proliferation [52] possibly contributing to the heart valvulopathies associated with chronic use of fenfluramine [53]. As there is little known value for antagonists to this subtype and a high level of caution regarding the effects of 5-HT_{2B} agonism, there have been no reports of selective 5-HT_{2B} ligands in the recent past. The interested reader is directed to a recent comprehensive report discussing the interest in selective antagonists for this subtype [54].

3.3. 5-HT_{2C} receptor ligands

It is well established from the past decade that the potential uses for 5-HT_{2C} ligands include anxiety, depression, obesity and cognitive dysfunction [55–59]. Since the initial report of the 5-HT_{2C} knockout mouse [60], the interest in selective ligands had been very high but, until recently, unrealized. It has only been in the last few years that truly selective ligands have been discovered and activity in animal models been reported. In the recent past there has been a great deal of interest in 5-HT_{2C} agonists in particular for a variety of uses. Several reviews report on a flurry of activity in areas ranging from obesity to schizophrenia and depression [61–63].

There are several noteworthy reports of selective 5-HT_{2C} agonists. The tricyclic furanoindole YM-348 (**17**), shows good potency for 5-HT_{2C} but only modest selectivity over the closely related 5-HT_{2A} and 5-HT_{2B} receptors (15-fold and 3-fold, respectively) [64]. YM-348 has been shown to be orally active in both the rat penile erection model (typical for 5-HT_{2C} agonists) as well as in reducing weight gain in obese Zucker rats. Three additional potential antiobesity compounds have recently been reported. WAY-629 (**18**) has been shown to be a potent 5-HT_{2C} agonist with demonstrated oral efficacy in a rat model of feeding behavior [65]. This tetracyclic indole possesses excellent selectivity over a number of monoamine receptor subtypes. The discovery of novel benzazepines as 5-HT_{2C} agonists, with efficacy in an acute feeding paradigm, highlights **19** as a potent (Ki 3nM) ligand with excellent selectivity over the closely related 5-HT_{2B} and 5-HT_{2A} receptor subtypes [66]. The selectivity over 5-HT_{2B} in particular has been a difficult, and quite important, goal for most efforts in this area. The very recent disclosure of the selective 5-HT_{2C} partial agonist A37215 9 (**20**) (Ki 3 nM) underscores this point. The identification of this biaryl indoline with >100 fold selectivity, relative to the 5-HT_{2B} receptor, was the result of an extensive SAR study [67]. This analog was shown to be active in reducing weight gain (3, 10 mg/kg p.o.) in rats in a chronic feeding study that was conducted for 14 weeks without any indication of tolerance or adverse effects.



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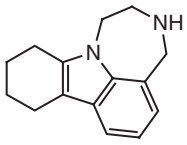

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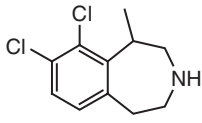

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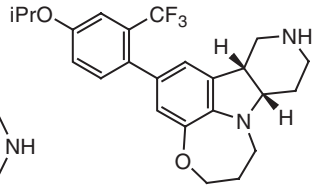

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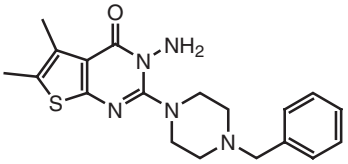
4. 5-HT_3 RECEPTOR FAMILY

In contrast to all other known 5-HT receptors, the 5-HT_3 receptor is a ligand-gated ion channel [68]. 5-HT_3 antagonists are well known in the literature and several are currently on the market for the treatment of chemotherapy-induced emesis and/or irritable bowel syndrome [69]. The chemistry and pharmacology of selective 5-HT_3 agonists is less well understood, although the state of the art in that area has

been reviewed recently [70]. Efforts continue to identify structural variations that are tolerated within the basic 5-HT_3 antagonist pharmacophore. SAR studies on fused heterocyclic thiophene analogs resulted in the identification of **21**, which displayed good potency for rat 5-HT_3 receptors (Ki 3.92 nM) and excellent selectivity over 5-HT_4 [71]. In a series of benzoisoindolones [72], compound **22** displayed good 5-HT_3 affinity (Ki 1.2 nM), *in vitro* antagonist activity (IC_{50} 12 nM) and blocked the Bezold-Jarisch reflex (ID_{50} 2.8 μg/kg i.v.). Compound **22** also prevented scopolamine-induced amnesia in a passive avoidance test at doses of 0.01–1.0 mg/kg i.p. New thienopyrimidines, represented by **23** [73], displayed moderate affinity for 5-HT_3 receptors (Ki 33 nM) and >100-fold selectivity for 5-HT_4. Functional studies suggest that **23** may act as a noncompetitive antagonist.



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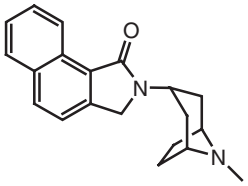

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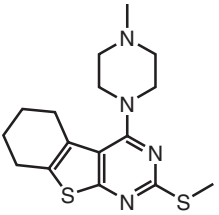
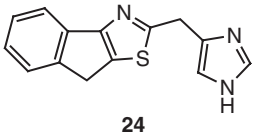


23

YM-31636 (**24**) is the most potent 5-HT_3 agonist in a series of indenothiazoles [74]. The compound displayed potent affinity for human 5-HT_3 receptors (Ki 0.2 nM) and excellent selectivity for a number of biogenic amine receptors, although data for 5-HT_4 were not presented. This compound demonstrated agonist activity in isolated guinea pig colon and anticonstipation effects in ferrets at doses of 0.03–3 mg/kg p.o.

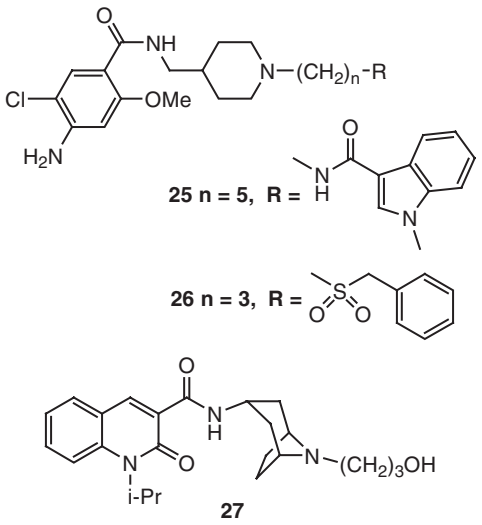


24

5. 5-HT_4 RECEPTOR FAMILY

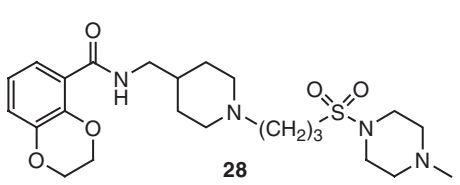
Significant advancement in the 5-HT_4 field has been realized in the past five years. Numerous reports provide evidence that 5-HT_4 agonists and partial agonists are useful in the treatment of irritable bowel syndrome [75]. A role in cognitive processes has been implicated for 5-HT_4 receptors as well [76–78]. Reviews on the 5-HT_4 receptor, its known ligands and therapeutic potentials have appeared recently [76,79–82]. Benzamide derivatives continue to generate interest as 5-HT_4 agonists. The poor oral bioavailability seen with the 5-HT_4 agonist Y-34959 (**25**) has been improved. Y-36912 (**26**) showed good affinity for guinea pig 5-HT_4 receptors and >500-fold selectivity for 5-HT4 over 5-HT_3 and D_2 receptors [83]. The compound demonstrated agonist activity in isolated guinea pig ascending colon (ED_{50}

10.8 nM) and enhanced gastric mobility and defecation in mice (MED 0.3–3 mg/kg p.o.). The oral bioavailability of **26** in dogs (76%) was significantly better than that seen with **25** (5%). A quinolone derivative [84], TS-951 (**27**), showed good 5-HT_4 affinity (Ki 11.8 nM), agonist activity *in vitro* (ED_{50} 32 nM) and good oral activity in canine gastrointestinal motility assays (0.003–0.3 mg/kg).



27

Side chain modification led to the identification of **28**, the third in a series of structurally similar 5-HT_4 antagonist clinical candidates [85]. This compound demonstrated strong affinity for the human cloned 5-HT_4 receptor (pKi 9.6), *in vitro* and *in vivo* antagonist properties, and an acceptable pharmacological profile in dogs. SAR within a related series of benzoates led to the identification of **29** [86]. Compound **29** displayed good affinity for four cloned human isoforms of the 5-HT_4 receptor (Ki's 2.47–8.1 nM) and antagonist activity in two *in vitro* models. Rhodopsin-based models of the 5-HT_4 receptor and site-directed mutagenesis have been employed to generate computational models of the interaction of the third transmembrane helix with several known 5-HT_4 antagonists [87–90].



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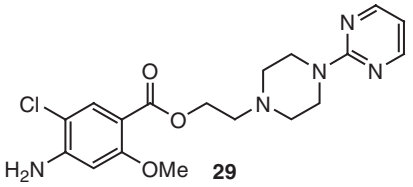
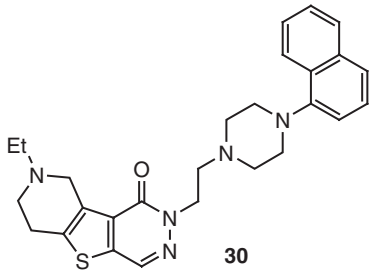


29

6. 5-HT_5 RECEPTOR FAMILY

Like 5-HT_{1E}, the 5-HT_5 receptor remains poorly understood. 5-HT_{5A} is present in human but the 5-HT_{5B} subtype appears to have been lost during evolution [91]. Review articles on this subject have appeared in the recent literature [92–94].

However, the lack of selective pharmacological tools continues to hinder progress in the area. A recent patent discloses 5-HT_5 ligands [95]. Compound **30** displayed affinity for recombinant human 5-HT_5 receptors (Ki 124 nM) and reduced infarct volume by 34% in a rat permanent middle cerebral artery occlusion model when given as an i.v. bolus followed by infusion, starting 90 minutes post-occlusion. Selectivity for 5-HT_5 versus other 5-HT receptors and agonist activity were inferred but data were not presented.



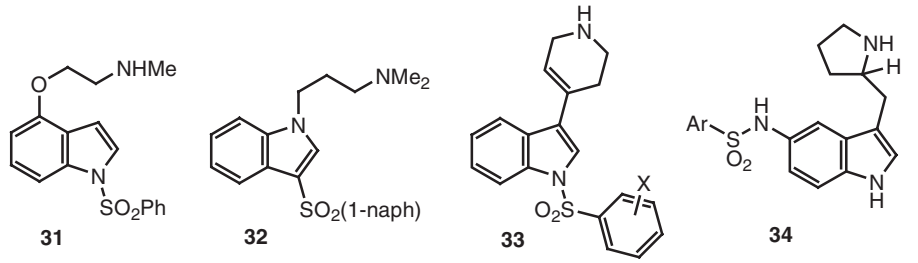
30

7. 5-HT_6 RECEPTOR FAMILY

The 5-HT_6 receptor is another GCPR in the serotonin family that positively couples to adenylyl cyclase through the G-protein Gs [96–98]. By mRNA, antibody mapping and radioligand binding, the receptor distribution in the CNS of humans and rats is most evident in the striatum with densities also noted in several other important brain regions [99–101]. The localization of 5-HT_6 receptors to limbic regions and the high affinity of therapeutic antipsychotics and antidepressants, have resulted in significant efforts to identify selective 5-HT_6 ligands for use in bipolar disorders, Parkinson's disease, and other affective disorders [102,103]. Furthermore, there has been a plethora of reports in the last five years implicating 5-HT_6 therapeutics in the modulation of cholinergic neurotransmission [104]. The application of these discoveries has led to significant effort in identifying selective 5-HT_6 ligands as potential therapeutics for Alzheimer's disease and other cognitive disorders.

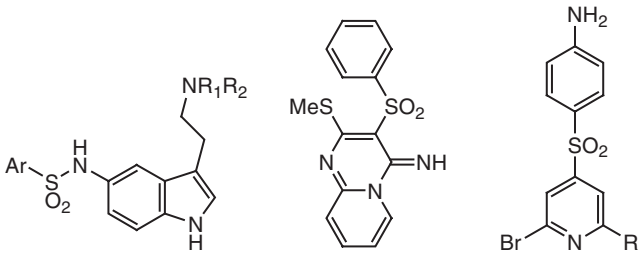
Reports of substituted indole ligands with excellent binding potency, represented by **31** and **32**, were taken as examples from their respective SAR studies [105,106]. Although limited selectivity data are provided, analogs from these series were shown to be full antagonists with modest efficacy in a cell based assay of 5-HT_6 function. Two additional reports by this same group have resulted in the identification of related indole ligands. From the first study, several examples of subnanomolar functional antagonists, such as **33**, were disclosed, with demonstrated selectivity against a panel of related monoamine receptors [107]. Finally, 3-pyrrolidinylmethyl analogs **34** have been shown to be potent agonists or antagonists for 5-HT_6, dependant upon the chirality of the pyrrolidine appendage [108]. In a functional assay of cAMP production, the S-enantiomers, i.e. **34** (Ar = 4-Br-phenyl), were shown to possess antagonist efficacy, while the related R-enantiomers, **34** (Ar = 2-Cl-phenyl), exhibited excellent potency as full agonists. Examples of both enantiomeric series were reported to be selective over a panel of

serotonin and dopamine receptor subtypes. An independent report of related 5-arylsulfonamidoindoles **35** has recently shown that functional variations can be achieved with a multitude of derivatives [109]. High affinity antagonists, as well as agonists and even partial agonists, were prepared and shown to be potent selective ligands for the 5-HT_6 receptor subtype.



31 **32** **33** **34**

Two non-indole derived entities have recently been reported to be potent selective ligands for this receptor. **36** was shown to be a full antagonist with excellent potency (Ki 4 nM) and selective for 5-HT_6 when profiled against a commercial screening package of receptors [110]. The disclosure of 2-substituted pyridine derivatives with subnanomolar potency and excellent selectivity for the 5-HT_6 receptor, has resulted in the identification of **37** (R = pyrrolidine) as a brain penetrant orally bioavailable antagonist [111]. **37** was shown to produce a 2-fold increase in intracellular levels of acetylcholine in the rat frontal cortex when dosed orally at 30 mg/kg. In addition, single oral administration showed efficacy in a rat behavioral assay of cognitive function at 10–100 mg/kg. These results contribute additional evidence supporting the potential therapeutic use of 5-HT_6 antagonists for the treatment of cognitive dysfunction.

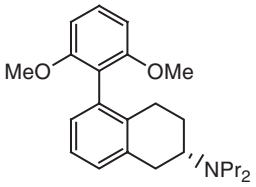


35 **36** **37**

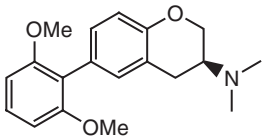
8. 5-HT_7 RECEPTOR FAMILY

First cloned in 1993 [112], the human 5-HT_7 receptor is the newest member of the 5-HT receptor family. The pharmacology, medicinal chemistry and therapeutic potential of 5-HT_7 ligands were extensively reviewed in 2004 [113–119]. In addition to migraine, depression and schizophrenia, 5-HT_7 antagonists may find use in the treatment of sleep disorders and cognitive deficits. The lack of selective 5-HT_7 agonists has made the identification of the biological effects of 5-HT_7 receptor

stimulation difficult. Aminochromans **38** [117] and **39** [118] displayed potent 5-HT_7 affinity (Ki's 7.9 nM and 6.44 nM, respectively), >27-fold selectivity for 5-HT_7 vs. a number of biogenic amine receptors and full agonist activity *in vitro*. SAR in a series of arylpiperazines [119] resulted in the identification of **40**, which displayed potent 5-HT_7 affinity (Ki 0.22 nM) and elicited a full agonist response in guinea pig ileum (ED_{50} 2.56 nM). The nature of the ortho substituent on the phenyl ring of the aryl piperazine is crucial, since changing the thiomethyl group to a hydroxyl moiety yielded a potent 5-HT_7 antagonist.



38



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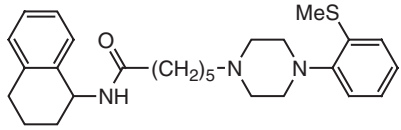
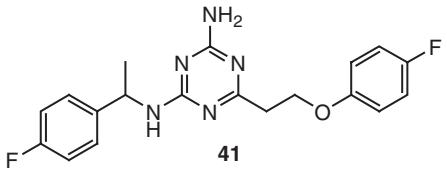


40

Many of the recent reports on 5-HT_7 antagonists describe extensions of earlier work on known sulfonamide, tetrahydrobenzindole and apomorphine scaffolds. However, a new class of aminotriazine 5-HT_7 antagonists has been reported [120]. Compound **41** displayed good 5-HT_7 affinity (Ki 2 nM), selectivity for 5-HT_7 vs. 5-HT_6, α_1 and 5-HT_{2C}, and antagonist activity *in vitro*. The compound also demonstrated good oral bioavailability in the rat (F 51%).



41

9. CONCLUSION

Interest in the discovery of selective 5-HT ligands remains high despite the long history of research in this field. Additional indications have been identified for well known receptor subtypes such as 5-HT_{1A}, and significant progress in less well understood subtypes such as 5-HT_4, 5-HT_6 and 5-HT_7 has led to clinical candidates and pharmacological tools that can be used to more fully map these receptors' therapeutic potential. Only time and significant clinical research will determine whether these exciting new advances are fruitful in the identification of new drugs. Nevertheless, the progress made over the last 5 years suggests the search for selective 5-HT receptor subtype ligands will continue.

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BACE Inhibitors for the Treatment of Alzheimer's Disease

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Contents

1. INTRODUCTION

Alzheimer's disease (AD) is a debilitating neurodegenerative disorder which imparts tremendous suffering upon more than 20 million people worldwide [1]. Current marketed therapy treats the symptoms and not the etiology of the disease, with cholinesterase inhibitors prescribed for mild to moderate AD and the NMDA antagonist memantine for moderate to severe AD [2]. There is broad consensus that amyloid peptides are involved in the progression of the disease [3–8]. This is supported by genetic mapping of the minor familiar forms of AD to mutations that either increase the overall production of β-amyloid$_{1\text{-}40(42)}$ (Aβ) or produce increased amounts of β-amyloid$_{1\text{-}42}$ which is more prone to aggregation. Oligomeric β-amyloid$_{1\text{-}42}$ (Aβ) and related peptides are neurotoxic in cell culture. In this chapter the focus will be on BACE (BACE-1, β-secretase, memapsin-2, Asp-2: Figure 1), an aspartic protease that has captured the attention of the pharmaceutical industry because of the important role it plays in processing the Type I transmembrane amyloid precursor protein (APP) to form β-amyloid peptides [2,9–11]. BACE is

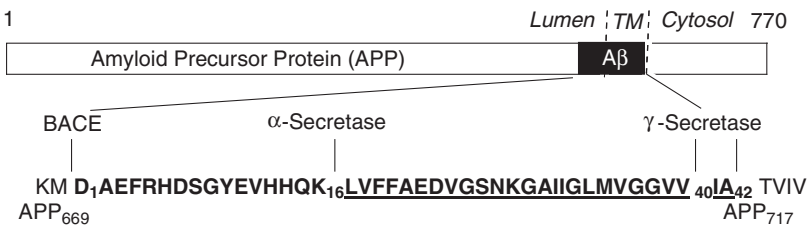

Figure 1. β-Amyloid$_{1\text{-}40(42)}$ is shown in bold, with the transmembrane region underlined.

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responsible for the initial cleavage of APP at aspartic acid D_1 of the N-terminus of the nascent Aβ peptides to give C terminal fragment C99 (β-CTF) which is subsequently cleaved by the membrane bound γ-secretase complex [12]. Alternative cleavage of APP by α-secretase provides innocuous peptide fragments. There are now more than 15 publications and 75 patent applications disclosing structures that inhibit BACE, and this review seeks to capture the field as of May, 2005.

2. BIOLOGICAL CHARACTERIZATION AND INTERPRETATION

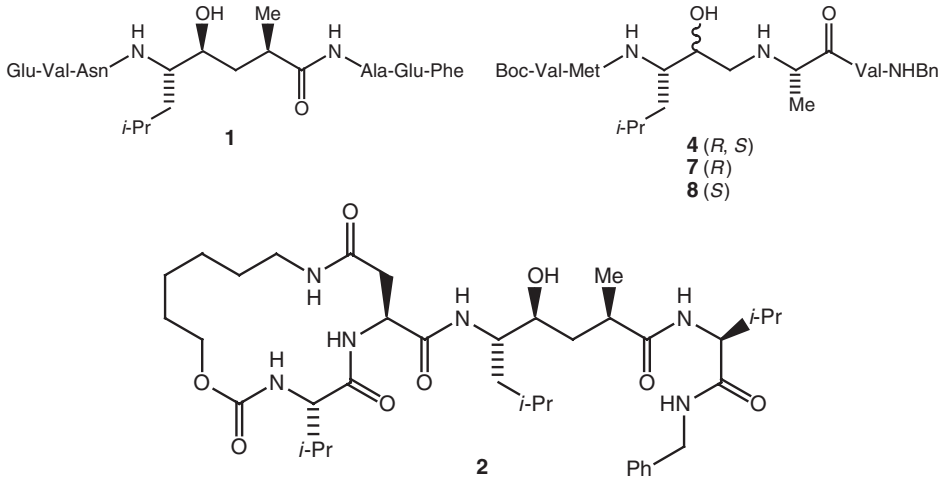
BACE is a Type I glycosylated transmembrane homodimer with two aspartic acids (Asp_{32} and Asp_{228}) at the active site and the active catalytic region extending out into the lumenal side of the membrane [13]. BACE and BACE-2, a related protein of relatively unknown function, constitute a new class of aspartic proteases closely related to the pepsin family of which there are only a small number of other members in humans, including renin and cathepsin D. BACE has three disulfide bonds in the catalytic domain, with Cys_{330}/Cys_{380} being the most sensitive to loss of function when removed [14]. There are four splice variants of BACE that are known, with different abilities to process APP, so that their relative expression may play a role in individual variability in the general population [15,16]. BACE is itself metabolized by furin/PC5 and an unknown enzyme into four characterized smaller metabolites [17]. The activity of BACE is enhanced by interaction with glycosylphosphatidylinositol (GPI)-anchored proteins and BACE accumulates in lipid rafts in the CNS [18], suggesting that there could be non-BACE molecular targets that may regulate BACE activity. The cleavage of APP by BACE depends upon the specific neuronal domain where Aβ is generated, and BACE overexpression alters the subcellular processing of APP and inhibits Aβ deposition in vivo [19], possibly explaining differences in amyloid deposition in BACE over-expressing mice when compared with sex- and age-matched controls [20,21]. In addition to APP, BACE has been shown to cleave the sialyltransferase ST6Gal I [22], the P-selectin glycoprotein ligand 1 [23], and the non-amyloidogenic APP like protein 2 [24].

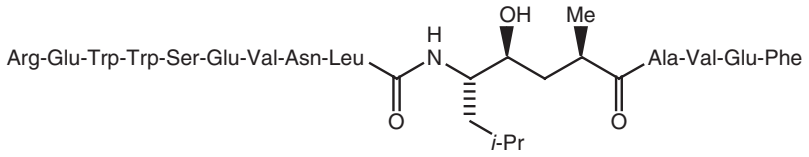
Levels of BACE mRNA and protein expression are reported to be higher in the brains of sporadic AD patients after death, and there is also a correlation of this upregulation with the levels of $Aβ_{1-x}$ and $Aβ_{1-42}$, suggesting that elevation of BACE may lead to increased Aβ production and enhanced deposition of amyloid plaques in the sporadic AD brain [25,26]. There is also increased expression of BACE in rats following transient ischemia, suggesting that BACE inhibitors may prove beneficial for the prevention of dementia following a stroke [27]. Although the BACE mouse knock-out showed only mild phenotypic changes [28], subsequent analysis revealed subtle behavioral changes characteristic of neurotransmitter modulation [29]. Importantly, when the BACE knock-out was incorporated in a mouse model of APP overexpression (Tg2576) not only were cerebral $Aβ_{1-40}$ and $Aβ_{1-42}$ levels lower but the behavioral deficits found in the Tg2576 mice were dramatically absent. This

suggests that inhibition of BACE could provide improvement in the cognitive symptoms of AD as well as alter the course of the disease [30]. When a BACE inhibitor was covalently linked to a carrier peptide that promoted transport into the brain, significant lowering of Aβ was observed both in plasma and brain upon i.p. administration, suggesting that an orally active BACE inhibitor that penetrated into the brain would lower amyloid levels there [31,32].

3. INHIBITORS AND MODULATORS OF BACE

Following the identification of Stat-Val [33] and then OM99-2 (**1**) as potent BACE inhibitors [34,35], researchers continue to prepare new analogs which feature a statine motif in which the hydroxyl group acts as a transition state mimic. Ghosh and Tang have patented the synthesis of analogs of OM99-2 which have low nanomolar potency [32,36,37]. The X-ray crystal structure of **1** ($K_i = 1.6\,nM$) indicated that the hydroxyl group of the "statine" moiety forms four hydrogen bonds to the two aspartic acids in the active site. Additionally, a hydrogen bond formed between the side chain of the glutamic acid and the aspartic acid residue which suggested that the three *N*-terminal amino acids of OM99-2 could be replaced by a macrocycle. A number of potent macrocyclic analogs have been prepared; in particular, urethane **2** has $K_i = 25.1\,nM$ and improved cellular inhibition ($IC_{50} = 3.9\,\mu M$) over **1** ($IC_{50} = 45\,\mu M$) [38]. An extended length peptide, OM03-4 (**3**), which occupies the S_5, S_6, and S_7 subsites in the BACE active site is extremely potent ($K_i = 0.03\,nM$) [39]. Compounds **4**, **5**, and **6**, which are analogs of GT1017, a truncated OM99-2 derivative, were prepared and feature modifications of the statine portion [40]. Hydroxyethylamine **4** and hydroxyethylsulfide **5** have 0.12 μM and 1.85 μM IC_{50}s, respectively, while *N*-benzyl urea **6** shows only 38% inhibition at 10 μM. The pure (*R*)-isomer **7** has a 0.014 μM IC_{50} while the (*S*)-alcohol **8** has a 1.57 μM IC_{50}. Analogs **7** and **8** have 0.005 and 0.161 μM IC_{50}s at cathepsin D, respectively.





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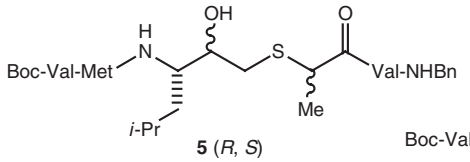
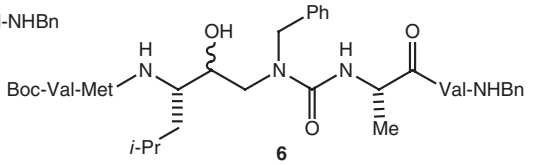


5 (*R*, *S*)



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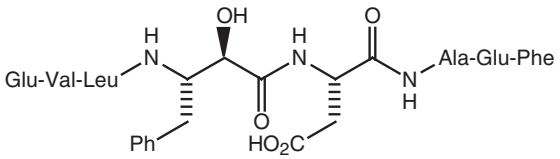
Many of the initially reported BACE inhibitors were peptides containing a statine-like moiety although significant efforts have been directed toward reducing the number of amino acid residues relative to OM99-2. The design of truncated analogs of octapeptide KMI-008 (**9**) ($IC_{50} = 413\,nM$) [41,42] led to the discovery of potent tetrapeptides KMI-358 (**10**) ($IC_{50} = 16\,nM$) and KMI-370 (**11**) ($IC_{50} = 3.4\,nM$) [43]. The *N*-oxalyl group is prone to migration from the β-amino group to the α-position; replacement of the carboxyl group with a tetrazole provided KMI-420 (**12**) ($IC_{50} = 8.2\,nM$) and KMI-429 (**13**) ($IC_{50} = 3.9\,nM$) which maintain potency [44]. Pentapeptide inhibitors **14** ($IC_{50} = 42\,nM$) and **15** ($IC_{50} = 45\,nM$) feature a Phe-Ala containing a hydroxyethyl group as a transition state mimic [45]. These compounds also show improved cellular activity ($IC_{50}s \sim 400\,nM$) relative to **1**. In addition, compounds **16** and **17** in which the NHBoc group is replaced with a hydroxyalkylamide moiety have 35 and 45 nM IC_{50} values at BACE, respectively, with cellular activity in the 1 μM range [46]. Analog **18**, which features a Phe-Glu hydroxyethyl transition state mimic is a modest BACE inhibitor with a 1.7 μM IC_{50} and 70–80-fold selectivity over cathepsin D and renin [47,48]. An even shorter peptide **19** that contains a 3,5-difluorobenzylhydroxyethyl isostere in place of a statine, has a 1 nM IC_{50} at BACE, but a 1,000 nM EC_{50} in HEK-293 cells [49]. Related structures are disclosed in a patent, but little biological information is reported [50]. A series of tetra, penta, and hexapeptides containing statine itself have been reported to have IC_{50} values $< 10\,\mu M$ [51]. Compound **20** with a bis-statine like motif has a 21 nM IC_{50} [52]. The docking of a number of statine peptidomimetics into BACE has been described [53–56].



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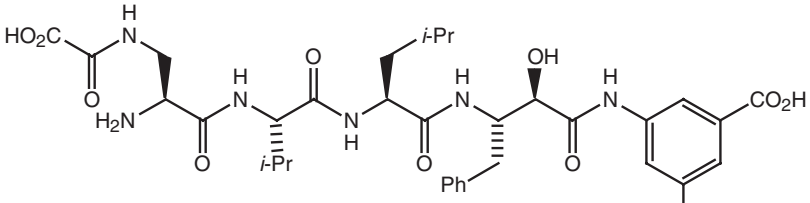
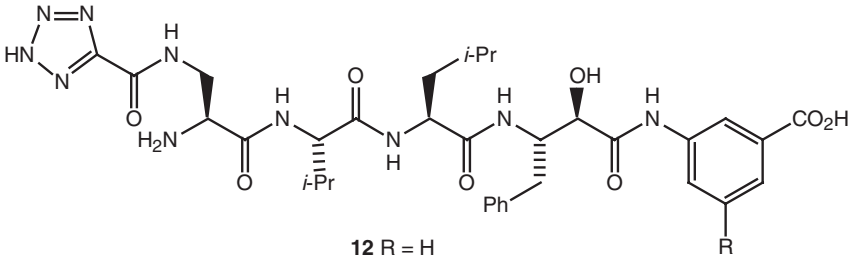
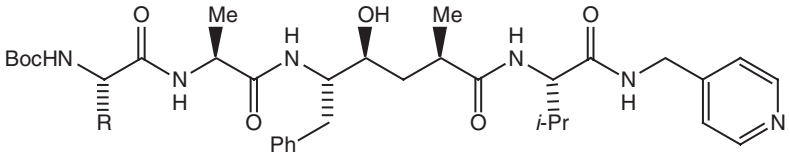


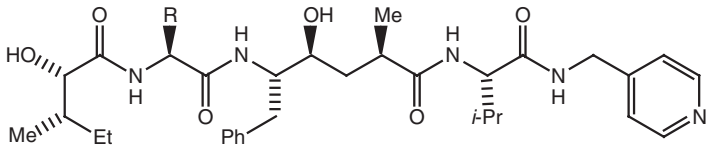
10 R = H
11 R = CO_2H



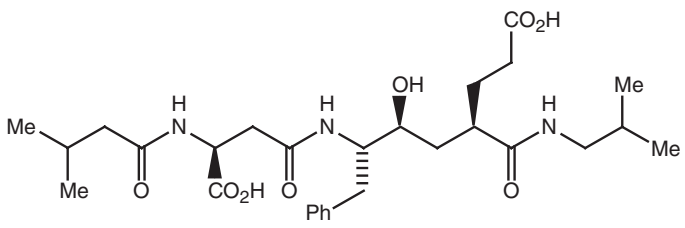
12 R = H
13 R = CO_2H



14 R = (*S*)-MeCHEt
15 R = CH_2CHF_2



16 R = Me
17 R = CH_2CHF_2



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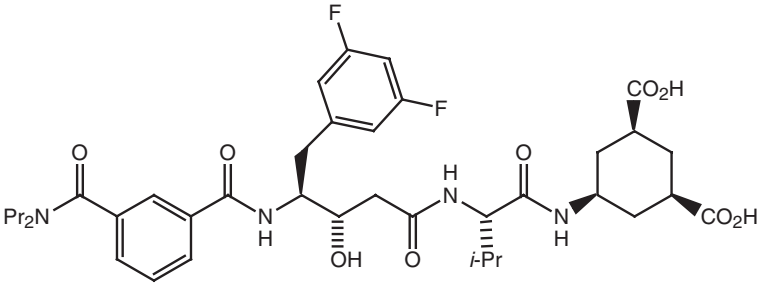

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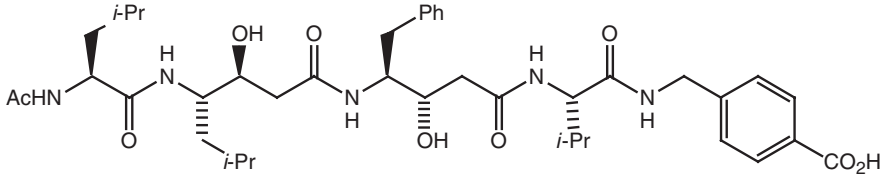

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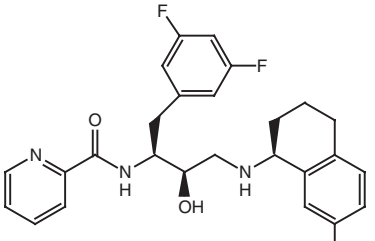
In addition to statine derivatives, 1,3-diaminopropan-2-ols have been utilized extensively as a transition state mimic in BACE inhibitors. Extensive patents have appeared on this class of structures, but little biological information is supplied [57–64]. Compound **21** is a representative structure. A virtual library of 1,3-diaminopropan-2-ol derivatives has also been patented [65]. Sulfonamides **22**, **23**, and **24** have 1–10 nM [66], and 1–100 nM [67], and $<1{,}000$ nM [68] inhibition, respectively. Compound **25**, which was designed independently, has an 11 nM IC_{50} [69,70]. Related structures containing a 1,3-dihydroxy-2-aminopropane scaffold have also been reported [71]. A 1,3-diaminopropan-2-ol containing a γ-lactam **26** has <100 nM potency [72]. Compounds **27** and **28** in which one of the nitrogens of the 1,3-diaminopropan-2-ol has been incorporated into a ring are potent BACE inhibitors with 1.4 nM [73] and 1 nM IC_{50}s, respectively [74,75]. Finally, structures containing 1,4-diaminobutan-2-ol [76,77] and a bis-(hydroxymethyl)aminomethane moiety [78] as transition state mimics have been patented, but little biological information is provided.



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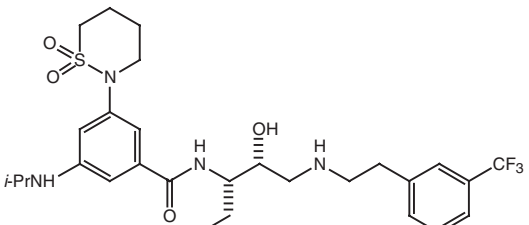

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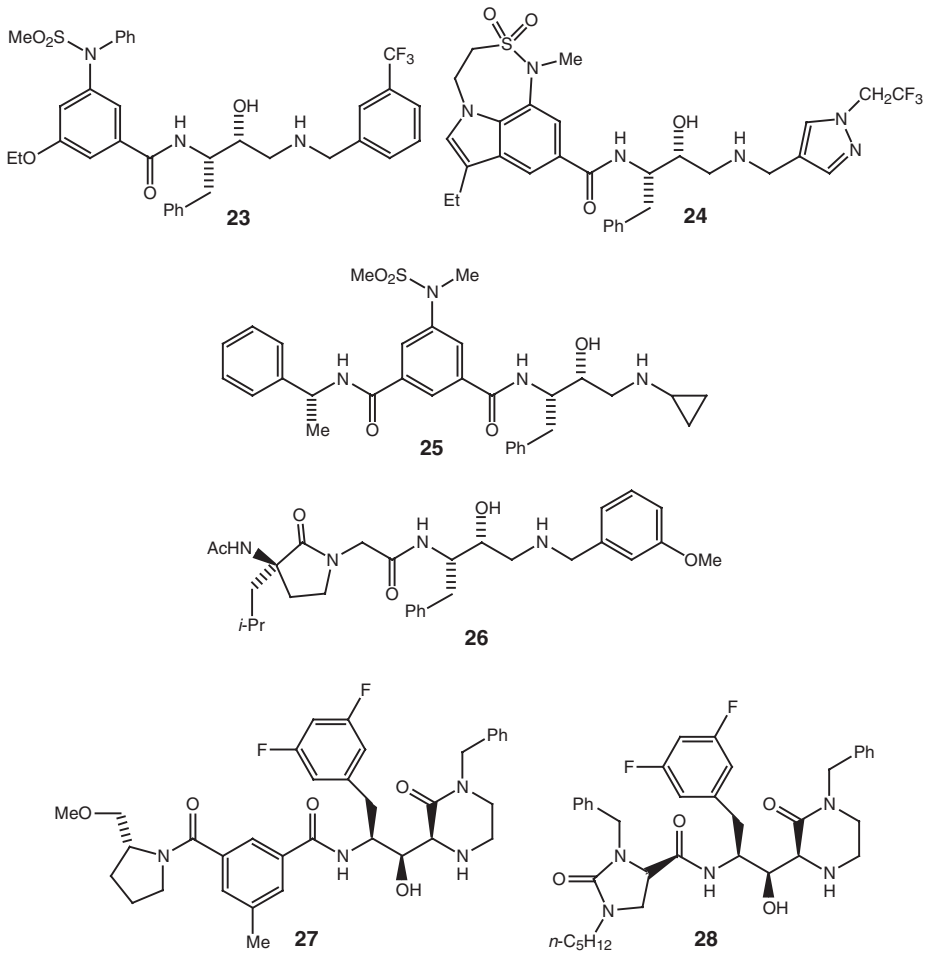
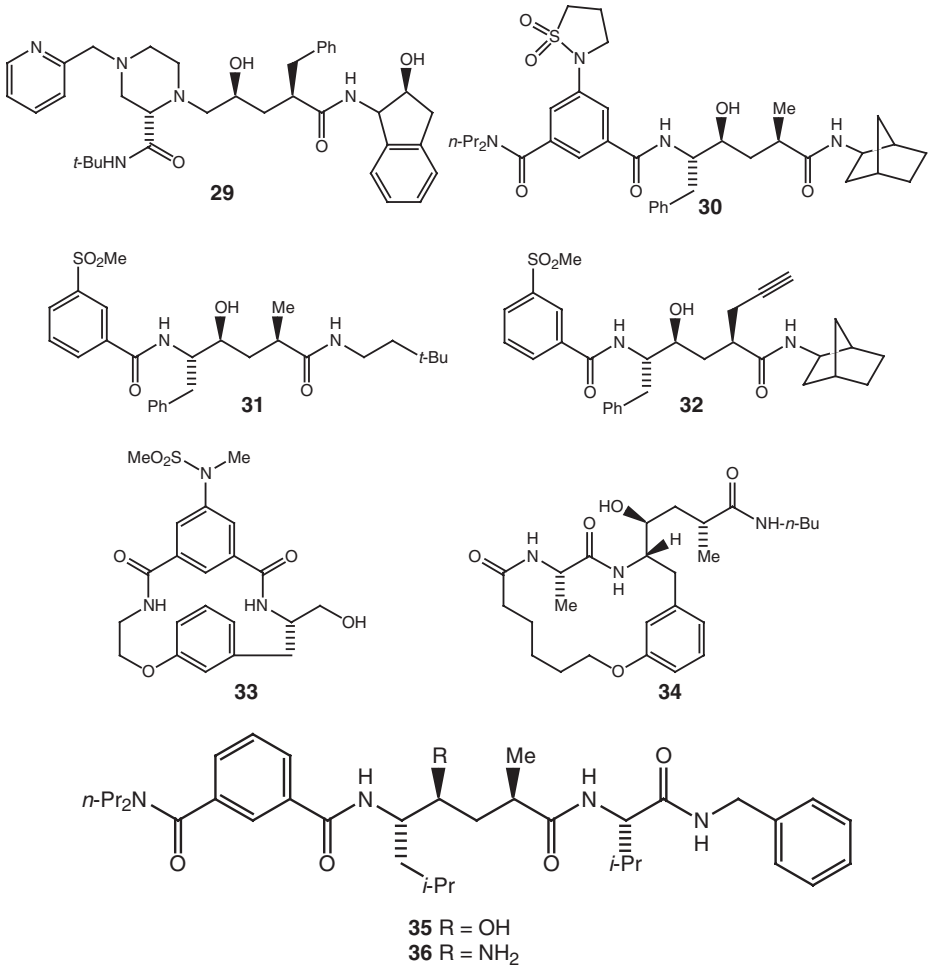
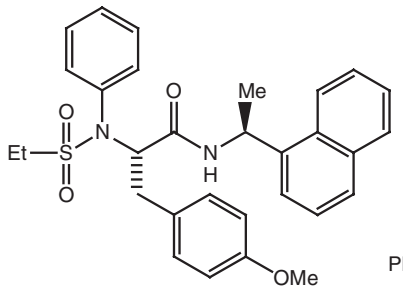


A simpler transition state mimic which has been incorporated into BACE inhibitors is the 2-aminoethanol scaffold, as exemplified by **29** but little biological activity is described [79–81]. Compound **30** has BACE inhibition in the 1–200 nM range [82]. Related structures which contain either a 2-aminoethanol or 3-aminopropanol motif have been recently reported [83]. Sulfones **31** and **32** have 400 and 330 nM IC_{50}s at BACE and 7700 and 5570 nM IC_{50}s at BACE-2, respectively [84]. Aminoethanol derivatives which contain a dibenzooxepine rather than an arylsulfonamide or arylsulfone substitution have been disclosed [85]. Macrocyclic inhibitors, such as **33**, have been described and have IC_{50}s = 1–1,000 nM [86]. Presumably the hydroxyl group is interacting with the aspartic acid residues in the active site. Compound **34** has BACE inhibition, but no biological data is provided [87]. Peptide **35**, which contains a 2-hydroxyethylamine scaffold, has modest BACE activity (K_i = 150 nM), potent cathepsin D inhibition (K_i = 20 nM), poor activity in H4 cells (IC_{50} = 2,000 nM), and little metabolic stability (0% recovery in liver

microsomes after 60 min). Analog **36**, in which the hydroxy has been replaced with an amino group, has comparable BACE activity ($K_i = 180$ nM), but reduced cathepsin D inhibition ($K_i = 610$ nM), improved activity in H4 cells ($IC_{50} = 740$ nM), and enhanced metabolic stability (43% recovery in liver microsomes after 60 min) [88–90]. Related structures containing a 1,2-diamino ethane scaffold have also been patented, but no biological activity has been reported [91]. Additionally, related compounds that have a 1,2-disubstituted diaminoethane moiety have been disclosed [92,93] as well as a macrocyclic variant [94]. Finally, amides of 3-hydroxypropionic acids have been recently reported [95].



Peptide inhibitors of BACE have been reported recently [96], including one that binds to an exosite with a 3 nM K_d [97]. In addition, substituted aminoacid sulfonamides such as **37** ($IC_{50} = 13\,\mu M$) are weak inhibitors [98]. Hydroxysuccinamides [99] and succinamic acids [100] have micromolar activity.



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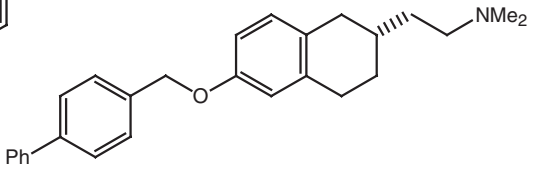

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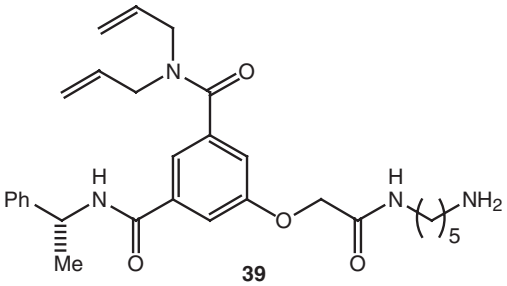

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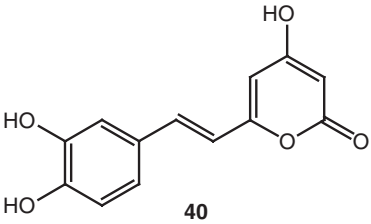

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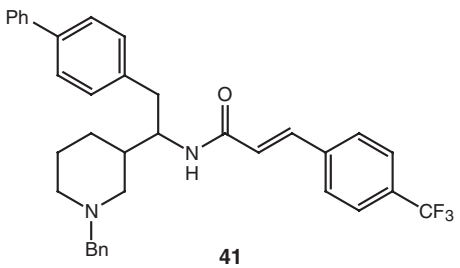
In addition to compounds containing transition state mimics as well as peptide and amino acid derived structures, several carbocyclic and heterocyclic BACE inhibitors have been disclosed. In general, these compounds do not contain an obvious transition state mimic. Tetralin **38** has an 857 nM IC_{50} at BACE [101,102]. An X-ray structure of trisubstituted phenyl analog **39** ($IC_{50} = 25\,\mu M$) indicates that the amide N-H of the 1,5-diaminopentyl side chain interacts with the active site aspartyl acid residues via a water molecule [103]. Hispidin (**40**) ($IC_{50} = 4.9\,\mu M$) was isolated from the mycelial culture of *Phellinus linteus* [104].

Piperidines, as exemplified by structures **41** and **42**, are BACE inhibitors, but more specific information is not available [105,106]. Disubstituted piperazines **43** ($IC_{50} = 2.8\,\mu M$) [107] and **44** ($IC_{50} = 3\,\mu M$) [108] are modest BACE inhibitors. In addition, 2-aminotriazole **45** is a patented BACE inhibitor [109], and the related triazinoindoles **46** ($IC_{50} = 10.6\,\mu M$) [110] and **47** ($IC_{50} = 3.1\,\mu M$) [111] have weak activity.



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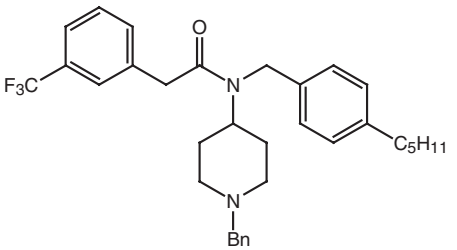

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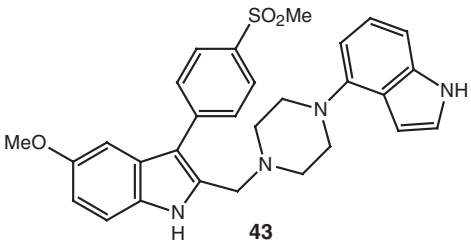

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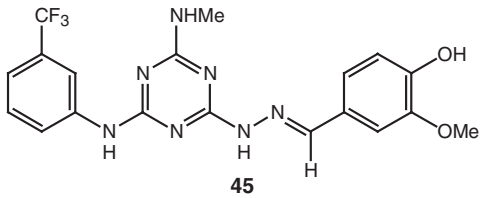

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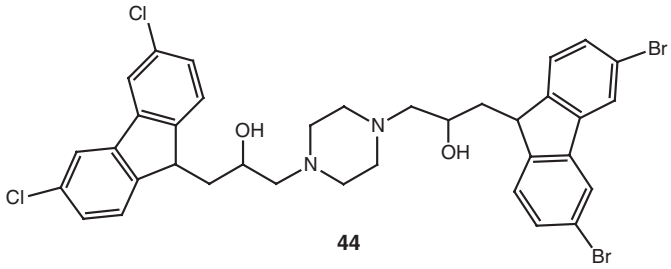

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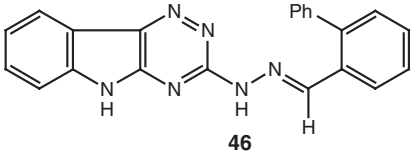

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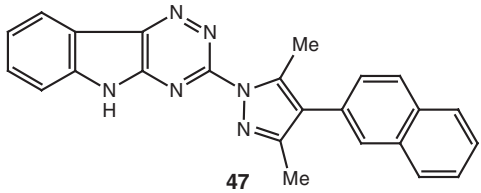

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4. STRUCTURAL BIOLOGY

The crystal structure of an inhibitor bound into BACE was first reported in 2000 by Tang, Ghosh and co-workers [34]. The use of X-ray crystallography has proved to be invaluable in designing inhibitors with improved potency as seen in the 50-fold increase in activity of **3** ($K_i = 0.03$ nM) from OM99-2 (**1**) ($K_i = 1.6$ nM) [39]. The active site has a ca. 55° bend with a flap that extends over it, although a crystal structure of BACE without an inhibitor has been obtained in an open flap form [112]. An X-ray structure of diaminopropanol **25** shows that the hydroxyl group forms a hydrogen bond with Asp_{32} in the active site while the protonated α-amino group interacts with Asp_{228} [69]. The sulfonamide oxygens interact with Arg_{235} in BACE which is substituted by a valine in cathepsin D and by a serine in renin. This inhibitor has no renin inhibition ($IC_{50} > 50\,\mu M$) and has modest cathepsin D activity ($IC_{50} = 7.6\,\mu M$), indicating that structural biology can be valuable to improve selectivity over other aspartic protease inhibitors. The hydroxyl group of related Phe-Ala mimics also interacts with the catalytic aspartic residues in BACE and the inhibitor side chains occupied the expected binding pockets [45,46]. An X-ray structure of analog **39**, which lacks an obvious transition state mimic, has been

published [103]. The amide carbonyl interacts with the active site aspartic acids via hydrogen bonding through a water molecule.

5. CONCLUSIONS

Many laboratories throughout the world are actively searching for inhibitors of the aspartyl protease BACE for the treatment of Alzheimer's disease, and only limited aspects of this research have been publically disclosed. Many of the structures reported so far as BACE inhibitors are transition state mimics with a statine-like hydroxyl group. In the future it will be possible to see what novel and unexpected BACE inhibitory series have emerged from corporate screening libraries. Nevertheless, it is clear from the present data that it is possible to achieve potent inhibition of BACE, and new pharmacology continues to validate this as a compelling target in drug discovery.

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Positron Emission Tomography Agents for Central Nervous System Drug Development Applications

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Contents

1. INTRODUCTION

The application of non-invasive imaging, particularly positron emission tomography (PET), has proven useful in accelerating drug discovery and development [1–5]. The Society of Non-Invasive Imaging in Drug Development (SNIDD) has supported the use of imaging in drug development and maintains a website dedicated to these applications (www.snidd.org). PET imaging, in particular, offers a unique role in drug development because of its ability to quantify drug properties *in vivo*. The advantages of the PET imaging method are that it employs radiotracer principles and is capable of quantitatively measuring a variety of *in vivo* processes without perturbing the biochemistry of systems that are easily saturable or operate at low capacity, such as receptors and enzymes. There are several classes of PET drug discovery and development studies. One class with demonstrated usefulness in central nervous system (CNS) applications involves the use of well-established radiopharmaceuticals, such as

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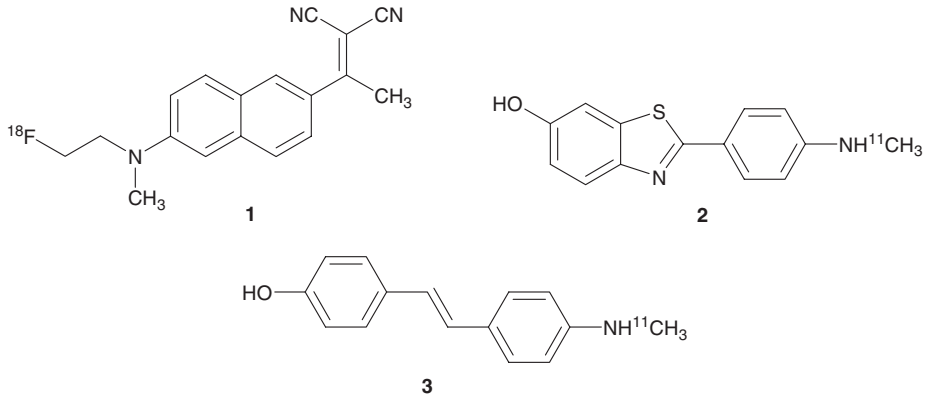
[^{15}O]water for cerebral blood flow and 2-[^{18}F]fluoro-2-deoxyglucose (FDG) for cerebral glucose metabolism, to measure the indirect effects of drugs on CNS targets [4,6]. Another approach is the utilization of well-established PET radioligands to measure direct or indirect effects of drugs on specific neurotransmitter systems, such as the use of [^{11}C]raclopride for drug occupancy studies of the dopamine D_2 and D_3 neuroreceptor systems and for the assessment of endogenous cerebral dopamine levels [7,8]. Studies with [^{11}C]raclopride have proven useful in determining effective drug doses for clinical trials for new D_2/D_3 drugs, for determining the duration of various drug actions on the D_2/D_3 system, and for examining potential drug interactions. The ability of new PET radiotracers to assist in a meaningful way with CNS drug development is largely dependent on how carefully and thoroughly the *in vitro* and *in vivo* properties of the PET radiotracer, as well as their intended target(s), have been characterized. The essential properties of CNS PET radioligands have been described in several reviews [9–11]. Some of the major issues include:

- Radiosynthesis considerations
  - Radiolabeling position (choice of radiolabeled parent compound or structural analog)
  - Radiolabeling yield (typically $>10\%$ at end of synthesis)
  - Specific activity requirements (typically >500 Ci/mmol (>18.5 TBq/mmol))
- *In vitro* characteristics
  - Binding selectivity/specificity (>100-fold for target site(s))
  - Binding affinity (typically <1 nM for neuroreceptor sites)
  - Lipophilicity (logP typically in range of 1–3)
- *In vivo* characteristics
  - Brain uptake ($>0.10\%$ ID-kg/g at early times post-injection)
  - Binding selectivity/specificity (should be demonstrated *in vivo* with blocking or displacement studies)
  - Pharmacokinetics of specific binding (should be reversible and the binding rate should be much less than the brain uptake rate ($k_{on} < K_1$))
  - Pharmacokinetics of non-specific binding (off-rate should be rapid; brain clearance $t_{1/2} < 30$ min for ^{11}C- (20.3 min half-life) and ^{18}F-labeled (109.8 min half-life) agents
  - Metabolism (absence of radiolabeled metabolites in brain)
  - Protein binding (should be reversible)
- Toxicology
  - Injected drug masses are typically $<10\,\mu$g
  - Limited toxicology packages are required by the FDA for new agents
- Radiation dosimetry
  - Limits dependent on regulatory approval route
  - Human measurements usually required for IND

Some recent advances in PET CNS radiopharmaceuticals of potential benefit to drug discovery and development efforts are highlighted in this report, and the examples given are intended to provide updates of progress made in selected PET research areas.

2. AMYLOID PLAQUE

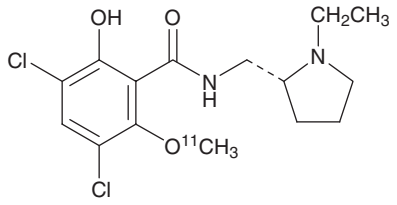
Over the past three years considerable progress has been made in the development and application of PET radiotracers for imaging amyloid-beta (Aβ) deposits in the CNS [11]. The application of Aβ imaging agents that satisfy the radioligand criteria listed above will likely facilitate evaluation of the efficacy of a variety of anti-amyloid therapies currently under intense development. The ability to assess CNS Aβ deposition pre- and post-treatment with anti-amyloid therapies in cognitively impaired human subjects could significantly benefit the development of a variety of promising experimental treatments. The use of PET Aβ radiotracers for this purpose follows naturally from the ability of PET to quantify the regional concentration of the radioligand throughout the brain. The relative regional concentrations of an amyloid-selective radioligand would reflect the regional density of Aβ plaques, which are the very targets of the anti-amyloid therapies. In addition, Aβ imaging agents could also serve as surrogate markers in early diagnosis and neuropathogenesis studies of Alzheimer's disease and other aging-related neurodegenerative disorders. Longitudinal studies of Aβ deposition also could help test the "amyloid cascade hypothesis." Several recent studies demonstrate the feasibility of PET imaging of Aβ plaques *in vivo* in human subjects using [^{18}F]FDDNP (**1**), [^{11}C]PIB (**2**), or [^{11}C]SB-13 (**3**) [12–14]. While **2** and **3** are Aβ selective radioligands, **1** binds to both Aβ plaques and neurofibrillary tangles comprised of hyperphosphorylated tau protein.



3. DOPAMINE RECEPTOR

3.1. Dopamine D_2/D_3

[^{11}C]Raclopride (**4**) has been used in imaging studies to evaluate the *in vivo* D_2-receptor occupancy of a variety of antipsychotics, including clozapine, risperidone, and olanzapine [15–17]. The concept that the dopaminergic system plays a role in the etiology of schizophrenia has led to efforts to develop a variety of PET imaging radioligands for the D_2-like family of receptors.



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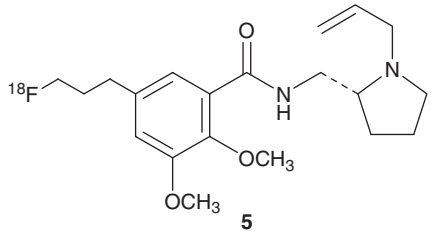
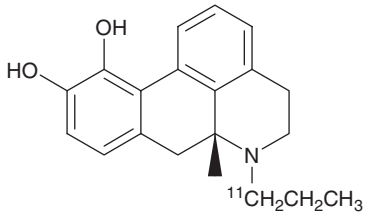


5

Fallypride, (5-(3-fluoropropyl)-2,3-dimethoxy-N-[(2S)-1-(2-propenyl)-2-pyrrolidinyl]methyl (**5**)), is a D_2/D_3 dopamine antagonist ligand that has been used to image D_2/D_3 receptors *in vivo*. Fallypride's high affinity, selectivity, and rapid non-specific binding clearance rate permit imaging of both striatal and extrastriatal brain regions [18]. Fallypride has been radiolabeled with both fluorine-18 and carbon-11 [19]. While antagonists bind indiscriminately to both the high- and low-affinity states of G-protein coupled receptors, an agonist that bound potently to only the high-affinity state might facilitate the selective imaging of this state of D_2 and D_3 receptors. Several compounds have been developed as potential D_2/D_3 receptor agonist radioligands for PET. Most of these fall into either the apomorphine or aminotetralin structural classes, such as (-)-N-[^{11}C]propylnorapomorphine ((-)-[^{11}C]NPA (**6**)) and (*R*)-[^{11}C]-2-methoxy-N-n-propylnorapomorphine or (*R,S*)-5-hydroxy-2-(N-propyl-N-(5′-[^{18}F]fluoropentyl)amino-tetralin ([^{18}F]-5-OH-FPPAT) and racemic 2-(N-phenethyl-N-1′-[^{11}C]propyl)amino-5-hydroxytetralin [20–23].



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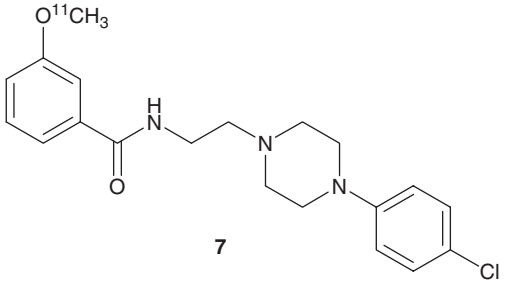
3.2. Dopamine D_4

The development of selective dopamine D_4 receptor ligands for use in PET likewise has been driven by interest in schizophrenia research related to the atypical antipsychotics such as clozapine, as well as the potential involvement of the D_4

receptor subtype in attention deficit hyperactivity disorder, Parkinson's disease, and depression. To date, there has been no convincing demonstration of D_4 receptor imaging *in vivo* using PET. Two groups have evaluated N-[2[4-(4-chlorophenyl)piperazin-1-yl]ethyl]-3-[^{11}C]methoxybenzamide ([^{11}C]PB-12 (**7**)), a selective dopamine D_4 receptor antagonist, in non-human primates and were unable to detect specific binding to D_4 receptors *in vivo*. In addition the radioactivity distribution of **7** was unaffected by pretreatment with unlabeled PB-12 [24,25].



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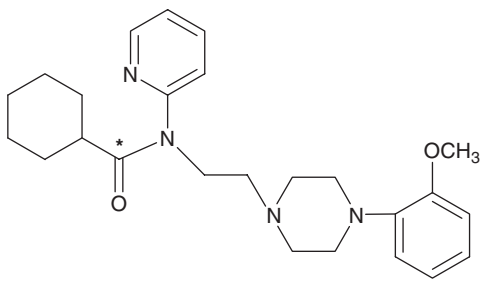
4. SEROTONIN 5-HT$_{1A}$ RECEPTOR

The 5-HT$_{1A}$ receptor has been implicated as a target for the treatment of anxiety and depression. {*Carbonyl*-[^{11}C]}WAY100635 (**8**) is a highly selective PET radiotracer for 5-HT$_{1A}$ receptors and has demonstrated utility in drug development applications. The occupancy of 5-HT$_{1A}$ receptors following oral administration of pindolol was determined using **8** in humans [26]. In other studies, **8** has been used to determine occupancy values for varying doses of robalzotan (NAD-299), a selective 5-HT$_{1A}$ receptor antagonist and putative antidepressant drug [27]. Considerable effort has been directed towards the development and validation of an F-18-labeled derivative of WAY100635. One such derivative is 4-[^{18}F]fluorocyclohexyl-WAY100635 ([^{18}F]FCWAY (**9**)). *In vivo* studies with **9** demonstrated that the compound was rapidly metabolized and that some of the metabolites resulted from defluorination, which led to problematic bone uptake of [^{18}F]fluoride [28]. Another radiofluorinated analog of WAY100635, 6-[^{18}F]fluoro-WAY100635 (**10**), also has been reported recently, and the fluorine-18 radiolabel was attached to the pyridine ring [29,30].



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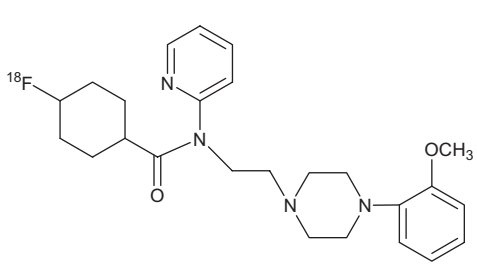

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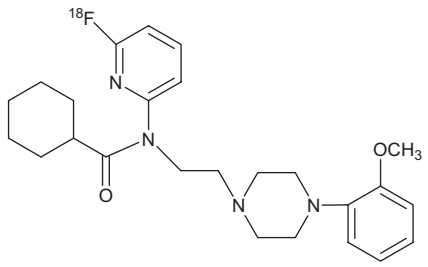
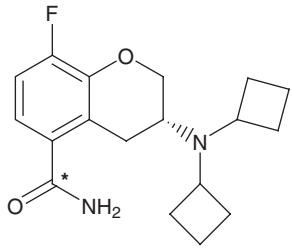


10

Robalzotan (NAD-299, (*R*)-3-N,N-dicyclobutylamino-8-fluoro-3,4-dihydro-2H-benzopyran-5-carboxamide (*2R,3R*)-tartrate monohydrate) possesses both high affinity (K_i 0.59 nM) and selectivity for the 5-HT_{1A} receptor. The radiosynthesis of [^{11}C]NAD-299 (**11**) has been reported, and preliminary results indicated that the compound had potential as a 5-HT_{1A} receptor PET radioligand [31,32]. The fluorinated piperazine, 4-(2′-methoxyphenyl)-1-[2′-[N-(2″-pyridinyl)-4-fluorobenzamido]ethyl]-piperazine (p-MPPF (**12**)), has been radiolabeled with fluorine-18, and human studies supported the utility of this selective 5-HT_{1A} antagonist for PET imaging studies [33].



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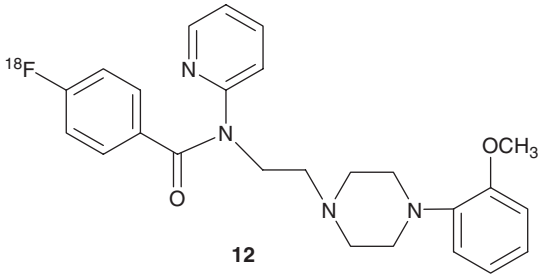


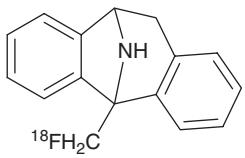
12

5. GLUTAMATE RECEPTOR

The N-methyl-D-aspartate (NMDA)/PCP ion channel has a role in a variety of neurological functions, including neurodegeneration, memory, and cognition. The NMDA/PCP ion channel has been implicated in the pathophysiology of several disorders including Parkinson's disease, schizophrenia, Huntington's chorea, and stroke, and this system is an attractive target for PET radioligand development.

Several candidate radioligands have been evaluated as potential PET imaging agents for the NMDA/PCP ion channel [34]. Some of these have been examined in non-human primate models, including derivatives of MK801 and adamantine. [^{18}F]Fluoromethyl-MK801 (**13**) and (+)-3-[^{11}C]-cyano-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine ([^{11}C]MKC) both demonstrated little specific binding *in vivo* [35,36]. A fluorine-18-labeled adamantine derivative, [^{18}F]memantine or [^{18}F]AFA (**14**), was examined in normal human subjects and likewise did not

demonstrate a radioactivity distribution in the brain consistent with the known regional distribution of NMDA receptors [37].



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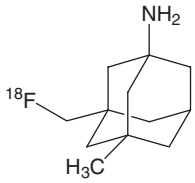
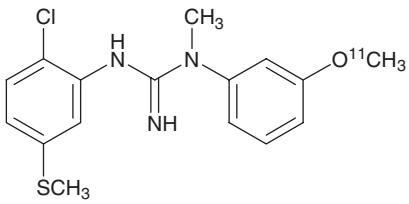


14

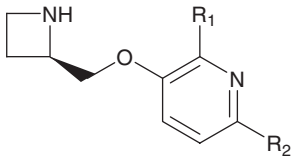
More recent work has focused on a group of selective NMDA/PCP ligands consisting of trisubstituted N-methyl guanidines, such as [^{11}C]N-(2-chloro-3thiomethylphenyl)-N′-(3-methoxyphenyl)-N′-methylguanidine ([^{11}C]GMOM (**15**)) [38]. While the regional brain distribution of **15** in awake rats was promising, non-human primate studies did not demonstrate a saturable binding component. However, anesthesia may have an effect on these studies, as both ketamine and isoflurane are known to reduce NMDA ion channel activation [39]. Another trisubstituted N-methylguanidine derivative, CNS-5161, has been radiolabeled with carbon-11 and demonstrated increased unilateral uptake in rat brain following brain injury compared to the contralateral side, indicating the potential usefulness of this radioligand for NMDA imaging studies [40].



15

6. NICOTINIC/MUSCARINIC RECEPTORS

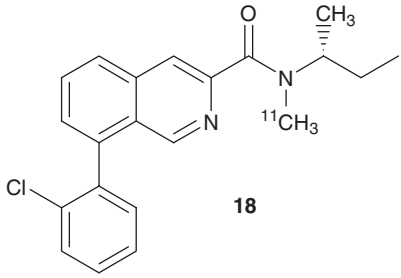
Nicotinic cholinergic receptors (nAChR) have been the target of PET radioligand development for several years. Early studies with [^{11}C]nicotine indicated that high levels of non-specific binding complicated image interpretation [41]. Several analogs of epibatidine, such as [^{18}F]norchlorofluoroepibatidine, have been radiolabeled as potential PET agents. However, concerns related to the extreme toxicity associated with these derivatives, even at microgram levels, have limited their use in human PET studies. Radiofluorinated derivatives of the $\alpha_4\beta_2$-selective compound A85380 (2-[^{18}F]fluoro-A85380 (**16**) and 6-[^{18}F]fluoro-A8530 (**17**)) have facilitated quantitative imaging of the nAChR system *in vivo* [42,43]. For muscarinic cholinergic receptors (mAChR), a number of non-selective radioligands for the four receptor subtypes of mAChR have been prepared and evaluated, but the lack of subtype selectivity has limited their application. An M_2-selective agonist, [^{18}F]FP-TZTP, has been radiolabeled and showed promise [44].



16: $R_1 = {}^{18}F$, $R_2 = H$
17: $R_1 = H$, $R_2 = {}^{18}F$

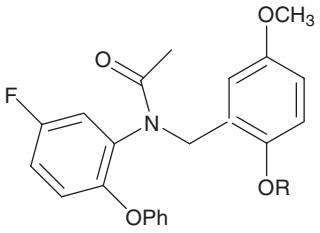
7. PERIPHERAL BENZODIAZEPINE RECEPTOR

The isoquinoline carboxamide derivative, 1-(2-chlorophenyl)-N-methyl-N-(1-methylpropyl)-3-isoquinolinecarboxamide (PK11195), is a potent and selective peripheral benzodiazepine receptor (PBR) antagonist with well-characterized pharmacology [45–49]. Racemic [*N*-methyl-^{11}C]PK11195 was shown to be rapidly and highly extracted from blood into brain and, in the absence of activated CNS microglial and macrophage pathology, distributed in a uniform manner throughout brain tissue [50,51]. While the PK11195 racemate was first radiolabeled with carbon-11 as a potential agent for imaging PBR expression in human myocardium, the R-enantiomer (*R*-[*N*-methyl-[^{11}C]PK11195, (**18**)) has higher affinity for PBR than the racemic mixture and allows improved detection of specific binding [52]. To date, **18** is the most widely used PET PBR imaging agent, largely as the result of the absence of suitable alternative PBR radioligands.



18

N-(2,5-Dimethoxybenzyl)-N-(5-fluoro-2-phenoxyphenyl)acetamide (DAA1106) is a novel ligand with sub-nanomolar affinity and excellent selectivity for the PBR. Radiolabeling of DAA1106 with carbon-11 has been reported [53]. Co-injection of mice with [^{11}C]DAA1106 (**19**) and a blocking dose (1 mg/kg) of either unlabeled DAA1106 or PK11195 resulted in a significant reduction of radioactivity throughout the brain that was greatest in olfactory bulb (14% of control) and cerebellum (16% of control), with moderate reductions in other cortical and subcortical areas (20–54% of control) [53]. These results suggested that a dominant portion of brain radioactivity following the injection of [^{11}C]DAA1106 was specifically bound to constitutive PBR receptors in brain.



19, R = $^{11}CH_3$
20, R = $CH_2{}^{18}F$
21, R = $CH_2CH_2{}^{18}F$

Two analogs of DAA1106 radiolabeled with fluorine-18 have been developed as imaging agents for the PBR [54]. Preliminary studies of these analogs, a fluoromethyl derivative (N-5-fluoro-2-phenoxyphenyl)-N-(2-[^{18}F]fluoromethyl-5-methoxybenzyl)-acetamide ([^{18}F]FMDAA1106, (**20**)) and a fluoroethyl derivative (N-5-fluoro-2-phenoxyphenyl)-N-(2-[^{18}F]fluoroethyl-5-methoxybenzyl)acetamide ([^{18}F]FEDAA1106, (**21**)), indicated that they possessed similar binding characteristics and brain distributions to that observed for [^{11}C]DAA1106 [53,54]. The *in vivo* properties of [^{11}C]DAA1106 and its radiofluorinated analogs remain to be fully characterized, but the promising preliminary *in vivo* and *in vitro* properties of these ligands in rodent and monkey brain warrant further study and potential development for human PET imaging.

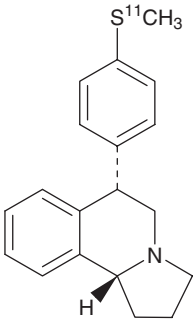
Another potential PET radioligand for PBR is the carbon-11-labeled neuroprotective agent vinpocetine [55]. Vinpocetine is a synthetic derivative of the Vinca minor alkaloid vincamine, whose mechanism of neuroprotective action is not completely understood. Earlier studies of this compound, radiolabeled with [^{11}C]ethyl iodide demonstrated the utility of PET in drug distribution studies in man [56]. While recent studies supported the binding of [^{11}C]vinpocetine to the PBR with much higher initial brain entry compared to **18** (4% vs. 0.8%), the affinity of [^{11}C]vinpocetine is sufficiently low to cause concern regarding the utility of the radioligand in PET imaging applications [57].

8. BIOGENIC AMINE TRANSPORTERS

8.1. Serotonin transporter

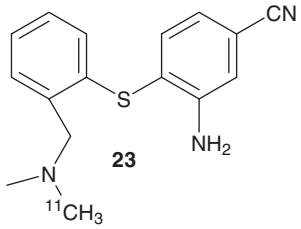
A number of PET radioligands have been examined as potential imaging agents for the serotonin transporter (SERT). These include derivatives of selective serotonin reuptake inhibitors, such as phenyl nortropanes and pyrroloisoquinoline derivatives [58–63]. The majority of these ligands are unsuitable for PET imaging applications as a result of high lipophilicity, unfavorable *in vivo* pharmacokinetics, or high

non-specific binding. An example of the pyrroloisoquinoline series is carbon-11-labeled (+)McN5652 (**22**), which has a long history of *in vivo* use in human PET studies. However, this radioligand is limited by a relatively modest signal-to-background ratio that results from high non-specific binding [64,65]. A wide variety of phenyl nortropane analogs have been examined as potential SERT imaging agents, yet few have demonstrated high signal-to-background ratios. A promising example from this class is [^{11}C]ZIET, but the ability of this compound to image SERT *in vivo* in human subjects remains to be demonstrated [66].

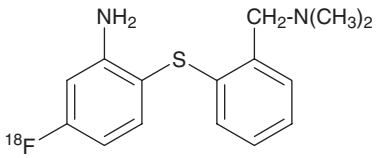


22

Recently, a new class of potential PET SERT agents, based on a diaryl sulfide structure, has been introduced. Several different radioligands from this class have been examined in both animals and humans, including [^{11}C]DASB (**23**), [^{11}C]AFM, [^{11}C]EADAM, [^{11}C]MADAM, [^{11}C]DAPP, [^{11}C]DAPA, and [^{11}C]HOMADAM [67–73]. Non-human primate and human imaging studies using **23** demonstrated that the compound reached a quasi-equilibrium binding state in the thalamus within a relatively short time frame (40 min) and demonstrated good specific-to-nonspecific binding ratios in brain regions known to contain high densities of SERT [69,74]. In non-human primates, [^{11}C]HODAM reached a state of quasi-equilibrium in less than one hour and demonstrated higher thalamus/cerebellum, mid-brain/cerebellum, and cortex/cerebellum ratios than those achieved using **23** [73]. [^{18}F]F-ADAM (**24**) and [^{18}F]AFM have demonstrated promising *in vivo* properties in rodent studies, but additional evaluations of brain pharmacokinetics and *in vivo* metabolism in non-human primates are required to assess the suitability of these ligands for SERT imaging [75–77].



23



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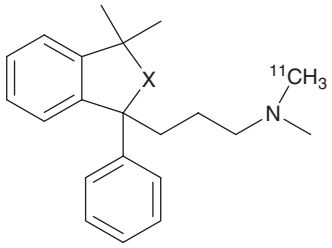
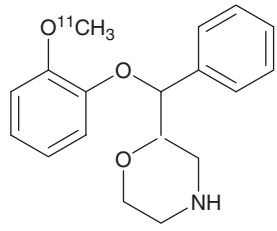
8.2. Norepinephrine transporter

The development of PET radioligands to image the norepinephrine transporter (NET) remains an area of active research. [^{11}C]Nisoxetine demonstrated high non-specific retention throughout the brain in rodent studies, and [^{11}C]desipramine failed as a CNS NET imaging radioligand as well [78,79]. While some NET radiotracers, such as {*N*-methyl-[^{11}C]}*m*-hydroxyephedrine, have demonstrated utility for imaging cardiac sympathetic innervation using PET, the same radioligand has not proven useful for imaging NET in the brain [80]. A series of benzo[*c*]thiophene and benzo[*c*]furan compounds have also been examined as potential lead candidates for the development of PET radioligands for the NET. Talopram (**25**) and talsupram (**26**) possess high affinity and selectivity for the NET and have been radiolabeled successfully with carbon-11. Both agents, however, demonstrated poor CNS uptake in rodents (<0.07% ID/g) and in non-human primates [81]. [^{11}C](*S*,*S*)-2-[(2-Methoxyphenoxy)phenylmethyl]morpholine (**27**), an analog of reboxetine, has demonstrated some promise in preliminary animal imaging studies [82,83]. The radioligand provided a hypothalamus-to-striatum ratio of 2.5-to-1 at 60 min post-injection in rats. The compound was also rapidly metabolized in rats, and evaluation of rat whole brain extracts demonstrated that greater than 95% of the extractable radioactivity was unmetabolized parent compound [82]. PET imaging studies in baboons of the (*S*,*S*)-isomer demonstrated a regional brain radioactivity distribution consistent with that known for NET, while studies utilizing the (*R*,*R*)-isomer showed a distribution consistent with only non-specific binding [83,84]. Another recent report of fluorine-18-radiolabeled analogs of reboxetine, ((*R*,*R*)- and (*S*,*S*)-2-[α-(2-(2-[^{18}F]fluoroethoxy)phenoxy)-benzyl]morpholine, [^{18}F]FRB), in non-human primates indicated that these analogs also may prove useful for NET imaging [85].



25 X = O
26 X = S



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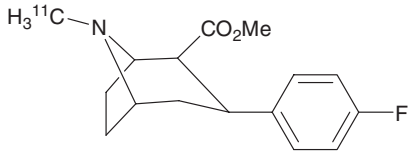
8.3. Dopamine transporter

The majority of dopamine transporter (DAT) PET radiotracers belong to the tropane family, and [^{11}C]cocaine was among the first DAT radioligands developed [86,87]. The relatively low affinity of cocaine for the DAT and its rapid metabolism led to the development of a variety of cocaine analogs. 2β-Carbomethoxy-3β-(4-iodophenyl)-tropane radiolabeled with iodine-123 ([^{123}I]β-CIT) was one of the first cocaine analogs used as a single photon emission computed tomography (SPECT) DAT radioligand, even though the compound demonstrated little selectivity for DAT (Ki 1.4 nM) compared to SERT (Ki 2.4 nM) [88,89]. In addition, [^{123}I]β-CIT demonstrated slow brain pharmacokinetics, so that delayed images (24 h post-injection) were required to obtain estimates of DAT concentrations in the striatum [90]. While β-CIT can be radiolabeled with carbon-11, it is not a useful PET radioligand as a result of its slow kinetics [91,92]. N-fluoroethyl and N-fluoropropyl derivatives of β-CIT demonstrated more rapid pharmacokinetics than the parent β-CIT, and carbon-11- and fluorine-18-labeled versions of these radiotracers have been evaluated. However, these radioligands also demonstrated a lack of selectivity for DAT relative to SERT [93].

The cocaine analog, 2β-carbomethoxy-3β-(4-fluorophenyl)tropane (WIN35,428, CFT, **28**), has been radiolabeled with both carbon-11 and fluorine-18 and has demonstrated utility as a PET radiotracer for DAT imaging [94,95]. However, the *in vivo* kinetics are relatively slow, with peak striatal uptake values reached approximately four hours post-injection [96]. 2β-Carbomethoxy-3β-(chloro-phenyl)-8-(2-fluoro-ethyl)nortropane (FECNT) has been radiolabeled using the two carbon synthon [^{18}F]fluoroethyltosylate [97]. [^{18}F]FECNT demonstrated faster peak striatal uptake (<2 h) compared to [^{11}C]WIN35,428 in non-human primate studies as well as higher striatum-to-cerebellum ratios [97].



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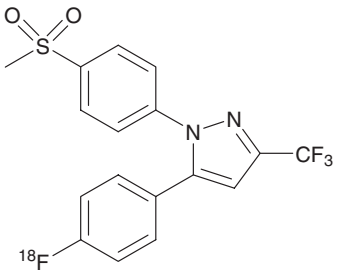
Bromo- and iodo-N-allyl cocaine analogs ((*E*)-N-(3-bromoprop-2-enyl)-2β-carbomethoxy-3β-4′-methylphenyl-nortropane (PE2Br) and (*E*)-N-(3-iodoprop-2-enyl)-2β-carbomethoxy-3β-4′methylphenyl-nortropane (PE2I) have been evaluated as potential DAT PET imaging agents following radiolabeling with either carbon-11 [98] or bromine-76 [99]. Both compounds demonstrated relatively fast equilibration times in the striatum and substantia nigra with good signal-to-background ratios between the striatum and cerebellum (10 at 40–50 min post-injection for [^{11}C]PE2I and 8 at 60 min post-injection for [^{76}Br]PE2Br).

9. ENZYME SYSTEMS

9.1. Cyclooxygenase-2 (COX-2)

Cyclooxygenase (COX), an enzyme involved in the biosynthesis of prostaglandins and thromboxanes, exists in two isoforms. The COX-2 isoform can be induced in response to inflammatory stimuli, is overexpressed in a variety of tumor types, and may also be involved in neurodegenerative diseases such as Alzheimer's and Parkinson's disease.

Fluorine-18-labeled analogs of DuP-697, as well as [^{18}F]SC58215 (**29**), have been evaluated as potential PET COX-2 imaging agents [100,101]. However, neither radioligand demonstrated increased radioactivity retention in animal models of COX-2 upregulation. Recently, a rofecoxib analog has been radiolabeled with fluorine-18 utilizing the Stille reaction with 4-[^{18}F]fluoroiodobenzene, but no *in vivo* data have been presented [102].



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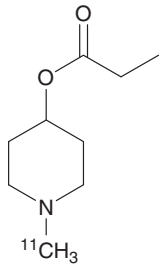
9.2. Acetylcholinesterase

Acetylcholine has been implicated in the function of memory and cognition, and its involvement in the loss of memory associated with aging and Alzheimer's disease has been suggested in clinical and postmortem studies [103]. Several recent reviews have focused on imaging the acetylcholinesterase (AChE) system and its use in drug design and therapy evaluations [104,105].

In vivo imaging studies of AChE have utilized two approaches, either radiolabeled inhibitors or radiolabeled substrates for AChE. Several inhibitors of AChE have been radiolabeled in order to visualize the brain distribution of AChE using PET, including [^{11}C]donepezil and [^{11}C]physostigmine. While animal imaging studies with [^{11}C]donepezil failed to demonstrate a radioactivity distribution consistent with the known regional brain distribution of AChE, PET imaging studies using [^{11}C]physostigmine in normal human subjects successfully displayed a regional distribution of radiotracer similar to that of AChE in postmortem human brain [106]. However, the non-specific binding of [^{11}C]physostigmine is relatively high,

limiting its usefulness. Several lactam benzisoxazole derivatives have demonstrated excellent specificity and selectivity for AChE. One of these, CP-126,998, has been radiolabeled with carbon-11 and demonstrated a high striatum-to-cerebellum ratio in mice. In addition, the striatal retention was specific for AChE, as demonstrated by blocking studies in mice [107]. [^{11}C]CP-126,998 imaging studies in human control subjects demonstrated a brain radioactivity distribution consistent with that of the known distribution of AChE [108]. Another AChE inhibitor has recently been radiolabeled with fluorine-18, 2-[^{18}F]fluoro-CP-118,954, and preliminary studies in mice suggest that this compound binds specifically and selectively to AChE [109].

An alternative approach to the non-invasive measurement of AChE is the use of radiolabeled acetylcholine analog substrates that are able to cross the blood-brain barrier, are selectively hydrolyzed by AChE, and are subsequently trapped in the brain [110]. A variety of N-methylpiperidyl esters have been radiolabeled with carbon-11 and evaluated as potential AChE substrates, including 1-[^{11}C]methylpiperidin-4-yl propionate ([^{11}C]PMP or [^{11}C]MP4P (**30**)) and 1-[^{11}C]methylpiperidin-4-yl acetate ([^{11}C]MP4A) [111,112]. [^{11}C]MP4A and **30** have both demonstrated utility as PET radiotracers to measure regional brain AChE activity in normal subjects and subjects with Alzheimer's disease [113–115]. A series of fluoroalkyl analogs of PMP has also been described, and one of these, (*R*)-N-[^{18}F]fluoroethyl-3-pyrrolidinyl acetate, exhibited similar characteristics to those of **30** in both mouse and non-human primate studies [116].



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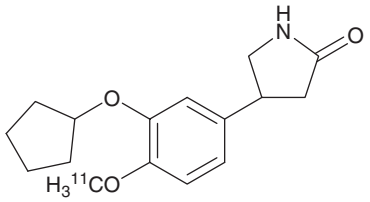
10. SECOND MESSENGER SYSTEMS

Efforts to image post-receptor signal transduction have focused on the development of PET radiotracers for the cyclic adenosine monophosphate (cAMP), phosphoinositide (PI), and arachidonic acid pathways [117,118]. One approach to the non-invasive monitoring of the cAMP system has been the development of radioligands based on compounds that inhibit the enzyme responsible for the hydrolysis of cAMP. Cyclic AMP is inactivated through hydrolysis via phosphodiesterase 4 (PDE4) enzymes that are comprised of four different subtypes. Rolipram, a specific inhibitor of the PDE4 family that does not display any sub-type selectivity, has been radiolabeled with carbon-11 in both the *R*(-)- and *S*(+)-forms (**31**) and demonstrated high specific brain uptake in an *ex vivo* rat study, as well as *in vivo* behavior

in porcine brain that correlated well with the known *in vitro* affinities of *R*(-)- and *S*(+)-rolipram [119,120].



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Phosphoinositide turnover is closely connected to the modulation of synaptic function and studies have demonstrated the incorporation of 1-[^{11}C]butyryl-2-palmitoyl-glycerol ([^{11}C]DAG) into the downstream components of the rat PI system, including phosphatidic acid and phosphotidylinositol [121]. This radiotracer has been utilized in imaging studies in normal control human subjects, as well as in Parkinson's disease, Alzheimer's disease, and stroke subjects [122]. [^{11}C]DAG has also been utilized for the evaluation of PI turnover in ischemic brain using PET [123]. However, the relatively high lipophilicity of the radioligand resulted in high non-specific binding and relatively slow pharmacokinetics in the brain.

11. CONCLUSIONS

While considerable progress has been made over the past five years in applying PET radiotracers to CNS drug discovery and development efforts, much work remains to be accomplished in many areas. It is worth emphasizing that the ability of new imaging agents to assist in a meaningful way with CNS drug development will depend largely on how carefully and thoroughly the properties of the imaging agents and their intended *in vivo* target(s) have been characterized. The ultimate usefulness of these agents will depend on the accurate interpretation of non-invasive imaging data, which will be possible only following the complete characterization of the behavior of the imaging agent both *in vitro* and *in vivo*.

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Emerging Topics in Atherosclerosis: HDL Raising Therapies

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Contents

1. INTRODUCTION

Atherosclerosis is an abnormal remodeling of the vasculature, generally seen in areas of high turbulent blood flow and in the presence of elevated serum lipid concentrations and high blood pressure. Early lesions, known as fatty streaks, are characterized by an influx of inflammatory cells and the accumulation of cholesterol. These fatty streaks evolve into fibrous plaques consisting of lipids, smooth muscle cells and connective tissue. Over time, the plaques may grow and calcify, thereby narrowing or completely blocking the affected blood vessel. Rupture of an atherosclerotic lesion can trigger an occlusive clot leading to heart attack or stroke. The atherosclerosis related conditions of coronary heart disease (CHD), cerebrovascular disease and peripheral vascular disease are major causes of morbidity and mortality in the U.S. and other parts of the Western world. As high total serum cholesterol and elevated low density lipoprotein-cholesterol (LDL-C) levels are risk factors of CHD, most current approaches to the treatment of dyslipidemias focus on lowering LDL cholesterol. Clinical trials have established that use of HMG

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Co-A reductase inhibitors (statins) can reduce LDL-cholesterol by up to 55% and lower the incidence of heart attack and stroke by up to 30% [1]. While significant, there remains a need to develop therapies to further reduce the burden of this disease.

There is a growing body of evidence showing an inverse correlation between high density lipoprotein-cholesterol (HDL-C) levels and CHD. Analysis of the Framingham Heart Study, Helsinki Heart Study and VA-HIT trials led to an estimate of a 3% decrease in death or heart attack for every 1% increase in HDL-C [2]. While the beneficial effect of HDL has been attributed primarily to its role in reverse cholesterol transport (the movement of cholesterol from the periphery to the liver for excretion) there is an increasing awareness of other atheroprotective roles of HDL, particularly its anti-oxidant and anti-inflammatory effects [3]. The role of HDL in atherosclerosis, its potential as a therapeutic target and approaches to increasing HDL have been the subject of many recent reviews [4–16].

Lipid biochemistry and physiology are complex and the biological pathways invoked in the effort to raise HDL-C vary widely. Agents that raise HDL include niacin, the statins and fibrates. Niacin therapy is the most effective method of increasing plasma HDL-C concentrations (up to 35%) and has been shown to improve clinical outcomes [17,18]. Statins, used as primary therapy for lowering LDL, modestly increase HDL-C (5–10%). Fibrates also raise HDL-C (5–15%) in addition to lowering triglycerides. This report will describe recent advances in the identification of the physiological target of niacin as well as some newer targets for HDL-C raising therapy.

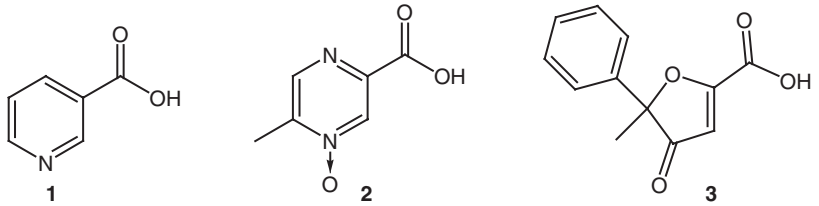
2. NICOTINIC ACID RECEPTOR AGONISTS

2.1. Niacin

Niacin, **1**, (nicotinic acid) has been used clinically since 1955 for the treatment of dyslipidemias. Niacin therapy has a beneficial effect on all blood lipid parameters, resulting in lower triglycerides, lower VLDL/LDL and increased HDL. The exact mechanism by which niacin exerts this effect is not precisely understood but it is known that administration of niacin reduces cAMP levels in adipocytes thereby inhibiting lipolysis by hormone sensitive lipase. The resulting decrease in free fatty acid release by adipocytes leads to decreased hepatic triglyceride synthesis and VLDL production. VLDL is the primary recipient of cholesteryl esters originating in HDL and transferred by the cholesteryl ester transfer protein. It is postulated that HDL-C levels increase as the amount of VLDL acceptor diminishes. Recently, a G-protein coupled receptor – termed HM74A – was identified as the high affinity target of niacin by three separate groups [19–21]. The reported dissociation constants for niacin were in good agreement and ranged from 63 to 95 nM. It was shown that niacin robustly stimulates binding of [^{35}S]GTPγS in HEK293 T cells expressing HM74A together with the G protein $G\alpha_{o1}$ ($EC_{50} = 250$ nM), thus recapitulating the same effect observed in rat adipocyte and spleen membranes.

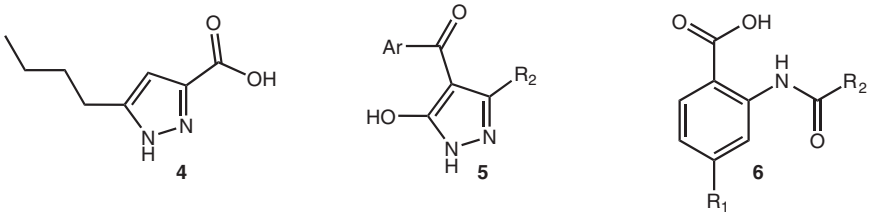
Additionally, both acipimox, **2**, and acifran, **3**, two niacin analogs that have beneficial effects on lipid profiles in humans, were also shown to have a high affinity for this receptor and to stimulate [^{35}S]GTPγS binding in the system described above.

Despite its attributes, niacin therapy suffers from a lack of compliance on the part of the patient due to the commonly experienced side effect of intense flushing. Repeated use of niacin results in less frequent and milder bouts of flushing but the problem may persist for the duration of treatment. Current approaches to the clinical use of niacin favor extended release formulations that attenuate the peak plasma levels achieved upon administration of this high-dose (> 1 g/day) drug and thereby lessen the severity of flushing.



2.2. Other nicotinic acid receptor agonists

Surprisingly little recent work has been published in the area of niacin analogs. A series of pyrazole-3-carboxylic acids has been reported as partial agonists for the nicotinic acid receptor [22]. It was postulated that partial agonism might result in tissue selectivity. The most potent member of the class, 5-butyl-pyrazole-3-carboxylic acid, **4**, had the greatest affinity to the nicotinic acid receptor as measured by a competitive binding assay using rat spleen membranes ($K_i = 72$ nM). In the assay measuring agonist induced stimulation of [^{35}S]GTPγS binding to rat adipocyte membranes, **4** exhibited a 4.12 μM EC_{50} and 75% activity relative to nicotinic acid. Tissue selectivity was not observed, however, as similar results were obtained in the same assay using rat spleen membranes ($EC_{50} = 2.26$ μM, activity relative to nicotinic acid = 81%). Recent patents claim similar 4,5-dialkyl-pyrazole-3-carboxylic acids, [23,24], hydroxypyrazoles, **5**, wherein Ar = heteroaryl [25], and substituted 2-amino benzoic acids, **6**, [26,27] as nicotinic acid receptor agonists.



3. CETP INHIBITORS

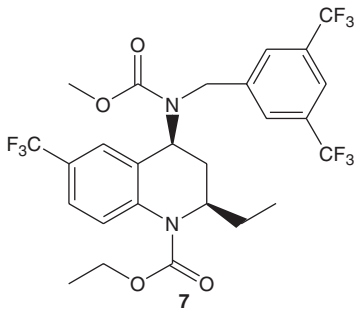
3.1. Background

Cholesteryl ester transfer protein (CETP) mediates the exchange of cholesteryl esters (CE) and triglycerides (TG) among lipoprotein particles. The process is driven by the substrate concentration gradient between the lipoproteins, and the net effect is the transfer of cholesteryl esters from the CE-rich HDL particles primarily to VLDL and the reciprocal movement of triglycerides from TG-rich VLDL particles to HDL. It has been postulated that CETP is pro-atherogenic as it directly decreases plasma HDL-C and increases LDL-C. Genetic studies of populations with reduced or absent CETP expression show markedly increased HDL-C levels but have yielded contradictory evidence as to whether the diminished CETP activity results in fewer cardiac events. It has been established in clinical studies that pharmacological inhibition of CETP results in increased plasma HDL-C concentrations, although the effect of this inhibition on coronary heart disease has yet to be determined. The use of CETP inhibitors as a method for treating dyslipidemias and atherosclerosis has been the subject of several recent reviews [28–31].

A number of diverse structure types have been reported as CETP inhibitors and have been reviewed [32,33]. It is not surprising that CETP inhibitors are generally highly lipophilic compounds, given the nature of the protein's physiological substrates (CE, TG). One consequence of this lipophilicity is loss of *in vitro* potency in whole serum assays relative to buffered systems, presumably due to non-specific serum protein binding. Another consequence is relatively poor bioavailability. Patent applications have published covering formulations for improving bioavailability for the two clinical candidates discussed below [34,35]. In a related vein, the observation has been made that multiple fluorine substituents are favored, likely due to imparted lipophilicity [33,36]. This observation is supported by the compounds highlighted below.

3.2. 4-Amino-tetrahydroquinolines

Torcetrapib, **7**, is a potent, reversible CETP inhibitor exhibiting an *in vitro* plasma IC_{50} of approximately 50 nM [37]. Phase I dose ranging studies showed dose dependent CETP inhibition as well as HDL-C elevation. The highest dose (120 mg bid) raised HDL-C concentrations 91% and reduced LDL-C 42%. ApoA-I increased to a smaller extent than did HDL-C. This fact was reflected in an observed size increase for the HDL particles and is consistent with lipoprotein profiles in CETP deficient populations. A phase II study in patients with low HDL-C showed the ability of **7** to markedly increase HDL-C when used alone or in combination with a statin (atorvastatin) [38]. A 120 mg/day dose of **7** for 4 weeks in patients receiving 20 mg atorvastatin saw an average HDL-C increase of 61% plus an additional 17% decrease in LDL-C. Extension of this class of CETP inhibitor to include 4-carbon substituted tetrahydroquinolines, **8** (X, Y = H), dihydroquinolines, **9** (X, Y = bond) and quinaxolines **10**, has been reported [39].



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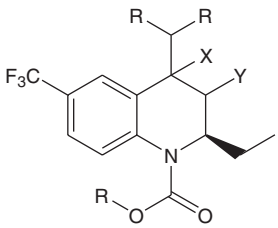

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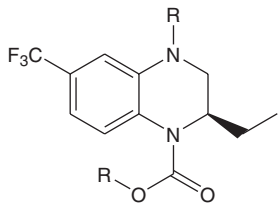

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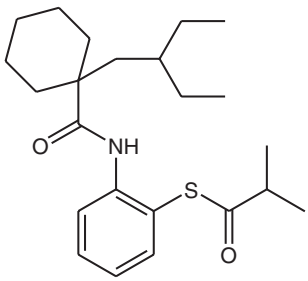
3.3. Acylaminobenzenethiols

A second CETP inhibitor has been studied in the clinic. Acylaminobenzenethioester **11** (JTT-705) is a significantly weaker inhibitor *in vitro* ($IC_{50} = 6\,\mu M$ in human plasma) than is torcetrapib [40]. In a 4 week phase II study, oral administration of **11** resulted in a dose dependent decrease in CETP activity and a concomitant increase in plasma HDL-C. The compound was well tolerated and the top dose of 900 mg resulted in a 37% increase in HDL-C [41]. Unlike torcetrapib, **11** is an irreversible inhibitor of CETP. Systematic structural modifications of the acylaminobenzenethiol scaffold showed that the sulfur atom is requisite for activity [40]. The free thiol, **12**, shows activity *in vitro* (3 μM IC_{50} in human plasma) and is considered to be the active species *in vivo*. Point mutation studies with recombinant CETP indicate that **12** forms a disulfide bond with cys13 residue located in the hydrophobic binding pocket of CETP [40]. As well, washout experiments with inhibited protein failed to restore activity. Thioester **11** exhibited improved oral bioavailability and stability relative to the free thiol **12** and so was selected for development [40]. Further SAR studies report that electron withdrawing groups on the acylaminophenyl ring can lead to improved activity. Thus, dichloro analog **14** (R = Cl) showed a 30-fold improvement in *in vitro* potency relative to the unsubstituted analog **13** (R = H). This gain in potency is attributed to acceleration of thioester hydrolysis [42].



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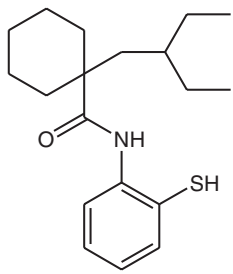

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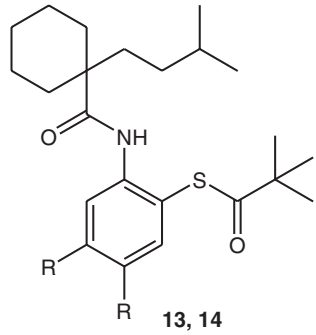
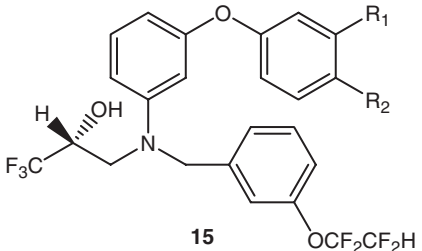


13, 14

3.4. Trifluoro-3-amino-2-propanols

A potent CETP inhibitor series based on an N-aryl-N-benzyl-trifluoro-3-amino-propanol scaffold has been the subject of several reports [43–46]. The starting point for this series was a 40 μM lead compound identified by screening of a combinatorial library. Sequential modification led to **15** (R_1, R_2 = H), which displayed an IC_{50} of 20 nM in a buffered assay system and a 30-fold shift in potency (IC_{50} = 600 nM) when assayed using human serum. The stereochemistry of the trifluoromethylamino-propanol side chain is critical with the R-enantiomer being roughly 40 times more active than the S-enantiomer. Further optimization of this series led to the subnanomolar inhibitor **16** (R_1 = ethyl, R_2 = Cl) having an IC_{50} of 0.8 nM in buffer and only a 7.4-fold serum shift (IC_{50} = 59 nM) [46]. In an *ex vivo* assay using transgenic mice expressing human CETP (hCETP mice), a single 30 mg/kg p.o. dose of **16** inhibited the CETP mediated transfer of radiolabelled cholesteryl ester from HDL to LDL by 38%. The same assay using Syrian golden hamsters gave comparable results. Five day studies of **16** at 30 mg/kg in hCETP mice and Syrian golden hamsters resulted in 12% and 6.2% increases in plasma HDL, respectively. The ability of **16** to only modestly raise HDL in mice and hamsters was not reflective of its *in vitro* potency. The compound has been shown to bind specifically to human serum albumin, and the modest efficacy in these models may be due in part to specific binding to plasma proteins in these species [46].



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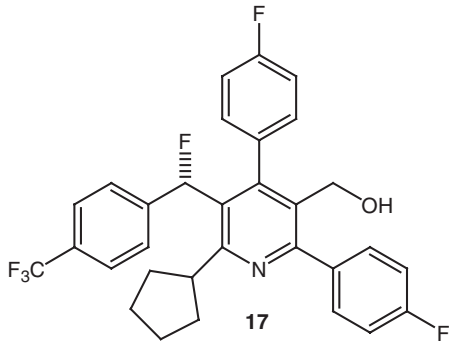
16

3.5. Pyridines

A series of pentasubstituted pyridine CETP inhibitors has been described [36]. A 1000-fold improvement in *in vitro* activity was realized as a 15 μM lead evolved into the low nanomolar inhibitor **17** (CETP IC_{50} = 13 nM). Citing insufficient metabolic stability attributed to the primary benzylic alcohol (data not given), the lead was further modified to a bicyclic scaffold **18** (X = N, R_1, R_2 = CH_3; CETP IC_{50} = 9 nM). Administration of **18** to hCETP mice at doses of 5 mg/kg and 10 mg/kg p.o. resulted in 35% and 50% increases in HDL-C, respectively. Noteworthy is the statement that New Zealand white rabbits maintained on a high fat diet and dosed (in food) for three months with **18** at 50 and 150 mg/kg showed reductions of atherosclerotic plaque areas of 40 and 70%, respectively (no further details given). In the course of the studies leading to **18** it was discovered that the tetrahydronaphthalene analogs were similarly potent. The tetrahydronaphthalene scaffold was

optimized to **19** (X = C, R_1, R_2 = -$(CH_2)_3$-; CETP IC_{50} = 3 nM). Administration of **19** to hCETP mice at 0.6 mg/kg p.o. elevated plasma HDL-C by 54%.



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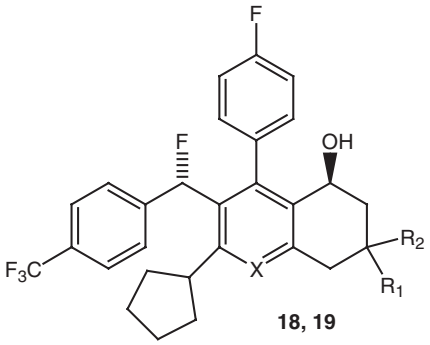
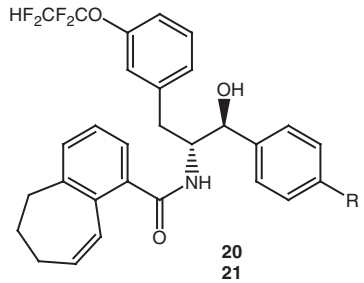


18, 19

3.6. Other CETP inhibitor scaffolds

CETP inhibitors based on a second aminoalcohol scaffold have been claimed [47]. Compounds **20** and **21** are among the most potent analogs listed and are equipotent in a buffered assay system using a fluorescence transfer assay (IC_{50} = 8 nM for each). However, in an assay containing 50% human serum, **20** (R = F) showed a 25-fold shift (IC_{50} = 200 nM), while **21** (R = phenoxy) showed only a 7.5-fold shift (IC_{50} = 60 nM). An N-heteroaryl-N,N- dibenzylamine scaffold has also been claimed [48]. Tetrazolyl derivative **22** was reported to have a plasma IC_{50} = 90 nM. A hamster *ex vivo* assay showed 55% inhibition of CETP 4 hours post a 3 mg/kg oral dose. HDL raising *in vivo* was demonstrated in hamsters at the same dose (31% increase, 8 h post dose).



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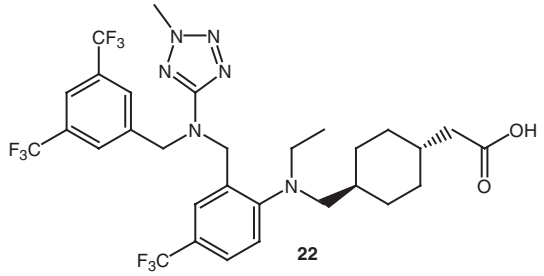

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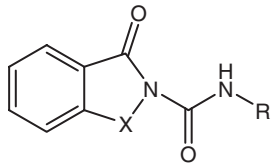
4. LIPASE INHIBITORS

Hepatic lipase (HL) and endothelial lipase (EL) are homologous hydrolytic enzymes involved in lipid metabolism and both have been suggested as therapeutic targets for raising HDL. As its name suggests, hepatic lipase is expressed primarily in the liver. Individuals with a common polymorphism possess a less active form of the enzyme and exhibit increased plasma HDL-C concentrations. It remains

unclear, however, as to whether the inhibition of hepatic lipase will be pro- or antiatherogenic and the topic has been reviewed [49,50]. Rather, studies indicate that the result of inhibition will likely depend on the background lipoprotein profile in which inhibition takes place. The case supporting endothelial lipase as a target for raising HDL is stronger and has also recently been reviewed [51]. Endothelial lipase is expressed in the vascular endothelium and it is active in hydrolyzing HDL associated lipids. Overexpression of endothelial lipase in mice results in markedly decreased HDL levels [52]. Functional loss of endothelial lipase in mice, either by genetic deletion [52,53] or antibody neutralization [54], leads to increased plasma HDL-C. One indication that endothelial lipase inhibition may be atheroprotective comes from a study showing that endothelial lipase/apoE double knockout mice were less susceptible to the development of atherosclerosis than were the mice lacking only apoE [55].

Although HL and EL may be useful targets for raising HDL, there are few reports of small molecule inhibitors of these enzymes. Patents claiming a homologous series of heterocycles as both hepatic lipase and endothelial lipase inhibitors have been published [56–58]. Inhibition of hepatic lipase activity for the benzoisoxazole, **23**, and indazole, **24**, derivatives were comparable (IC_{50}s = 62 and 67 nM, respectively) while the benzoisothiazole congener, **25**, was roughly 10-fold less potent (IC_{50} = 879 nM). Inhibition data for endothelial lipase were reported only for a small number of indazoles with the ethyl substituted benzyl urea **26** exhibiting the greatest potency (IC_{50} = 12 nM).



23 X = O, R = $(CH_2)_4CH_3$
24 X = NH, R = $(CH_2)_4CH_3$
25 X = S, R = $(CH_2)_4CH_3$
26 X = NH, R = CH_2-*o*-ethylphenyl

5. APO A-I AND MIMETICS

5.1. ApoA-I milano

ApoA-I is the constitutive protein of HDL. It is produced in the liver and intestines and is secreted along with phospholipid as pre-β1 HDL – a discoidal particle that becomes HDL after complex lipid remodeling of the particle in the plasma. ApoA-I Milano is a naturally occurring variant of apoA-I wherein arg173 is replaced by a cysteine residue. Individuals expressing this protein have significantly lower than average HDL-C. However, the subpopulation expressing apoA-I Milano shows a much reduced frequency of atherosclerosis than would be

anticipated based on their HDL levels [59]. In a small placebo controlled clinical trial, recombinant apoA-I Milano/phospholipid complex was administered intravenously once per week for 5 weeks to subjects with coronary artery disease. Intravascular ultrasound imaging studies were used to gauge the progress of the atherosclerosis. At the end of the trial the treated group had a small but significant reduction in atheroma volume while the untreated group had a small increase in plaque size [60].

5.2. ApoA-I mimetics

The approach mentioned above involving administration of exogenous apoA-I or HDL suffers from the limitation of requisite iv administration. An interesting variation on this approach is the effort to develop an orally bioavailable apoA-I mimetic, which is the subject of a recent review [61]. The preparation and characterization of a number of amphipathic peptides as potential apoA-I mimetics has been reported. One compound, D-4F, is an 18 residue peptide comprised of D-amino acids. Oral administration of D-4F to apoE knockout mice resulted in low (picomolar) plasma concentrations of the peptide. These animals showed an increase in preβ-HDL enriched in antioxidant activity. As a control, scrambled D-4F was administered and resulted in no detectable plasma levels nor any effects on blood lipids [62]. Studies on the effect of D-4F on atherosclerosis in apoE knockout mice resulted in a reduction of atherosclerotic plaque size in evolving lesions but had no significant effect on established atherosclerosis [63].

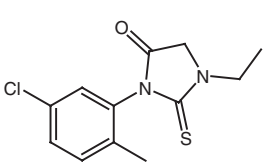
6. AGENTS WITH UNSPECIFIED MECHANISMS

Thiohydantoin **27** is one of a series of compounds that were shown, via *in vivo* profiling in diet induced hypercholesterolemic rats, to preferentially increase HDL relative to other lipoproteins [64]. In this model, 8 day dosing (in food) of **27** at 100 mg/kg led to a 132% increase in HDL-C. In a similar experiment using normal rats, administration of **27** at 100 mg/kg resulted in an HDL-C increase of 85% relative to control and apoA-I showed a 52% increase at this dose (as determined by ELISA). Evaluation of **27** in cholesterol fed hamsters resulted in a 54% increase in HDL-C as well as decreased LDL-C.

A second thiocarbonyl containing series has been reported and was also discovered through *in vivo* profiling [65]. When administered to cholesterol fed rats in chow (*ad libitum*) thiourea analog **28** increased HDL-C and apoA-I by 156% and 94%, respectively, relative to control. No mechanisms that would account for the observed HDL increases were proposed for either **27** or **28**.

It has been shown over the past three decades that long chain hydrocarbons can produce beneficial effects on lipid profiles in animal models, although the mechanism(s) by which this occurs remain unknown. Studies were reported recently wherein administration of dicarboxylic acid **29** to obese Zucker fatty rats for 14 days at 100 mg/kg/day led to 279% increase in HDL-C and a decrease in

triglycerides of 91% relative to pretreatment values [66]. Additionally, **29** was shown to inhibit fatty acid and sterol synthesis in rat hepatocytes and *in vivo* and it was proposed that this inhibition of lipid synthesis at least partially explains the beneficial effect of these compounds on lipid profiles. More recently, the series was extended to α-cycloalkyl-ω-keto dicarboxylic acids and analog **30**, when tested in obese Zucker fatty rats as described above, led to an increase in HDL-C of 171% and a decrease in triglycerides of 94% relative to pretreatment values [67]. Compound **30** also showed inhibition of fatty acid synthesis in rat hepatocytes.



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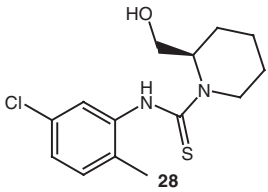
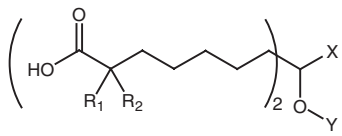


28



29 $R_1 = R_2 = CH_3$, X = Y = H
30 $R_1, R_2 = -CH_2CH_2-$, X, Y = bond

7. CONCLUSION

Niacin therapy and CETP inhibition are currently the two most clinically validated small molecule approaches towards raising HDL. Niacin therapy is the most established method of productively increasing HDL but suffers from lack of compliance. The discovery of the physiological target of niacin should aid the effort to identify potent nicotinic acid receptor agonists which lack the undesired side effects. CETP inhibitors have been shown to markedly raise HDL in humans but it is still unknown whether CETP inhibition will have a beneficial effect on atherosclerosis and its related conditions. That question, however, should be answered in the near future by the two current clinical candidates. There remains a need to reduce atherosclerosis associated morbidity and mortality. That fact, coupled with the increasing awareness of the beneficial effects of HDL, ensures continued efforts to identify effective HDL raising therapies.

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Small Molecule Anticoagulant/Antithrombotic Agents

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Contents

1. INTRODUCTION

The introduction of new anticoagulant or antithrombotic agents to treat both acute and chronic cardiovascular diseases has been stymied since the adoption of aspirin as an antiplatelet agent and coumadin as an oral anticoagulant agent. Both agents have gained widespread use, but were introduced several decades ago. Only since the recent introduction of the thienopyridine antiplatelet agents ticlopidine and clopidogrel has there been major impact on the treatment paradigms for patients with chronic thrombotic disorders. Coumadin remains the only oral anticoagulant. Significant resources have been expended in the search for the next generation agents during the last several decades [1–4] and there continues to be promise, but success has been fleeting.

2. ANTICOAGULANTS

2.1. Thrombin inhibitors

The serine protease thrombin occupies a central role in coagulation. The primary actions of thrombin are to activate platelets and to cleave fibrinogen to fibrin, which together constitute the primary components of vascular hemostasis. Inhibitors of thrombin have been recognized as potential therapeutic agents for the treatment of a variety of thrombotic disorders. Intravenous and oral thrombin inhibitors have

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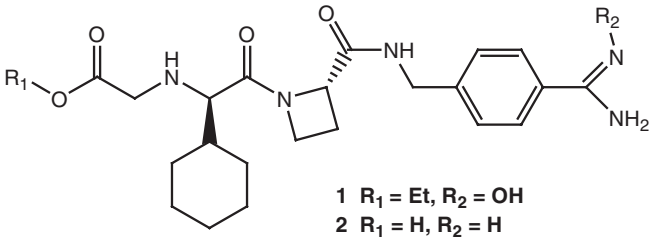
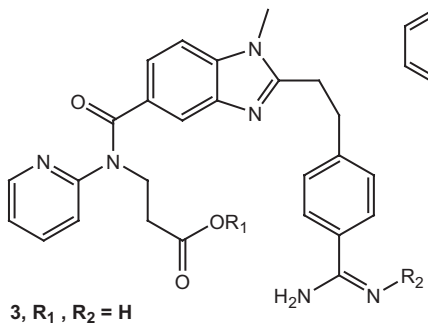
shown promising results in human clinical trials [5]. Recently, ximelagatran **1** (Exanta), the prodrug of oral direct thrombin inhibitor melagatran **2** was approved for short-term venous thromboembolism (VTE) prophylaxis following orthopaedic surgery. In humans the bioavailability of melagatran following oral administration of ximelagatran is about 20% and its half-life is approximately 3 h. A fixed dose of ximelagatran (without coagulation monitoring) is as effective as carefully monitored warfarin for the prevention of stroke and is associated with less bleeding [6–8]. However, in October 2004, the FDA decided not to approve ximelagatran for the prevention of stroke in patients with atrial fibrillation, for the prevention of VTE in patients undergoing knee replacement or for the long-term prevention of VTE because of liver toxicity concerns [6].

A series of thrombin inhibitors built around a 1,2,5-trisubstituted benzimidazole as the central scaffold has been reported [9]. The most potent and selective analog in this class is dabigatran **3** which inhibits thrombin with a K_i of 4.5 nM. Upon iv bolus administration to rats **3** (1 mg/kg) time-dependently prolonged the *ex vivo* activated partial thromboplastin time (APTT) up to 3 h after administration [9]. Dabigatran etexilate (BIBR1048, **4**), an orally active double prodrug of dabigatran, is in Phase III clinical trials [9,10].

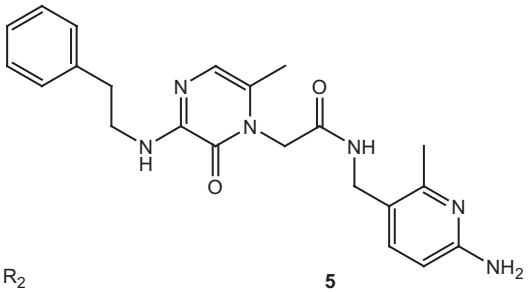


1 R_1 = Et, R_2 = OH
2 R_1 = H, R_2 = H

The pyrazinone acetamide **5** is a potent, orally bioavailable thrombin inhibitor ($K_i = 0.8$ nM) that has served as a starting point for further optimization [11]. Modification of **5** to reduce metabolism led to chloropyrazinone **6**, which had good potency ($K_i = 5$ nM), oral bioavailability and an improved half-life in dogs of 4.5 h [12]. Further modifications of the P1 group led to discovery of the tetrazole analog **7** with a K_i of 1.4 pM, one of the most potent thrombin inhibitors reported to date [11]. Tetrazole **7** doubled the APPT (2x APPT) in human plasma *in vitro* at a concentration of 0.13 μM.



3, R_1 , R_2 = H
4, R_1 = Et, R_2 = CO_2-hexyl



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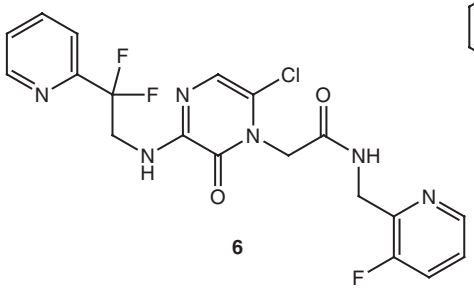

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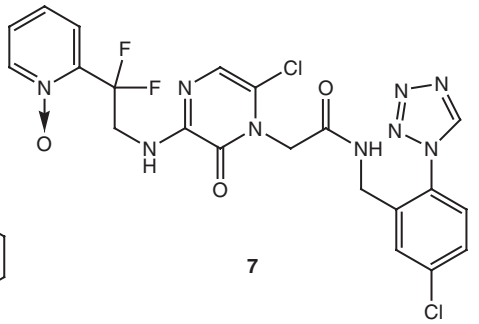
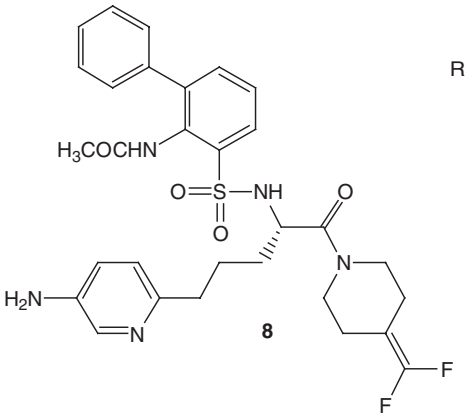


7

The orally active thrombin inhibitor **8** (SSR182289A) was reported to have a K_i of 31 nM [13]. In the arterio-venous shunt model in rats **8** strongly inhibited thrombus formation ($ED_{50} = 3.1$ mg/kg). Other proline P2 based thrombin inhibitors have been reported such as **9** ($K_i = 2.1$ nM) and **10** ($K_i = 3.7$ nM) which exhibited 2x APTT of 0.23 and 0.28 μM respectively [14]. Compound **10** possessed favorable pharmacokinetics in three species (dog: F = 81%, $t_{1/2} = 3.9$ h; monkey: F = 46%, $t_{1/2} = 3.5$ h; rat: F = 37%, $t_{1/2} = 2.0$ h).



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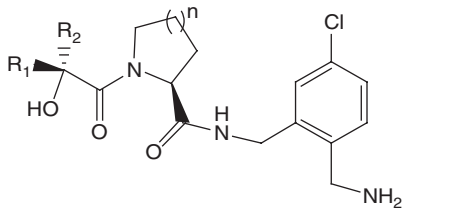
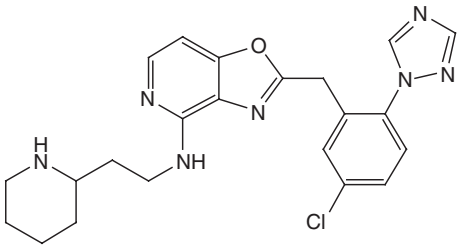


9, R_1 = H, R_2 = *t*-Bu, n = 1
10, R_1 = H, R_2 = C(Me)$_2$c-Pr, n = 0



11

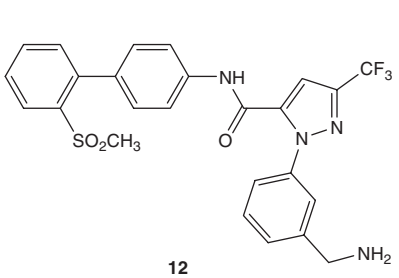
Novel benzoxazole P2 scaffold-containing thrombin inhibitors exemplified by compound **11** have been reported [15]. Compound **11** showed good potency

($K_i = 36$ nM, 2x APTT = 70 nM) and complete antithrombotic efficacy in an *in vivo* rat $FeCl_3$ model, but exhibited poor oral bioavailability in dog (F < 1%).

2.2. Factor Xa inhibitors

Factor Xa is another key serine protease in the coagulation cascade and is a promising target enzyme for prevention of arterial and venous thrombosis. Factor Xa inhibitors have demonstrated potent anticoagulant activity *in vitro* and antithrombotic efficacy in preclinical and clinical models *in vivo*. Several comprehensive review articles on factor Xa inhibitors have been published [16–18]. This section will focus on the most recent advances reported in late 2003 and 2004 towards the design and discovery of novel orally bioavailable factor Xa inhibitors.

Attempts to improve the oral bioavailability and plasma half-life of benzamidine-containing factor Xa inhibitors by direct replacement of the highly basic benzamidine in the P1 position with mimics or neutral residues have been the subject of many efforts. For example, benzylamine **12** (DPC423, $K_i = 0.15$ nM) is orally bioavailable in both dogs (57%) and rats (36%) with half-life in these species of 7.5 h and 4.6 h respectively [19]. Optimization in this series led to razaxaban (DPC906, **13**) as a clinical candidate [20,21]. The X-ray crystal structure of razaxaban complexed with factor Xa has revealed binding interactions similar to those of other benzamidine mimics [21].



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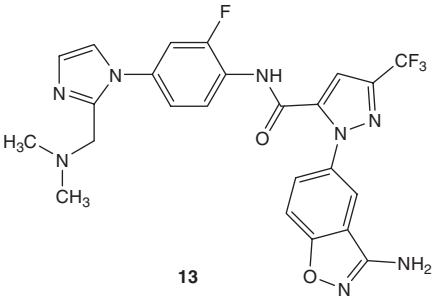

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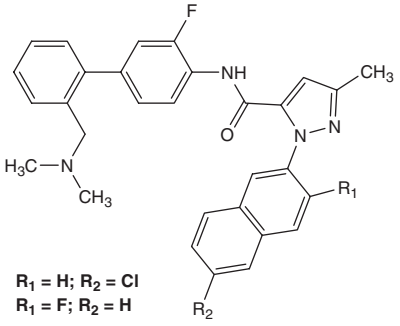
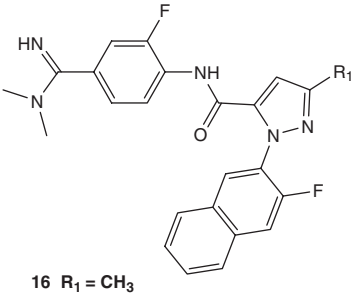


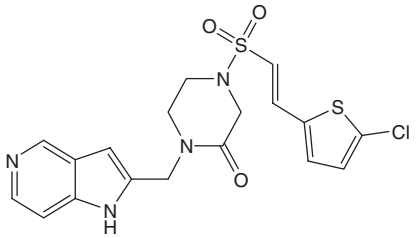
14 R_1 = H; R_2 = Cl
15 R_1 = F; R_2 = H



16 $R_1 = CH_3$
17 $R_1 = CF_3$

A 1-(2-naphthyl)-1H-pyrazole-5-carboxamide series of factor Xa inhibitors exemplified by compounds **14** ($K_i = 1.5$ nM) and **15** ($K_i = 2.4$ nM) has been reported. In rats, **14** and **15** possessed modest oral bioavailability of 35 and 23% respectively and half-lives of 3 to 7 h [22]. Replacement of the benzylamine moiety by an N,N-dialkylated benzamidine to improve oral absorption afforded **16** ($IC_{50} = 22$ nM) which displayed an oral bioavailability of 49% in rats. A more potent trifluoromethyl derivative **17** ($IC_{50} = 3$ nM) afforded an overall enhancement of rat pharmacokinetic parameters ($F = 47\%$, $t_{1/2} = 8.8$ h, $Cl = 14.7$ ml/ min /kg) [23].

A new series of factor Xa inhibitors containing the N-sulfonylketopiperazinone moiety has been identified, the most potent being **18** (RPR-209685) with a factor Xa K_i of 1.1 nM [24]. Sulfonamide **18** was orally bioavailable in dogs (5 mg/kg, $F = 97\%$), but displayed a short half-life of 52 min and a Cmax of 1.6 μM. In a canine model of arterial and venous thrombosis, dosing of **18** (20 mg/kg po) afforded a 1.9-fold prolongation in time-to-occlusion on the venous side and 1.8-fold on the arterial side. An X-ray crystal structure of **18** bound to factor Xa revealed a reversal of the expected binding orientation, with the chlorothiophene moiety binding in the S1 pocket and the azaindole occupying the S4 pocket [25]. The unique reversed-binding mode revealed that electrostatic interactions in the S1 subsite are not absolute requirements to maintain high affinity for factor Xa and selectivity against other serine proteases such as thrombin and trypsin. Another series of sulfonylpiperazine analogs incorporating neutral P1 moieties with basic N,N-dialkylbenzamidine P4 substituents was reported as potent factor Xa inhibitors [26]. The 6-chlorobenzo[*b*]thiophene and 5-chloroindole groups were found to be optimal S1 binding elements, and the N-methylimidazoline **19** ($K_i = 1.9$ nM) was identified as the most potent inhibitor. In a rabbit deep venous thrombosis (DVT) model, compound **19** produced 57% inhibition of thrombus growth and a 2-fold *ex-vivo* prothrombin time (PT) extension at a plasma concentration of 2.7 μM, but had poor oral bioavailability [26]. Novel cyclized variants of the sulfonylpiperazinone class of factor Xa inhibitors are represented by **20** (M55532, (-)-enantiomer). Compound **20** displayed a factor Xa IC_{50} of 2 nM and did not inhibit other serine proteases. In rats at 10 mg/kg po, **20** achieved a Cmax of 0.148 μg/ml, a half-life of 3 h and oral bioavailability of 53% [27]. The piperazine **21** substituted by a pyrrolidine carboxamide sidechain was reported to have a factor Xa IC_{50} of 0.70 nM [28].



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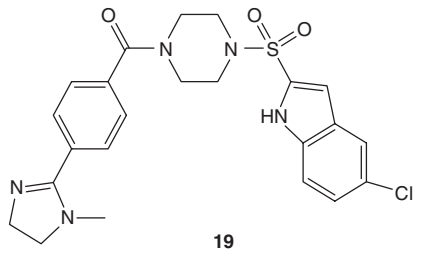

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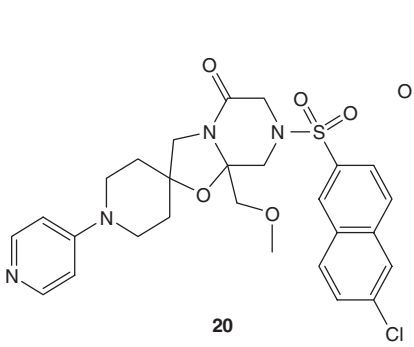

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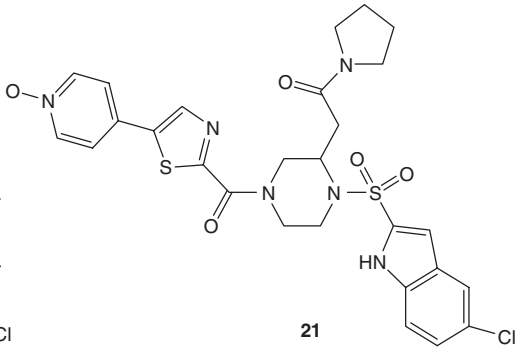
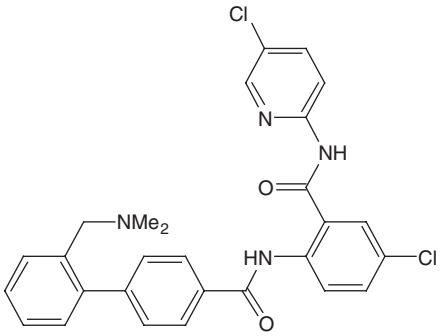
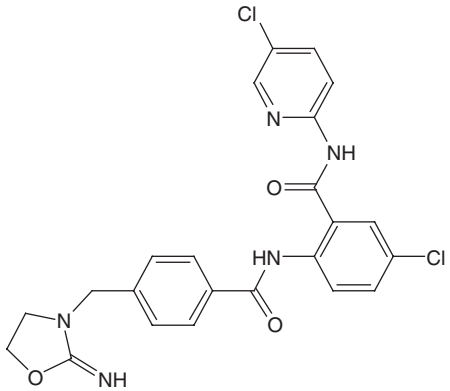


21

Potent non-benzamidine factor Xa inhibitors with a novel anthranilamide central scaffold have been disclosed [29–31]. Although potent factor Xa inhibitors were obtained, exemplified by **22** ($K_i = 0.1$ nM), members of this structural class lacked antithrombotic activity ($IC_{50} > 5\,\mu M$) in an *in vitro* PT assay [31]. To design less lipophilic inhibitors, the distal phenyl ring of **22** was replaced with various 4-dialkylaminomethyl substituents which were predicted to bind to the S4 pocket of factor Xa through a π-cation interaction. Extensive SAR exploration resulted in the optimized oxazolidine **23** ($K_i = 1.5$ nM). Compound **23** had a promising PK profile in rats, with 44% oral bioavailability, $t_{1/2}$ of 8.5 h and a volume of distribution of 13.6 L/Kg. In a rabbit DVT model, **23** exhibited 25 and 40% inhibition of thrombus growth at plasma concentrations of 0.25 and 0.83 μM respectively [32].



22



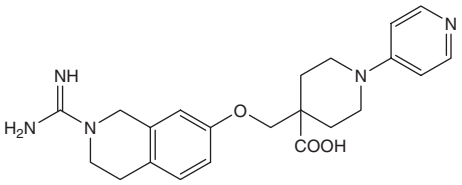
23

A series of tetrahydroisoquinoline derivatives was designed and synthesized, such as **24** (JTV-803; fXa $K_i = 40$ nM), which displayed good selectivity for factor Xa relative to other serine proteases [33]. In a rat venous thrombosis model, compound **24** produced a dose-dependent antithrombotic effect upon iv infusion at 0.3–1 mg/kg/h.

There have been several reports of factor Xa inhibitors incorporating a P1 chlorothiophenecarboxamide moiety [34]. Structure-activity studies around variations of an aminoacid core and the P4 residue resulted in **25** (EMD495235). Thiophene **25**

inhibited factor Xa with a K_i of 6.8 nM. The concentration required to double the APTT and PT was 1 μM. Pharmacokinetic evaluation in rats, dogs and monkeys showed rapid absorption from the GI tract, plasma elimination half-lives of 0.57–2.3 h, clearance between 0.25 and 1.3 L/h/kg and absolute bioavailability of 60–80%.

The oxazolidinone **26** (BAY59-7939; $IC_{50} = 0.4$ nM, $K_i = 2.1$ nM) displayed selectivity of > 10,000 for factor Xa versus other relevant serine proteases [35]. In rats and dogs, **26** had oral bioavailability of 60–80%, but a short half-life of 0.9 h, and clearance of 0.4 and 0.3 L/h/kg respectively. Phase I clinical trials with **26** showed dose proportional increases in AUC, Cmax was reached after 2.5 to 4 h, and the terminal half-life was 4 to 6 h. Oral doses of 1.25–80 mg were well tolerated with no signs of bleeding [36]. In a multiple dose escalation study, maximal factor Xa inhibition of 70% was achieved at steady state with the highest dose (30 mg bid), and no sign of bleeding was observed [37,38].



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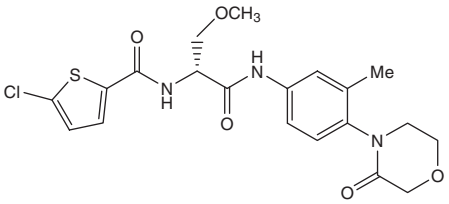

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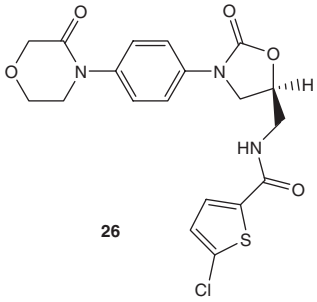

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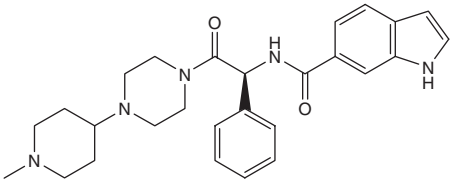
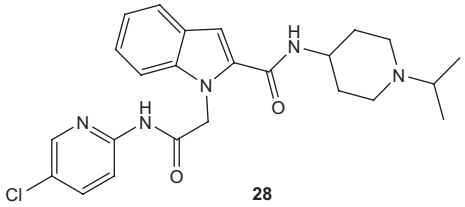
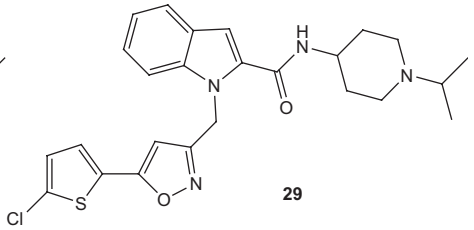


27

The indole **27** (LY517717, $K_i = 5$ nM) is in Phase II clinical trials for venous and arterial thrombosis. The compound was well tolerated in Phase I studies and proved to be suitable for once-daily administration [39]. A series of 2-carboxyindole-based factor Xa inhibitors such as compounds **28** and **29** has been described [40,41]. Analogs **28** ($K_i = 1$ nM) and **29** ($K_i = 3$ nM) doubled the plasma clotting time of APTT and PT at concentrations of 1 and 0.35 μM respectively.



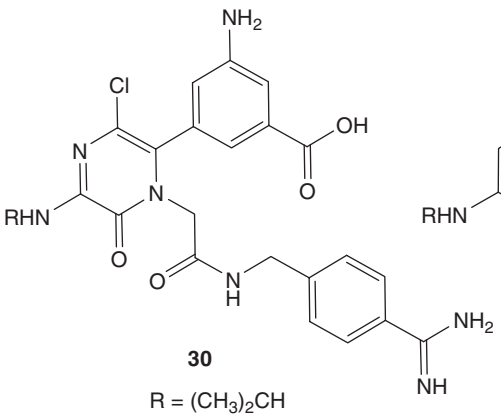
28



29

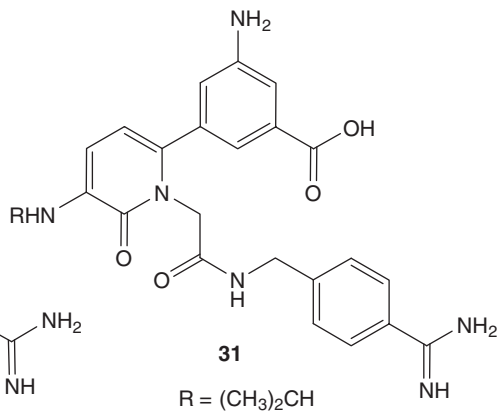
2.3. Factor VIIa/TF inhibitors

The factor VIIa/TF (tissue factor) pathway is recognized as the primary initiator of normal hemostasis. Upon vascular injury, TF in the vessel wall binds to circulating factor VIIa to form an activated factor VIIa/TF complex. This complex activates the coagulation cascade by activating both factors IX and X, ultimately resulting in the generation of thrombin and fibrin clots [42–44]. A review article on VIIa/TF inhibitors has been published [45]. Structure-based drug design was used to develop a series of VIIa/TF inhibitors containing a pyrazinone template. These efforts led to the potent inhibitor **30**, which exhibited a VIIa/TF IC_{50} of 16 nM and >6250-fold selectivity versus factor Xa and thrombin [46]. In an effort to modulate the pharmacokinetic properties of **30**, the pyrazinone core was replaced by a pyridone scaffold with a substitution pattern that would interact with the S1, S2 and S3 pockets of the VIIa/TF enzyme complex [47]. This effort led to analog **31** which had a diminished VIIa/TF IC_{50} of 52 nM but which maintained selectivity over thrombin and factor Xa (IC_{50}'s > 30 μM). The biphenyl analog **32** showed modest potency for VIIa/TF (IC_{50} = 340 nM) [48]. An X-ray crystal structure of **32** bound to VIIa/TF showed that the benzamidine moiety interacts with the Asp 189 in the S1 site, the peptide nitrogen of the acetamide linker forms a H-bond with the carbonyl oxygen of Ser 214 and the fluorine atom in the central ring accepts a H-bond from the amide nitrogen of Gly 216. Phenylglycine amide derivatives exemplified by compound **33** have been shown to have low nanomolar affinity for VIIa/TF (K_i = 2 nM) and 100-fold selectivity against factor Xa and thrombin [49].



30

R = $(CH_3)_2CH$



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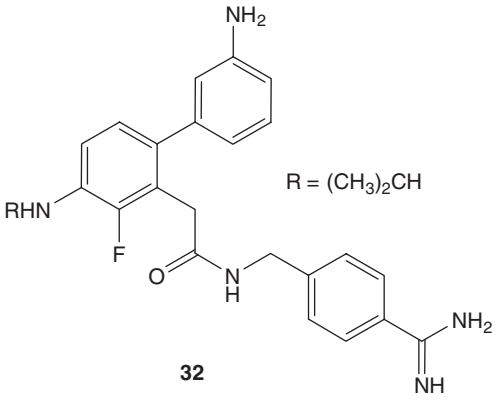
R = $(CH_3)_2CH$



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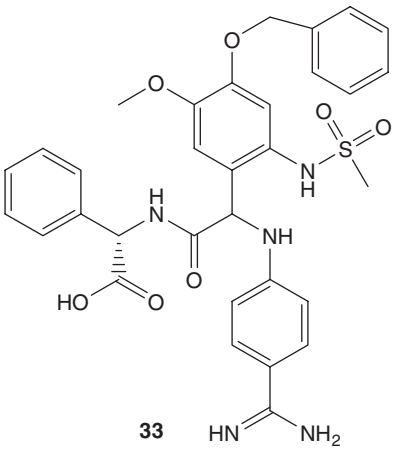

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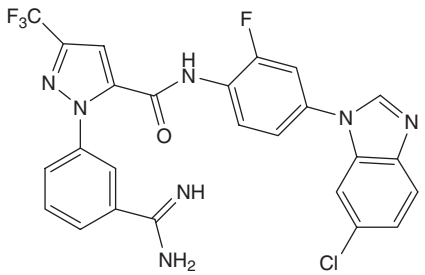
2.4. Factor IXa inhibitors

Inhibition of factor IXa, a newer target in the coagulation cascade, has received recent attention following *in vivo* validation using active-site blocked factor IXa [50] or anti-factor IXa antibodies [51]. High-throughput screening of existing libraries of thrombin and factor Xa inhibitors has been used to identify starting points for generation of dual inhibitors of factor Xa and factor IXa [52,53]. Examples of optimized inhibitors are the benzimidazole **34** with K_i's for IXa and Xa = 4.2 and 0.42 nM respectively, and the 5-amidinobenzothiophene **35** with K_i's for IXa and Xa equal to 0.12 and 0.18 μM respectively.



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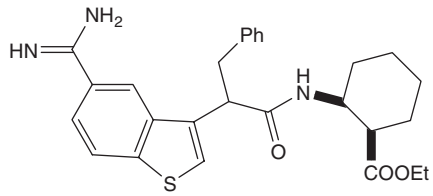

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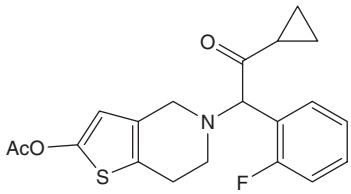
3. ANTIPLATELET AGENTS

3.1. P2Y$_{12}$ antagonists

The importance of ADP in platelet activation and aggregation resulting in arterial thrombosis, has been demonstrated both by antiplatelet agents (i.e. ticlopidine and

clopidogrel) that target the $P2Y_{12}$ receptor [54–58] and by patients with congenitally defective $P2Y_{12}$ receptors [59]. Clopidogrel, and its predecessor ticlopidine, are irreversible antagonists of $P2Y_{12}$, which manifests activity for the life-span of the platelet. These agents require metabolic activation by hepatic cytochrome P450-1A and 3A4 in order to generate active metabolites, transient intermediates that covalently modify and inactivate the receptor [60]. Despite its clinical success in treating arterial thrombosis [56–58], clopidogrel has several drawbacks, characterized by a slow onset of action, and weak and variable inhibition of the $P2Y_{12}$ receptor [61]. Furthermore, due to the nature of its irreversibility, clopidogrel in combination with aspirin use prior to coronary bypass surgery is associated with high rate of postoperative bleeding and morbidity [62]. Thus, recent research efforts are aimed at the discovery of rapid-onset, more efficacious and also reversible antagonists of the $P2Y_{12}$ receptor [63].

Prasugrel (CS-747, **36**), a new thienopyridine prodrug similar to clopidogrel, has been examined in several animal models of thrombosis and is currently under clinical evaluation in ACS patients [64,65]. At 0.5 h after dosing in SD rats (3 mg/kg), prasugrel produced more than 50% inhibition of ADP (3 μM)-induced platelet aggregation in platelet-rich plasma, while clopidogrel (30 mg/kg) had minimal effect, suggesting an early onset of the antiplatelet action of prasugrel. Maximum inhibition of 80% was observed for both agents 4 h after the dosing, but the effect of prasugrel was more potent than that of clopidogrel [65].



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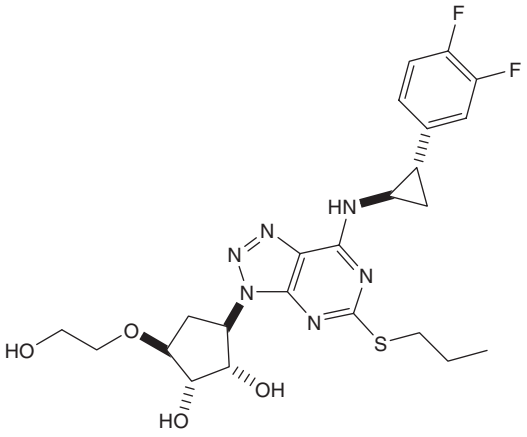

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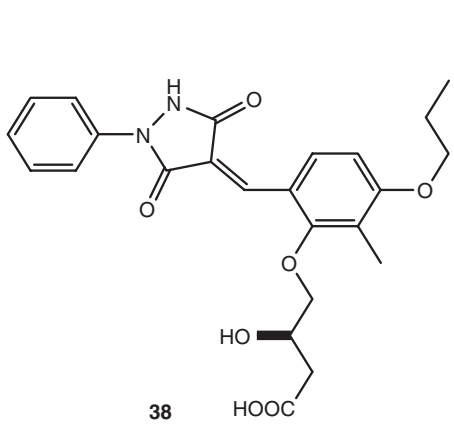
A number of reversible $P2Y_{12}$ antagonists have appeared in the recent literature, reflecting the desire to improve onset of action and allow more control over antiplatelet treatments. A successful approach to discovering reversible $P2Y_{12}$ antagonists is through modification of ATP, a competitive, albeit weak, antagonist of ADP-induced platelet aggregation. Replacing the ribose triphosphate of ATP by a substituted dihydroxypentane moiety and the purine ring by [1,2,3]triazolo[4.5]pyrimidine, led to the identification of **37** (AZD-6140) as a potent ($pK_i = 8.7$) and reversible antagonist of the $P2Y_{12}$ receptor. AZD-6140 has

38% and 86% oral bioavailability in rats and dogs, respectively, and pIC_{90} of 6.4 in human plasma against ADP-induced platelet aggregation [66].

A series of 4-(hetero)arylmethylidene-substituted pyrazolin-3,5-dione derivatives has been recently claimed in the patent literature as antagonists of the platelet $P2Y_{12}$ receptor [67]. The 2S-3-carboxy-2-hydroxy-propoxy substituted **38** displayed IC_{50} of 0.8 nM in the radioligand-binding assay with [^{3}H] 2-MeS-ADP. A short-acting nucleotide inhibitor **39**, (INS50589) has also recently been disclosed. It inhibits $P2Y_{12}$ with an IC_{50} of 4 nM and is currently in Phase I clinical trials [68].



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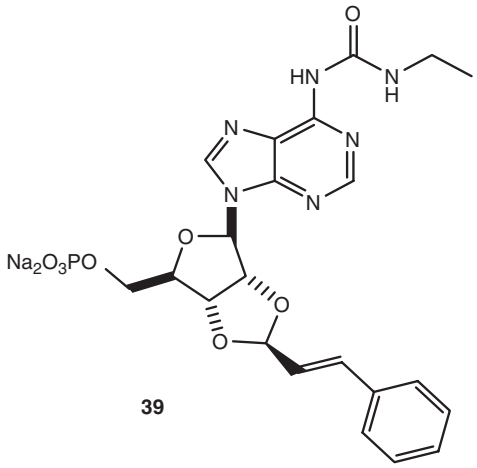

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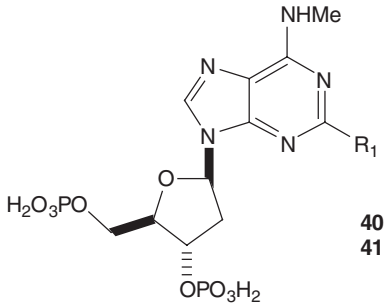
3.2. $P2Y_1$ antagonists

ADP also activates $P2Y_1$, a G_q coupled receptor that mediates platelet shape change and initiates ADP-induced platelet aggregation by mobilization of intracellular calcium [69]. The role of $P2Y_1$ receptors in thrombosis and as a potential therapeutic target for new antithrombotic agents has been supported by selective antagonists of this receptor and $P2Y_1$ deficient mice [70]. $P2Y_1$-null mice have increased bleeding time and are protected from ADP and collagen induced thromboembolism [71].

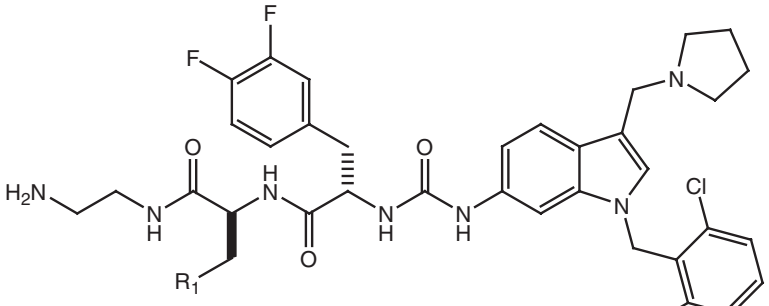
The early specific antagonists of the $P2Y_1$ receptor, such as **40** ($K_i = 110$ nM, $pA_2 = 6.55 \pm 0.05$), were derived from naturally occurring nucleotide like adenosine-3′,5′-bisphosphate [72]. The bisphosphonate **40** following i.v. administration, significantly increases the time-to-occlusion in a ferric chloride-induced arterial thrombosis model in mice [70]. Addition of 2-substituents to the purine ring have been found to further improve potency and selectivity of these bisphosphonate derivatives. The 2-ethynyl bisphosphonate **41** ($K_i = 10$ nM, $pA_2 = 7.54 \pm 0.10$) has been recently reported to be a highly potent $P2Y_1$ antagonist which is approximately 10-fold more potent than **40** in various assays [72].



40 $R_1 = H$
41 $R_2 = C{\equiv}CH$

3.3. Protease activated receptor antagonists

Since the discovery of the unique G-protein coupled receptor family encompassing the protease-activated receptors (PARs) in 1991, there has been considerable interest in developing novel antagonists to these targets. Of particular interest has been the identification of antagonists of the platelet thrombin receptor, PAR-1. Approaches to design of antagonists have been reviewed previously [73–77]. Of note is the recent activity improvement in the series of indole-based peptide mimetic antagonists described previously [74,77]. Indoles **42** and **43** which contain modifications of the dipeptide moiety are reported to be the most potent antagonists prepared from this series to date, with **42** and **43** having PAR-1 receptor binding IC_{50}'s of $0.025 \pm 0.004\,\mu M$ and $0.035 \pm 0.004\,\mu M$ respectively [78]. These antagonists also block thrombin-induced platelet aggregation with sub-micromolar activity ($IC_{50} = 0.22 \pm 0.07\,\mu M$ and $0.27 \pm 0.12\,\mu M$ respectively). Thrombin inhibitory activity has been difficult to obtain with previously reported antagonists [73–77].

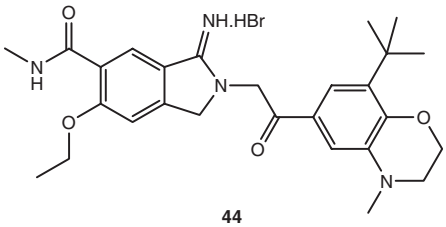


42 $R_1 = -CH_2SCH_3$

43 R_1 = 2-thienyl

However, a very advanced series of compounds are the 2-iminoisoindoline derivatives exemplified by structure **44**. Starting from the relatively weak HTS screening hit **45**, extensive SAR was conducted to obtain **44**. 2-Iminoisoindoline derivative **44** displayed an $IC_{50} = 0.014\,\mu M$ for inhibition of thrombin-induced

human platelet aggregation in platelet rich plasma. Oral administration of **44** to guinea pigs at 30 and 100 mg/kg showed antithrombotic effects consistent with its potent effects on thrombin-mediated platelet aggregation inhibitory activity measured *ex-vivo* [79]. Another advanced series of PAR-1 antagonists is the himbacine analogs first reported in 2001 [73,75] and exemplified by vinyl pyridine **46** (SCH-73754). This analog is reported to inhibit a high affinity thrombin receptor agonist peptide (haTRAP) ligand binding with an $IC_{50} = 15$ nM and to inhibit thrombin and haTRAP-induced aggregation of washed human platelets with IC_{50}'s of 0.10–0.30 μM. A further disclosure of a crystalline polymorph of a bisulfate salt of analog **47** (SCH-530348) from this series has been reported which has surprising *ex vivo* activity [80,81]. When ethylcarbamate derivative **47** was dosed orally at 0.1 mg/kg to conscious cynomolgus monkeys, *ex vivo* measured platelet aggregation was completely inhibited by exogenously added TRAP for 24 h, and afforded 65% inhibition of platelet function even at 48 h. More recently, **47** has been reported to bind to PAR-1 with a K_i of 8.5 nM and display oral bioavailability of 33 and 86% in rats and monkeys respectively, and is reported to be in Phase I trials [82]. Finally, novel pyrazoline antagonists of PAR-1, exemplified by pyrazoline **48** ($IC_{50} = 2$ nM) has been recently reported [83].



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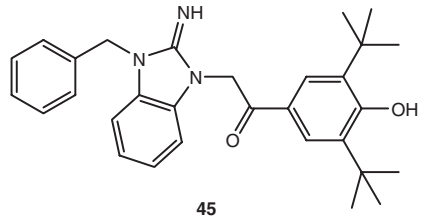

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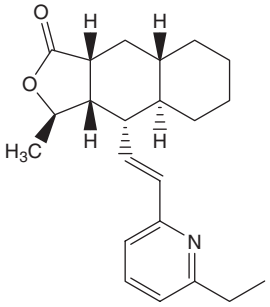

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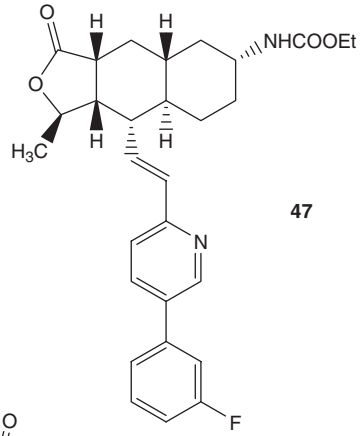

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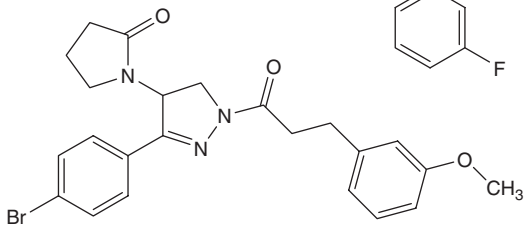

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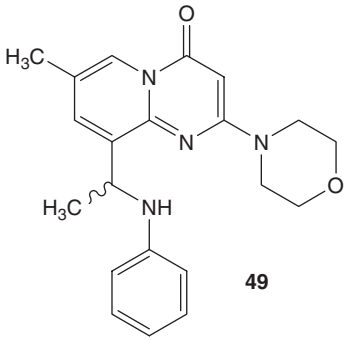
3.4. Isoform specific PI 3-kinase inhibitors

Two platelet receptors, GPIb/V/IX and integrin $\alpha_{IIb}\beta_3$ are capable of sensing rheological disturbances of high shear within the vasculature and can regulate platelet activation through critical signaling mechanisms. Recently, it has been shown that PI 3-kinase β (also known as p110β) is one of the elements of this signaling cascade, suggesting that novel antithrombotic agents may target this kinase. Because of the variety of PI 3-kinase isoforms, specificity is an important issue in designing novel antithrombotic agents targeting this pathway [84]. Using previously described isoform-nonspecific PI 3-kinase inhibitors as a template, novel, highly specific inhibitors of PI 3-kinase β have been recently disclosed [85,86]. Of most interest is the recently disclosed pyrido[1,2-a]-pyrimidine-4-one **49** (TGX221) [86]. This inhibitor displays an IC_{50} of 5 nM against PI 3-kinase β and is 20-fold more selective versus PI 3-kinase δ, 1000-fold less active against PI 3-kinase α and greater than 2000-fold more selective versus other unrelated kinases. The compound has also been studied in *in vitro* and *in vivo* models of pathological shear where it was found to be effective at 2 mg/kg in the rat electrolytic injury model of thrombosis. When administered at a dose 20-fold higher than the minimum therapeutic dose, it did not increase the rat bleeding times suggesting that this approach may afford inhibitors that do not affect hemostasis.



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CB1 Cannabinoid Receptor Antagonists

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Contents

1. INTRODUCTION

Interest in the pharmacology of cannabinoids (CBs) has rapidly increased after the cloning of cannabinoid receptors and the discovery of their endogenous ligands (endocannabinoids) in the early 1990's [1,2]. In this context, the discovery of the first cannabinoid antagonist, rimonabant (SR141716, **1**), in 1994, has provided researchers with an important tool for determining the physiological role of the endocannabinoid system. The interest in CB1 antagonists further increased when the first clinical results on the use of rimonabant for the treatment of obesity and related metabolic disorders were reported in 2001 [3]. Considering the important impact of obesity on public health, the dramatic increase of its worldwide prevalence and the lack of highly efficient and well-tolerated drugs to cure it, it is no surprise that CB1 antagonists are currently the subject of intense research in both industrial and academic groups.

Advances in cannabinoid ligands [4] and CB1 antagonists [5,6] have been reviewed recently. In this chapter, we will focus on important results published in the field of the medicinal chemistry of CB1 antagonists since the publication of the review by Xiang and Lee [7], with a special emphasis on very recently reported new structures and new potential clinical applications.

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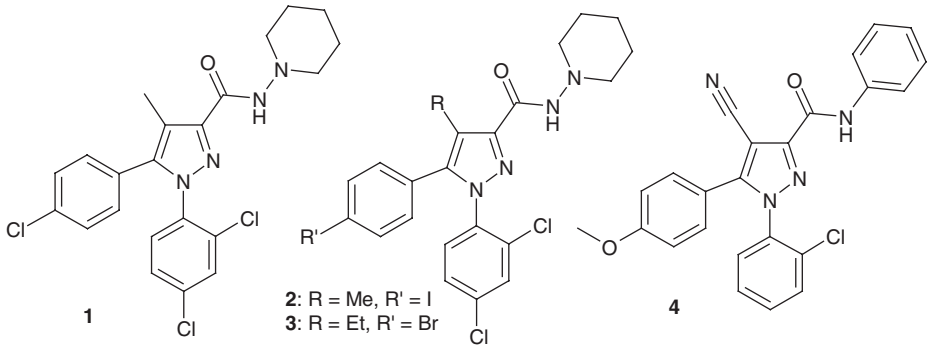
2. PYRAZOLE AND OTHER FIVE-MEMBERED RING PYRAZOLE BIOISOSTERE CB1 ANTAGONISTS

2.1. Pyrazole CB1 antagonists

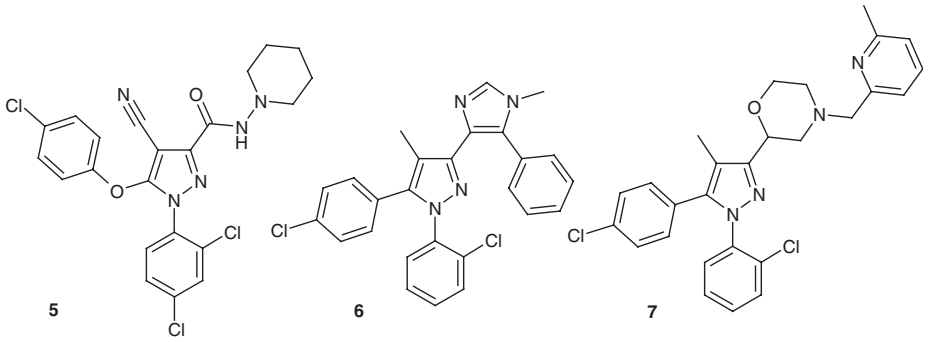
The first cannabinoid receptor antagonist, rimonabant was described in 1994 by researchers at Sanofi [8,9]. Rimonabant belongs to a chemical family distinct from previously known cannabinoid ligands: 1,5-diarylpyrazoles. Interestingly, most early attempts to identify a cannabinoid antagonist based on the structure of agonists such as classical cannabinoids (THC like compounds) or amino-alkyl indoles proved rather disappointing [10].

Several groups have recently described SR141716 analogues, leading to a good understanding of the structure-activity relationship (SAR) within this chemical series [11–15]. Based on this information, several three-dimensional pharmacophore models as well as models of receptor-ligand interactions were generated [13,16–18]. While most compounds described in these papers are less potent than SR141716, two of them deserve special attention. The first one is AM251 (**2**), obtained by replacing the 5-phenyl chloro substituent by iodo [19] and often cited in the literature as a close analog of SR141716. The second is SR147778 (**3**), obtained by replacing the 5-phenyl chloro substituent by bromo and methyl by ethyl at position 4 of the pyrazole ring. This compound was able to reduce food intake in fasted and non-deprived rats [20] and to selectively decrease alcohol intake in selectively bred Sardinian alcohol preferring (sP) rats [21]. It is currently undergoing phase I clinical trials.



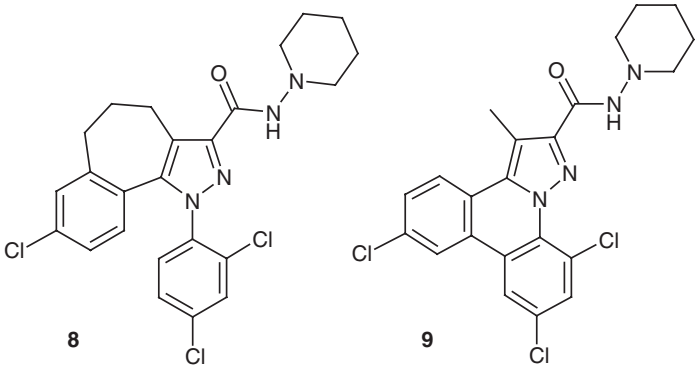
The pyrazole template was also used by an independent group who introduced the CB1 antagonist CP-272,871 (**4**) a 4-cyano pyrazole, which is however significantly less potent than SR141716 [22]. More recently, the same group filed a patent application for 5-aryloxypyrazoles such as (**5**) [23].

Based on variations on the 3 position of the pyrazole ring, several patent applications were published in which the carboxamide group was replaced by either a heterocyclic carboxamide bioisostere such as substituted 2- or 4-imidazole as in (**6**), or by an amino alcohol, ketone or morpholino ring such as (**7**). Compound (**7**) was reported to have a binding affinity of 79 nM [24,25].



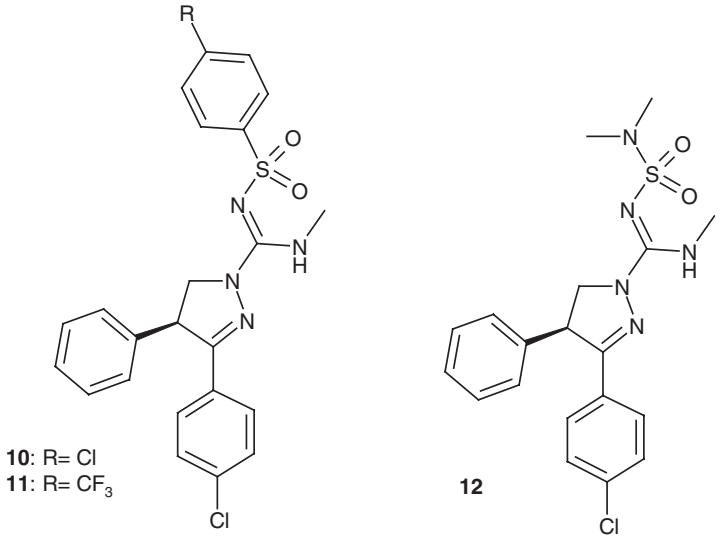
Several groups also developed conformationally restricted analogs of SR141716 by incorporating an additional ring in the diarylpyrazole structure. Two groups independently reported cyclisation between the 4 position of the pyrazole and the 5-aryl group. The first group described 2- and 3-membered bridges optionally incorporating a sulfur atom [26], while the second group focused on 3-membered bridges optionally incorporating an oxygen atom [27]. Among the best compounds was (**8**), which displayed an affinity for the hCB1 receptor of 125 nM (vs 25 nM for SR141716). Curiously, the same compound was described under the name NESS 0327 with sub-picomolar affinity for rCB1 receptors (0.25 pM vs 1.8 nM for SR141716) in another paper [28]. One carbon bridged compounds have also been synthesized, but this led to highly selective CB2 rather than CB1 ligands [29].

Cyclisation between the two *ortho* position of the phenyl rings at the 1 and 5 positions of the pyrazole ring has also been described. Despite being an entirely planar tetracyclic compound, (**9**) was reported to retain significant affinity for hCB1 receptor [30].



Structurally distinct from these pyrazoles is a series of 3,4-diarylpyrazolines [31,32]. Within this chemical series is SLV-319 (**10**), that is presently in phase I clinical trials. Based on *in vitro* and *in vivo* pharmacological data, (**10**) as well as its close analog (**11**) (SLV326) were characterized as potent CB1 antagonists which display *in vivo* activity similar to rimonabant in several pharmacological models.

Several patents were filed within this chemical series, including compounds with lower lipophilicity such as (**12**) [33].

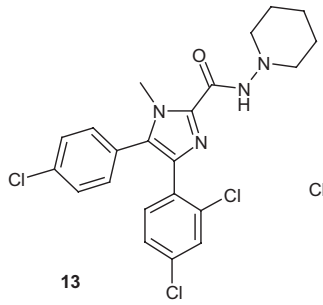


2.2. Five-membered ring pyrazole bioisostere CB1 antagonists

Ring bioisosterism, one of the most frequent relationships in drugs of different therapeutic classes has been widely used to design new cannabinoid antagonists. The first patent application describing analogs of rimonabant in which the central pyrazole ring was replaced by another heterocycle was published in 2003, in the imidazole series [34]. Since then, more than 25 patent applications have been published using this approach.

4,5-Diarylimidazoles were the first reported bioisosteres [34]. The synthesis and SAR of these compounds is discussed in a recent paper that introduced (**13**) as a potent CB1 antagonist (IC_{50}, hCB1 = 6.1 nM). Preliminary pharmacokinetic evaluation in rats indicated good oral absorption (F = 50%) and brain penetration for compound (**13**), which was also active in a food intake and weight-loss study in diet-obese rats [35].

Related patent applications for the regioisomeric 1,2-diaryl imidazoles were successively filed by three independent groups [36–38]. Some compounds in this series such as (**14**) displayed affinities for hCB1 similar to that of rimonabant and were orally active in mechanistic models [39,40]. Recently, 1,2-diaryl imidazoles such as (**15**) in which the 5 position is substituted by more polar groups were also reported [41]. A binding activity below 10 nM was found for the latter compound.



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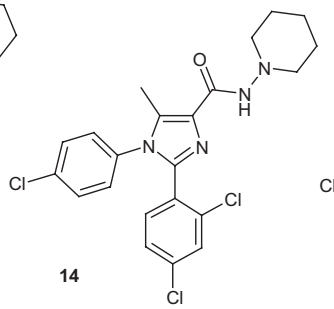

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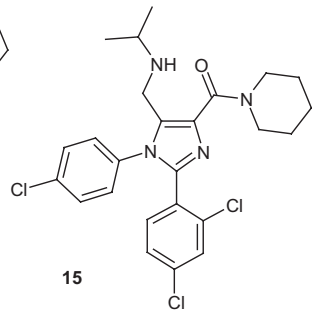

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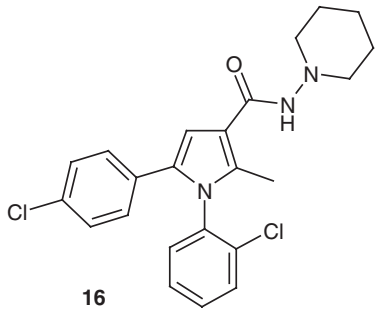
The corresponding triazoles have been described by two independent groups which concluded that replacement of a 5-methylimidazole by a triazole led to a loss in CB1 affinity by about ten-fold [39,40,42]. Interestingly, a triazole derivative in which an n-hexyl group replaced the carboxamide group was reported to behave as a CB1 antagonist both *in vivo* and in functional assays, despite a very moderate affinity for rCB1 receptors [43].

Similarly, 2,3-diaryl oxazoles [35] and thiazoles [39,44,45] were reported to be about one log less potent than the corresponding 5-methyl 1,2-diarylimidazoles. This difference in activity was attributed to the methyl group of the imidazole compounds, which may play a role in favorably orienting the amide nitrogen substituent, as the non-methylated imidazoles, as well as the triazoles, oxazoles and thiazoles lacking the orienting methyl group were all less potent than the 5-methylimidazoles. Similarly, in the 4,5-diaryl imidazoles series, the NMe compounds were found to be much more potent than the corresponding NH compounds [35].

Two patent applications concerning 1,5-diaryl pyrrole-3-carboxamides have also been filed [46,47]. Although one of these does not specifically claim use as CB1 antagonists, but rather for ''compounds treating obesity'', the compounds described are closely related to SR141716. This publication reports a significant decrease in food consumption following oral administration of compound (**16**). Conformationally restrained analogs of these molecules were also prepared by bridging the methyl group with the adjacent amide nitrogen, leading to compounds such as (**17**) [48]. Other patent applications describing pyrroles and imidazoles have also been published by an independent group. In these patents that each include more than 300 examples, the substituent in position 1 includes both aromatic and non-aromatic groups such as methylcyclohexyl, and the substituent in position 5 may be a substituted phenyl or thiazole ring [49,50].



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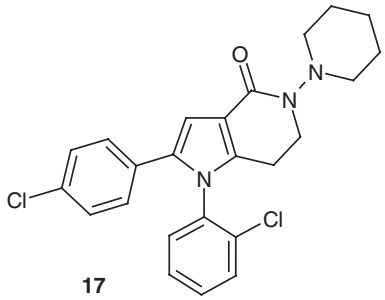

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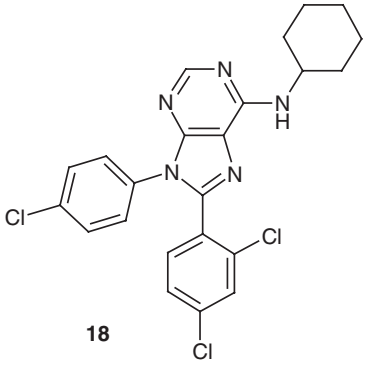
A large number of fused bicyclic derivatives of diaryl-pyrazole and imidazole were reported in a series of eight patent applications. Among these are the purine derivatives (**18**) and the pyrazolo-triazine (**19**) [51,52]. Although no specific biological data is available for these compounds, the patent applications claim affinities below 1 nM for some non-specified examples.



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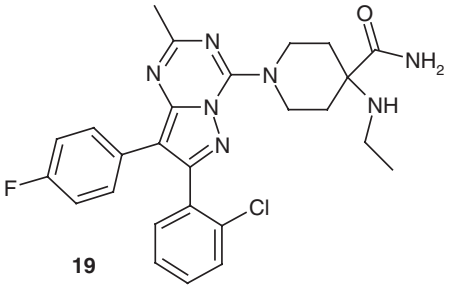

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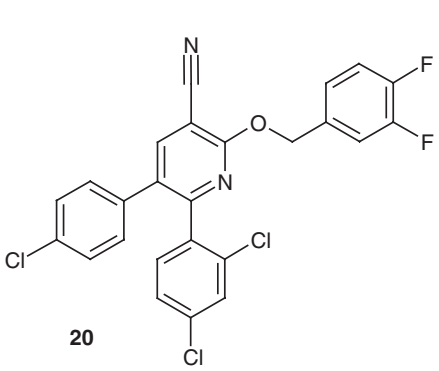
3. SIX-MEMBERED RING PYRAZOLE BIOISOSTERE CB1 ANTAGONISTS

Several research groups have expanded the ring bioisosterism strategy from 5-membered to 6-membered rings. 2,3-Diarylpyridine CB1 antagonists are claimed in two patents applications [53,54]. One of these patents is restricted to 6-carboxamides, while the other includes 5 and 6-carboxamides as well as compounds lacking the carboxamide function. For example (**20**) was found to be a potent and selective CB1 inverse agonist (IC_{50} hCB1 = 1.7 nM). The structure of this compound is interesting in that it demonstrates the possibility that the amide moiety of rimonabant could be split into a lipophilic (benzyloxy) and a polar (nitrile) functionality. Preliminary pharmacokinetic studies in rats with (**20**) indicated a moderate oral absorption (F = 27%), slow brain penetration and a low brain to plasma

ratio. The moderate effects observed in a food intake and body weight loss study using diet-obese rats were consistent with these pharmacokinetic parameters [55]. Other novel 2,3-diaryl-5-carboxamides were also recently disclosed [56].

By fusing a furan ring to the pyridine ring, a new series of furo[2,3-*b*]pyridines such as (**21**) was also developed [57]. However no biological data is available for these compounds.



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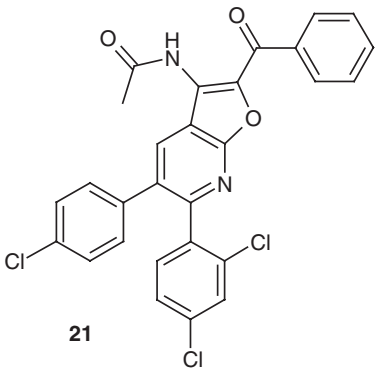

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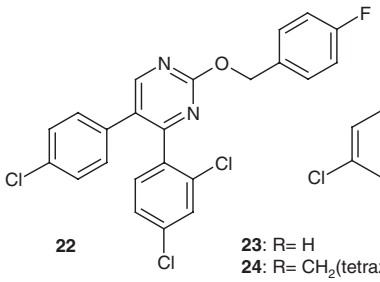
Pyrimidines, very similar to the pyridines (**20**), were also claimed in a patent including 136 examples such as (**22**) without any biological data [58]. Similar 2-carboxamido-1,3-pyrimidines were also claimed independently [59].

An important work has been devoted to the 2,3-diarylpyrazine series. Six patent applications disclosed strict rimonabant bioisosteres such as (**23**) as well as more functionalized compounds such as (**24**) [60,61]. The latter compounds displayed IC_{50} below 2 nM in a hCB1 receptor GTPγS assay. The introduction of more polar substituents on the pyrazine ring, as exemplified in (**24**) is expected to lead to less lipophilic, more bioavailable compounds.



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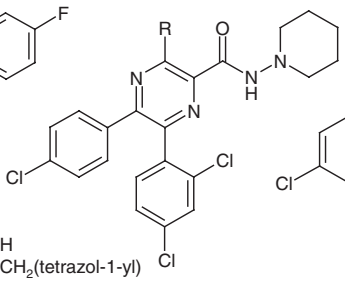
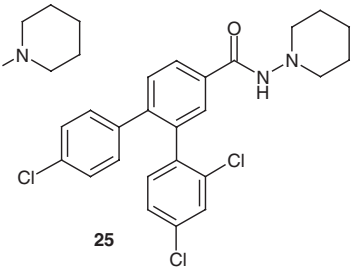


23: R= H
24: R= CH_2(tetrazol-1-yl)



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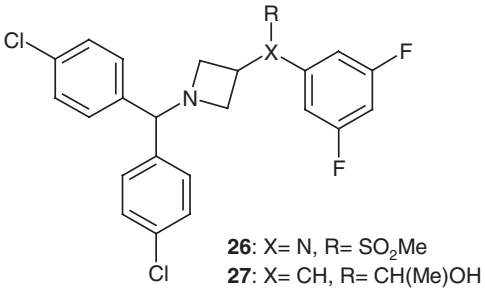
Interestingly, terphenyl compounds such as (**25**) with reported affinity of 113 nM for the hCB1 receptor suggest that the presence of a nitrogen atom in the central ring is not necessary to ensure CB1 binding [62,63].

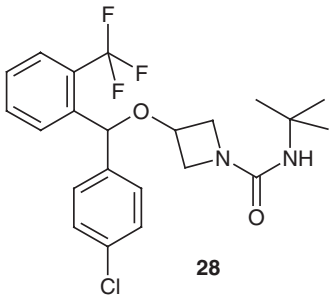
4. OTHER CYCLIC AND ACYCLIC CB1 ANTAGONISTS

While most five and six-membered ring analogues reported above are derived from the structure of rimonabant, several other families of structurally distinct CB1 antagonists have been reported. Many of them share as a common feature a 1, 1-diphenyl group, which may mimic the 1,5-diaryl motif of the diarylpyrazole and pyrazole bioisostere compounds.

Particularly important are the azetidines reported in 2000 [64]. A typical member of this class is the methylsulfonamide (**26**), but many analogs are claimed in several patent applications. Unfortunately, no information on the biological properties of these compounds is available. Closely related azetidines such as (**27**) have also been reported [65]. A more novel series of azetidine compounds has been independently disclosed. In this work, the nitrogen was moved to the opposite position of the four-membered central ring and an oxygen atom was incorporated next to the benzhydryl moiety, to obtain compounds such as (**28**) which are claimed to display nanomolar affinity for hCB1 receptors [66].



26: X= N, R= SO_2Me
27: X= CH, R= CH(Me)OH



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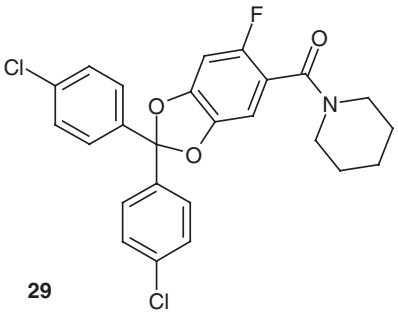
The 1,1-diaryl group pattern is also present in the benzodioxoles such as (**29**) reported in a patent application [67] which does not include any biological data, and in the hydantoins developed independently [68]. The best compound of the latter series, DML-20 (**30**), binds to the hCB1 and rCB1 receptors with K_i in the micromolar range and behaves as a neutral antagonist in rat cerebellum homogenates [69].



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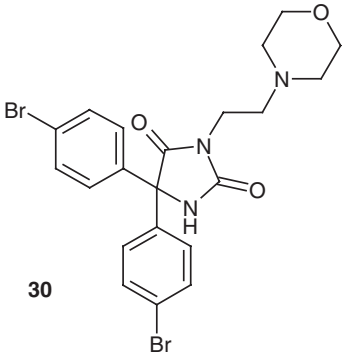

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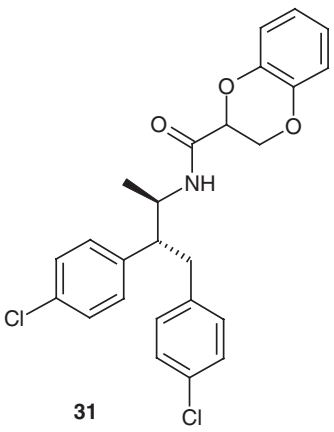
Acyclic CB1 antagonists have been reported by at least two groups. These compounds include a 1,2-diaryl motif which may be superposed with the 1,5-diaryl substituents of rimonabant and related molecules. Five patent applications for compounds similar to (**31**) were filed, in which two phenyl groups are linked to a saturated carbon framework, with an amide in the beta position [70]. Recently a patent application disclosed compounds such as (**32**), in which the aryl groups are part of a phenylbenzamide, and in which the nitrogen is further substituted by a benzothiazole ring. An IC_{50} of 730 nM has been reported for the latter compound [71].



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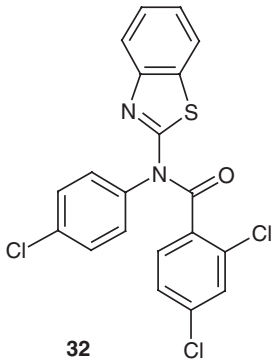

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5. POTENTIAL THERAPEUTIC APPLICATIONS

The endocannabinoid system, comprising cannabinoid receptors, endogenous ligands and enzymes for ligand biosynthesis and inactivation seems to be involved in an ever-increasing number of pathological conditions [2]. Based on available data, the main therapeutic application for CB1 antagonists clearly appears to be the treatment of obesity [72]. Many animal studies suggested that rimonabant and other cannabinoid antagonists are able to selectively decrease the intake of palatable food, and to decrease body weight gain in obese animals [73,74]. Interestingly, several lines of evidence suggest that rimonabant's action on body weight is mediated not only by a reduction of food intake, but also by an effect on energy expenditure or metabolism via a peripheral site of action [75,76]. It was recently demonstrated that CB1 receptors are present in rat adipocytes, and that treatment of obese Zucker rats with rimonabant increased adiponectin (Acrp30) expression in this tissue [77]. Adiponectin is a secreted protein which plays a major role in the regulation of glucose, insulin and fatty acids and which has anti-obesity effects [78]. Adiponectin modulation could therefore be involved in the anti-obesity effects of rimonabant.

The first results of phase III clinical studies of rimonabant in obesity were presented in March 2004 [79,80]. 1036 overweight or obese patients (BMI between 27 and 40 kg/m^2) with untreated dyslipidemia (high triglycerides and/or low HDL cholesterol) were randomized to receive either a daily, fixed dose of rimonabant (5 or 20 mg) or placebo along with a mild hypocaloric diet. Patients treated for one year with rimonabant (20 mg per day) lost 8.6 kg (versus 2.3 kg in the placebo group). In addition to weight loss, the study was designed to assess a number of important associated cardiovascular risk factors. Rimonabant (20 mg) was associated with a significant reduction in waist circumference, triglycerides and C-reactive protein and an increase in HDL-cholesterol. Importantly, the number of patients classified as having metabolic syndrome [81] was reduced from 52.9% at baseline to 25.8% at one year [82]. These robust data were replicated in another phase III study (RIO-Europe) involving 1507 obese patients with or without co-morbidities [83]. Rimonabant, which was well tolerated, could therefore become an important agent in the management of cardiovascular risk in obese patients.

The second therapeutic application for which clinical data are available is smoking cessation. Strong interaction between cannabinoids and brain reward function are well documented [84]. Several studies demonstrated that SR141716 was able to block the reinforcing effects of heroin [85], morphine [86], ethanol [87] and nicotine [88,89]. Cannabinoid antagonists were therefore suggested as potential treatment for nicotine and alcohol dependence. The STRATUS-US phase III clinical trial enrolled 787 tobacco smokers motivated to stop, who were randomized to placebo, or to 5 or 20 mg rimonabant once daily for 10 weeks. Among patients receiving 20 mg rimonabant, 27.6% were able to stop smoking compared to 16.1% of those taking placebo. Moreover, among patients who were not obese at baseline, there was a 77% reduction in post-cessation weight gain compared to placebo [79]. These results are highly encouraging, considering the need for effective pharmacotherapies for the treatment of tobacco dependence [90].

As suggested above, the treatment of alcohol dependence is often considered as another potential clinical indication for CB1 antagonists. Rimonabant has been shown to reduce voluntary alcohol intake in several animal models [87,91,92]. Recently these findings have been extended to SR147778 (**3**) a new CB1 antagonist [21]. Considering the high predictive validity of the model used in the later study, it is expected that blockade of CB1 receptors may constitute a novel approach in the treatment of alcoholism.

Based on the large number of pathological conditions in which the endocannabinoids seem to be involved, many other potential applications for CB1 receptors have been suggested. Several studies report the use of CB1 antagonists to improve memory performance in rodents and to reverse memory deficits seen in aged animals. SR141716 (**1**) improved olfactory memory as assessed by the social recognition test [93] and enhanced spatial memory in the radial-arm maze task [94,95] in rodents. Moreover, amnesia induced by icv injection of β-amyloid fragments was reversed by pre-treatment with SR141716 in mice [96].

A potential role in the treatment of psychosis as well as affective and cognitive disorders was also suggested based on biochemical and pharmacological evidence. *In vivo* microdialysis experiments were used to investigate the effects of CB1

antagonists on monoaminergic neurotransmission in specific rat brain areas. Administration of SR141716 selectively increased norepinephrine, dopamine and acetylcholine efflux in the medial prefrontal cortex [97]. Together with the fact that SR141716 has also been shown to enhance arousal [98], these observations suggested a possible role of CB1 antagonists in the treatment of attention and hyperactivity disorder (ADHD). Rimonabant was also evaluated in several models of anxiety and depression. While some authors reported an anxiogenic-like profile for the compound [99], opposite anxiolytic-like and anti-depressant-like effects were observed in several other studies [100,101]. A role in the treatment of schizophrenia was also suggested for CB1 antagonists, based on a pharmacological profile reminiscent of that of atypical antipsychotic drugs [102,103]. On the other hand, the failure of SR141716 to reverse disruptions in pre-pulse inhibition (PPI) and hyperactivity induced by apomorphine or d-amphetamine in rats suggested that blockade of the CB1 receptor is not sufficient for antipsychotic therapy [104]. Moreover, the results of a recent meta-trial evaluating the efficacy of four novel compounds for the treatment of schizophrenia and schizoaffective disorders indicated that the group receiving SR141716 did not differ from the group receiving placebo on any outcome measure [105].

Functional CB1 receptors are also present outside the brain, and particularly in the enteric nervous system of several species, including human [106]. Both *in vitro* and *in vivo* studies indicated that CB1 antagonists increased intestinal motility in rodents [107]. Moreover the impaired intestinal motility induced by ip injection of acetic acid in mice was restored by SR141716 [108]. These data open the possibility of the use of CB1 antagonists for the clinical management of paralytic ileus, an illness defined as long-lasting inhibition of gastro-intestinal transit in response to abdominal nociception.

The patent literature claims a number of other therapeutic applications for CB1 antagonists ranging from migraine to cancer, although they are not all supported by robust biological data. The use of CB1 antagonists for the treatment of sexual behavior dysfunction was recently claimed based on data showing a stimulatory effect of rimonabant on the sexual performance of naïve rats [109]. Another patent application claimed the use of CB1 and CB2 inverse agonists and antagonists for the treatment of bone disorders such as osteoporosis [110]. This claim was based on biological data showing that the CB1 antagonist AM251 (**2**) potently inhibited osteoclast survival *in vitro*, and was also effective *in vivo*, in reversing the ovariectomy-induced bone loss in mice.

6. PERSPECTIVES

As shown in Figure 1, the number of publications related to "cannabinoid CB1 antagonists" published each year as reported by Chemical Abstracts continued to rise during the last 10 years, approximately doubling every four years. The growth in the number of patent applications is even more spectacular. The dramatic rise observed in 2003 and largely confirmed in 2004 and 2005, is easily explained by the

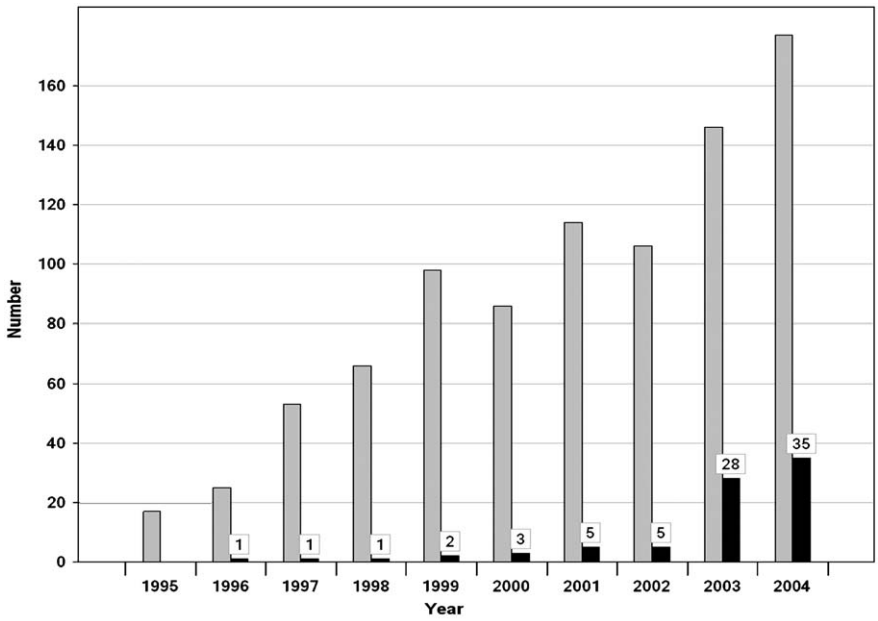

Fig. 1. Number of publications (gray) and patent applications (black) related to "cannabinoid CB1 antagonists" published each year from 1995 to 2004.

interest of the pharmaceutical industry following the publication of the first clinical study with rimonabant in obesity during 2001.

While most of the recently published compounds are derived from the diaryl pyrazole structure of rimonabant, the search for entirely new structures, often driven by biological screening of chemical libraries, is expected to increase chemical diversity of available CB1 antagonists.

Many efforts have been devoted recently to designing CB1 antagonists with reduced lipophilicity. In order to bind with a high affinity to cannabinoid receptors, cannabinoid ligands are usually highly lipophilic and this often leads to low aqueous solubility and poor oral bioavailability. Structures recently disclosed in the patent literature incorporate more polar groups in order to solve this problem. Several questions remains to be answered: will the newly discovered CB1 antagonists display the same clinical profile as rimonabant, especially in terms of improvement of the cardiovascular risk factors? Most of the CB1 antagonists known today also behave as inverse agonists [111]. Would a neutral antagonist display a different pharmacological profile? What is the effect of known and future cannabinoid antagonists on CB1 receptor isoforms [112,113] and on yet to be cloned potential other sub-types of cannabinoid receptors [114], and what is the pharmacological relevance of these receptors? Finally, which of the potential clinical indications based on animal models will be confirmed in humans and lead to new drugs?

The launch of rimonabant and the clinical advancement of other CB1 antagonists are expected to answer many of these questions in the coming years.

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Melanin-Concentrating Hormone as a Therapeutic Target

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Contents

1. INTRODUCTION

Among the worldwide population, the frequency of obesity has increased significantly over the last decade [1]. Approximately 30% of the current United States adult population is classified as obese [defined as a body mass index (BMI) > 30], while an additional 30% of U.S. adults are overweight (BMI > 25) [2]. Similar trends have also been observed in many industrialized countries, principally those adopting a western diet and sedentary lifestyle [3]. This epidemic is having a pronounced worldwide economic impact, with estimated annual direct and indirect costs of $117 billion in the U.S. alone [4].

Importantly, the ramifications of the obesity epidemic are not merely aesthetic or financial. Obese individuals have a significantly higher risk of mortality and related co-morbidities such as hyperlipidemia, cardiovascular disease, hypertension and Type 2 diabetes mellitus, as well as other health problems such as arthritis, sleep apnea and certain forms of cancer [1,5]. Approximately 47 million Americans are affected with metabolic syndrome (also termed Syndrome X, insulin resistance syndrome, Reaven syndrome or metabolic cardiovascular syndrome), which is defined as the clustering of obesity, insulin resistance, hypertension, and dyslipidemia [6]. Many affected individuals are at increased risk for developing Type 2 diabetes and mortality from cardiovascular disease [7,8].

As a first line of treatment for metabolic syndrome, the NCEP has suggested that weight reduction be primary focus, as this has been shown to reduce all risk factors of metabolic syndrome and delay or halt the development of Type 2 diabetes [9–11]. Maintaining weight loss solely by implementing changes in lifestyle remains

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difficult. Currently, two medications are approved for weight loss in the U.S.: orlistat (Xenical®), a pancreatic lipase inhibitor, and sibutramine (Meridia®), a serotonin and norepinepherine reuptake inhibitor. Both of these medications suffer from patient compliance issues and undesirable side effects which consequently limit their therapeutic potential. As a result, the search for anti-obesity therapies with improved pharmacodynamic profiles has been a major focus within the pharmaceutical industry. Among the pharmacological targets, regulation of melanin-concentrating hormone (MCH) has emerged as increasing genetic and preclinical evidence has demonstrated that antagonism of the MCH 1-receptor may provide an effective therapy for the treatment of obesity and related co-morbidities.

2. MELANIN-CONCENTRATING HORMONE (MCH)

2.1. Characterization of the MCH peptide

In rodents and humans, MCH (**1**) is a cyclic nonadecapeptide that is generated by cleavage from the C-terminus of a larger precursor, pre-pro MCH, the product of the *pmch* gene [12]. Pre-pro MCH is expressed predominantly within the lateral hypothalamus (LH) and zona incerta (ZI), and these neurons send diffuse MCH projections throughout the central nervous system, suggesting that MCH may be involved in many neuronal functions [13–15]. In addition to MCH, the pre-pro MCH peptide is processed to generate the post-translational products neuropeptide EI (NEI) and the putative protein neuropeptide GE (NGE).

H_2N-Asp-Phe-Asp-Met-Leu-Arg-Cys-Met-Leu-Gly-Arg-Val-Tyr-Arg-Pro-Cys-Trp-Gln-Val-CO_2H

1

H_2N-(I)Tyr-ADO-Arg-Cys-Met-Leu-Gly-Arg-Val-Phe-Arg-Pro-Cys-Trp-CO_2H

2

ADO = 8-amino-3,6-dioxyoctanoyl

In an effort to identify the critical residues involved in binding and activation of MCH, several groups have studied modifications of the parent peptide. Replacement of Arg^{11} with Ala resulted near complete loss of activity, confirming a key role for this residue; while Ala replacement of Arg^{14} showed no significant effects [16] Further studies employing truncated and/or modified forms of the MCH peptide have shown that deletion of several residues on each end of the termini has negligible effects on activity [17–21]. One of these truncated variants, (**2**) was identified as having improved solubility relative to endogenous MCH, and could be radiolabelled at the iodinated Tyr residue, facilitating further pharmacological studies [19].

2.2. MCH receptors

To date, two MCH receptors have been identified, both of which are members of the G-protein coupled receptor family. The MCH1-receptor (MCH1-R, also called SLC-1 or GPR24) was identified using a 'reverse pharmacology' approach, and shown to signal through $G_{\alpha i}$, $G_{\alpha o}$ and $G_{\alpha q}$ [22–27]. MCH1-R is a 353 residue peptide found in rodents and higher mammalian species where it is expressed in several brain regions including those associated with olfaction [28]. It is also expressed in several brainstem nuclei, including the locus coeruleus, hypoglossal, motor trigeminal and dorsal motor vagus [29]. Immunohistochemical analysis has also demonstrated that MCH1-R protein is present in the dorsomedial and ventromedial nuclei of the hypothalamus, areas which are involved in feeding behavior and energy homeostasis [23].

A second MCH receptor, MCH2-R, was identified based upon its homology (~37%) with the MCH1-receptor and is predominantly coupled to $G_{\alpha q}$ [30–35]. The profile of MCH2-R expression in the central nervous system of higher mammals is somewhat different from MCH1-R, being expressed at lower levels overall with a more restricted expression pattern. While present in higher mammals such as ferrets, dogs, rhesus monkey and humans, MCH2-R is not expressed in rodents and lagomorphs, unlike MCH1-R [28,36]. Localization of MCH2-R suggests that it may mediate MCH effects other than regulation of food intake and energy expenditure; however the species-specific expression pattern has limited the effort in defining the pharmacological role of MCH2-R, particularly with respect to metabolic homeostasis.

3. POTENTIAL THERAPEUTIC INDICATIONS

Initial studies showing higher expression of MCH in hypothalami of leptin deficient ($Lep^{ob/ob}$) and hypoleptinemic (fasted) mice, and that i.c.v. administration of MCH to rats stimulates food intake, established a role for MCH in feeding [37]. Several groups have since confirmed the hyperphagic effect of acute central administration of MCH in both mice and rats [38–41], as well as the over-expression of MCH in genetic models of leptin resistance [42,43]. Sub-chronic (7–14 days) central infusion of MCH to mice on a high fat diet induced persistent hyperphagia accompanied by increased adiposity, hyperinsulinemia and hyperleptinemia [44,45]; while i.c.v. infusion of a potent MCH1-R peptide agonist to rats produced similar effects [46]. Consistent with these findings, transgenic eutopic over-expression of MCH produces an obese, insulin resistant and hyperphagic phenotype in mice on a high fat diet [47]. Deletion of the *pmch* gene, which generates an animal null for MCH as well as NEI and NGE, results in a lean phenotype characterized by hypophagia and increased energy expenditure [48]. MCH1-R null mice are lean, and have decreased leptin and insulin levels, similar to the findings in the $pmch^{-/-}$ mice [49,50]. Additionally, they fail to respond to exogenously administered MCH and are resistant to diet-induced obesity. Unlike the MCH deficient mice, however, $mch1r^{-/-}$ mice are

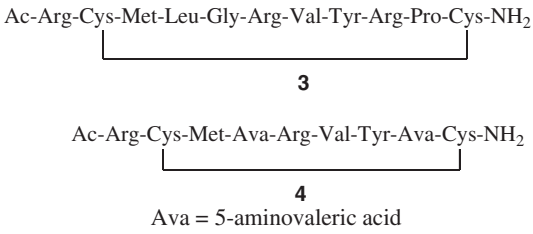
hyperphagic, and the leanness is due to a hyperactive and hypermetabolic phenotype. This hyperphagia is not explained by alterations in the expression of orexigenic (NPY, AgRP, orexin) and anorexigenic (CART, POMC) neuropeptides, nor in the tone of endogenous orexigenic signals as evidenced by a normal response to exogenously administered AgRP and NPY. Rather, it has recently been shown that the hyperactive phenotype of *mch1r*$^{-/-}$ mice is associated with an increased heart rate and an altered autonomic regulation of body temperature in response to fasting [51].

Taken as a whole, these studies convincingly demonstrate that MCH signaling plays a pivotal role in the regulation of both food intake and energy expenditure. Several preclinical studies suggest that small molecule MCH1-R antagonists will be efficacious for the treatment of obesity.

4. MCH1-R ANTAGONISTS

4.1. Peptidal MCH1-R antagonists

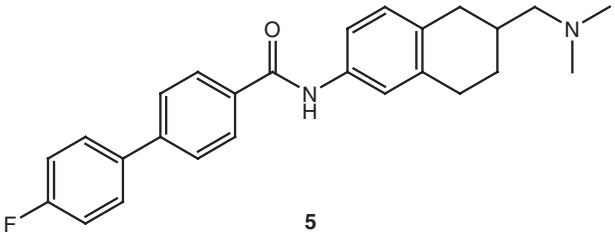
Initial progress toward peptidal MCH antagonists was recently described [21,46,52]. Replacement of Leu^{9}-Gly^{10} and Arg^{14}-Pro^{15} with 5-aminovaleric acid (5-Ava) in the peptidal MCH agonist (**3**) provided a potent MCH1-R antagonist (**4**). Administration (i.c.v.) of the modified peptide showed no influence on food intake over 6 h, but did reverse hyperphagia induced by treatment with a peptidal MCH1-R agonist [46]. Sub-chronic infusion of the antagonist (14 d) induced modest hyperphagia and reduced weight gain relative to controls. While enabling the study of physiological ramifications of MCH receptor modulation, peptidal ligands suffer from inherently poor intracerebral transitivity and oral bioavailability.



4.2. Small molecule MCH1-R antagonists

Non-peptidal MCH1-R antagonists have been the topic of several patents and publications in recent years, indicating the fervor with which research in this area has been pursued. Initial reviews in the area have documented these endeavors, which have laid a solid foundation for recent discoveries of MCH1-R antagonists which demonstrate *in vivo* efficacy [53–56].

The first non-peptide MCH1-R antagonist, T-226296 (**5**, $K_i = 5.5\,nM$) showed good selectivity over other homologous receptors such as MCH2-R, somatostatin (sst1-sst5), opioid, and urotensin II [57]. Oral administration (30 mpk) suppressed the orexigenic effect of exogenous MCH by >90% in lean rats, consistent with the *in vivo* results of peptidal antagonist studies. Structural variations in which the tetrahydronaphthyl group has been replaced with a *para*-substituted phenyl have been recently described [58].



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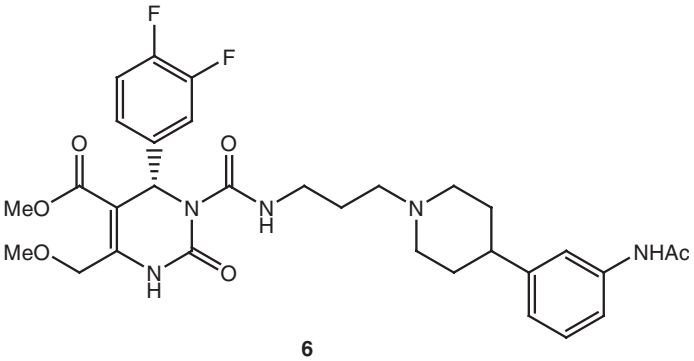
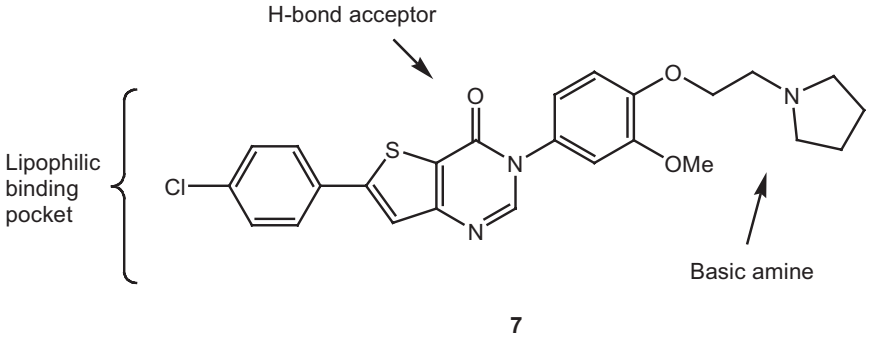


6

A second small molecule antagonist, SNAP-7941 (**6**, $K_b = 0.5\,nM$), demonstrated similar effects to those of T-226296 upon intraperitoneal injection [59]. Specifically, i.p. administration to lean rats suppressed the orexigenic effect induced by i.c.v. administration of MCH. Chronic administration to diet induced obese (DIO) rats (10 mpk, b.i.d.) suppressed food intake, providing a 26% weight reduction over 28 days (relative to controls). This contrasted with D-fenfluramine treatment wherein a pronounced hyperphagia and weight loss over 7 days was followed by a rebound in both by day 14. Though neither T-226296 nor SNAP-7941 were tested in MCH null mice to confirm that the observed effects are MCH1-R specific, radiolabelled SNAP-7941 was shown to specifically bind to MCH1-R in several brain sections. In conjunction with the anorectic effects, SNAP-7941 also exhibited anxiolytic and antidepressant properties in forced swim and Vogel Conflict tests [60]. Derivatives of SNAP-7941 lacking chirality have recently been disclosed [61,62].

These seminal contributions were followed by more recent reports of *in vivo* efficacy demonstrated by GW-803430 (**7**) [63,64]. Derived from a lead structurally similar to T-226296, a homology model was used to highlight key pharmacophore interactions as shown below (Fig. 1).

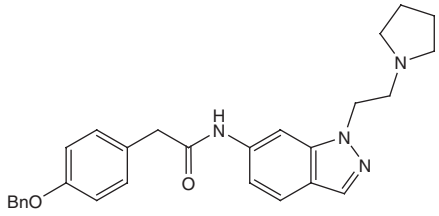


7

Fig. 1. Pharmacophore model for MCH1-R antagonists.

Biaryl surrogates wherein an isosteric amide replacement is fused to a heterobiaryl were explored, along with modifications at the distal amine. GW-803430 has shown oral efficacy in the AKR mouse, a model prone to diet induced obesity, causing a dose-dependent weight loss after 12 days of 7.4% and 13.3% (0.3 and 3 mpk, respectively) with no rebound observed upon prolonged dosing. In contrast, sibutramine induced a weight loss of 3.2% in the same study.

Indazoles such as **8** have also exhibited oral efficacy in diet induced obese mice (10 and 30 mpk, b.i.d.) over 14 days, providing an 8–15% dose dependent weight loss [65]. Comparable effects were initially seen with D-fenfluramine treatment, however a slight rebound in body weight change was observed during the final week of treatment. Consistent with the phenotype exhibited by MCH1-R, subjects treated with **8** did not have altered food intake relative to control, suggesting an alteration of energy expenditure as the causative factor in weight loss. DEXA (dual-energy X-ray absorptiometry) scanning analysis of body composition indicated a significant reduction in fat mass of the treated animals while lean mass was unaffected.



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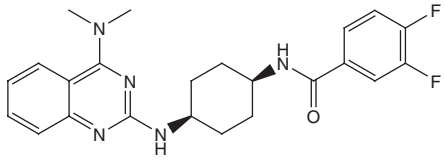

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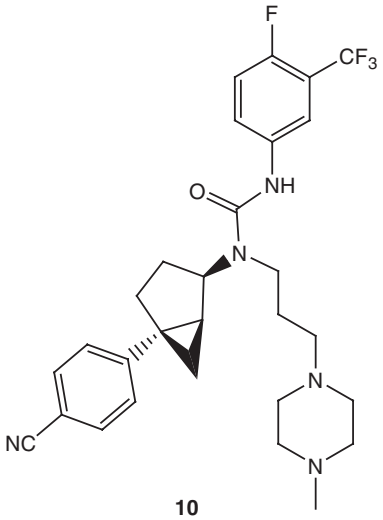
Oral efficacy in rodent models on a high-fat diet was also achieved by ATC-0175 **9**, which provided a 10% weight reduction during a 4-day feeding cycle (45 mpk) relative to a sibutramine control [66,67]. Anxiolytic activity was also demonstrated in a number of rodent anxiety models. In this and related structural series, the aminoquinazoline and aryl amide can be linked by a variety of structures including piperidyl and cyclohexyl moieties of differing chain lengths. The *cis*-1,4-cyclohexyl derivatives confer improved selectivity over Y5 and α_{2a} receptors.



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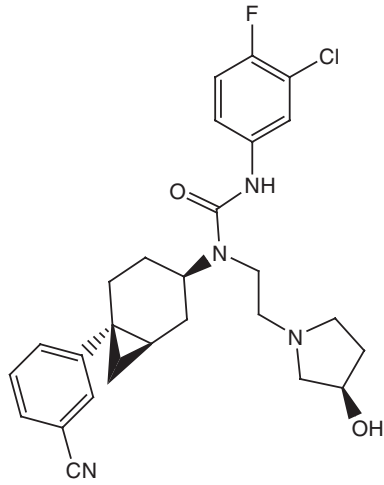
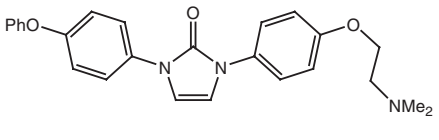


11

Recently, **10** ($K_i = 2.7$ nM) was disclosed as an orally active MCH1-R antagonist [68,69]. Employing a bicycloalkane as an aryl surrogate to circumvent potential mutagenic liabilities, improvements in degree and duration of receptor occupancy were also observed via an *ex-vivo* binding assay. This assay facilitated medium throughput screening of drug occupancy at MCH1-R in rodent models, and could be measured at several timepoints post-dose. Correlation between receptor coverage and efficacy in a DIO mouse model served as an important screening tool. Oral dosing of DIO mice with **10** (30 mpk, p.o.) provided a 22% reduction in food intake over 24 h relative to controls. Similar bicycloheptyl derivatives exhibiting less extensive receptor occupancy such as **11** failed to demonstrate efficacy in the DIO mouse model.

Another urea-derived MCH1-R antagonist is compound **12**, a diaryl imidazolone core appended with a sidechain containing a basic nitrogen atom [70–72]. Structural variations covered in this series of patent applications include benzimidazole, benzothiazole, benzofuran and indole derived ureas. IC_{50} values are reported between 1 nM and 1 μM, with the specified compound reducing milk consumption by 58% in a fasted mouse model (10 mpk, p.o.). In a related structural series, **13** was shown to reduce milk consumption by 64% in a similar model, though higher dosing was performed [73].



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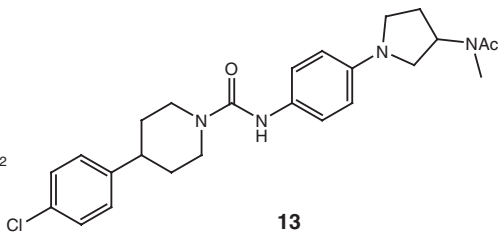
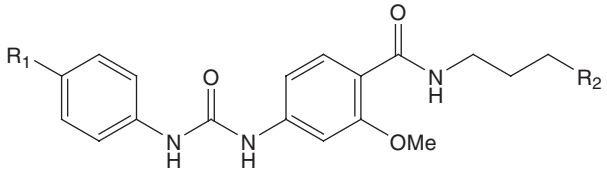
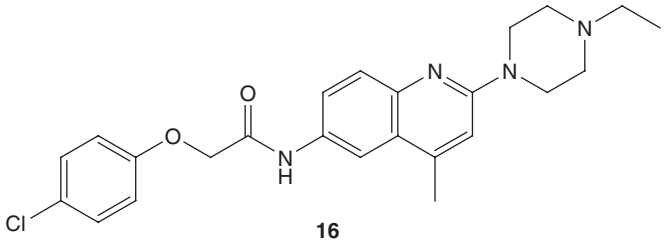


13

The biaryl urea motif has also been exploited as a structural feature in MCH1-R antagonists as demonstrated by **14** ($IC_{50} = 8\,nM$) which resulted from a combination of MCH1 receptor modeling and structural input from D_2 and D_3 receptor ligands as well as other known MCH1-R antagonists such as T-226296 [74]. The dopamine ligands were chosen due to the physicochemical similarity of the D_2 and D_3 binding sites to that of MCH1-R. Considerable structural tolerance was observed in the aliphatic amine region, with side chain homologation and steric congestion at the terminus improving affinity. Amides and oxadiazoles served as urea replacements, however disruption of planarity in the core was detrimental, as was methylation of the urea nitrogen atoms. *In vivo* activity of a related truncated amide **15** was demonstrated in rats (10 mpk, i.p.), with a reduction in cumulative food intake over 6 h [75].



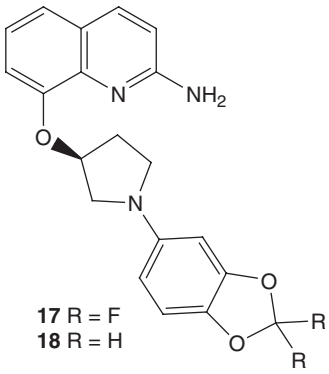
14 R_1 = NHPh, R_2 = N-piperidyl
15 R_1 = OCF_3, R_2 = NEt_2



16

Another group of patents details an aminoquinoline series which has shown efficacy in rats [76,77]. Piperazinyl quinoline **16** reduced cumulative food intake by 20% over 6 h in rats (50 mpk). Variations such as acyclic diamines in lieu of piperazines, and homologated phenoxy acetamides with electron poor *para*-substituents were also efficacious.

Though no *in vivo* efficacy has been reported, differentially substituted aminoquinolines such as **17** have been recently discovered as MCH1-R antagonists [78]. Compound **17** was the culmination of SAR studies in which the pyrrolidyl sidechains exhibit an optimal combination of functional activity and CNS penetration relative to the acyclic benzylamine or benzamide derivatives. Hydrophobic substituents on the terminal aryl group imparted enhanced activity relative to derivatives such as acetamides. The (*S*)-enantiomer ($IC_{50} = 0.9\,nM$) provided a 40-fold increase in binding affinity relative to the (*R*)-configuration. Importantly, the pharmacokinetic profile of **17** in DIO mice was shown to be excellent, with a brain AUC $> 17\,\mu M\,h$ (20-fold relative to plasma AUC) at 10 mpk p.o.. This contrasts with 10-fold lower brain levels exhibited by **18**, which is devoid of the geminal difluoro group.



Aminoquinoline **19** was discovered as the result of a virtual screening approach involving substructure, similarity and homology models based on a set of published MCH1-R antagonists [79]. Hits obtained via screening of over 615,000 commercial entities were then narrowed to a subset based on assessments of druglikeness such as molecular weight, ClogP and polar surface area as well as synthetic facility. Upon assay of this subset, **19** was identified as having an $IC_{50} = 55\,nM$ along with favorable physicochemical properties. Further analysis of **19** in terms of proposed binding mode was performed using a homology model derived from the crystal structure of rhodopsin, which showed good similarity with the transmembrane helical region of MCH1-R (Fig. 2). The following three interactions between **19** and the postulated binding site are deemed crucial: (1) a salt bridge between the distal piperazine nitrogen atom and Asp^{172}, (2) a hydrogen bond between the amide carbonyl and Gln^{325}, and (3) an aromatic binding interaction between the

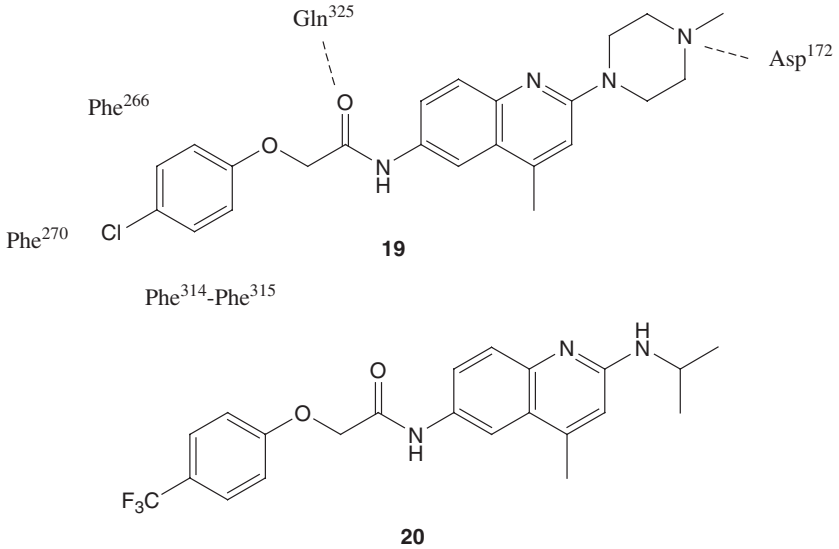
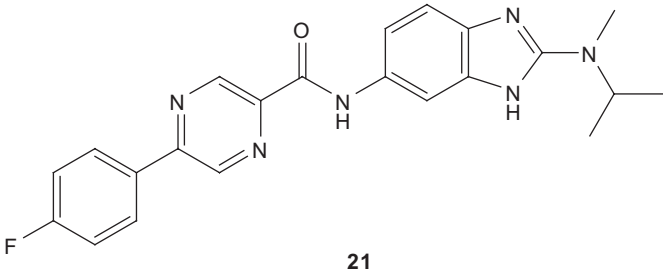


Fig. 2. Pharmacophore developed using Rhodopsin derived homology modeling.

chlorophenyl moiety and several Phe residues from helices 5 and 6. Consistent with the model of key pharmacophore interactions indicated by studies using **7** (*vide supra*), the importance of these receptor binding interactions for other MCH1-R antagonists is evident. Subsequent lead optimization was performed via conventional synthesis-based SAR, including probes of electronic and steric requirements on the aryl ring (relatively large, electron withdrawing *para* substituents were preferred) [80]. Piperazine replacements such as pyrrolidines and acyclic amines (**20**) improved potency ($IC_{50} = 11$ nM) and selectivity versus other GPCRs such as 5-HT subtypes, D_2 and α1a. Though *in vivo* data has yet to be reported, these results demonstrate the utility of ligand-based virtual screening as an efficient approach to hit generation for GCPR targets.

Ring contracted variants of the quinazoline heteroaryl derivatives containing benzimidazoles have also exhibited feeding effects [81,82]. In rats, a dose-dependent (10–30 mpk) decrease in MCH-stimulated food intake was observed upon administration of **21**.



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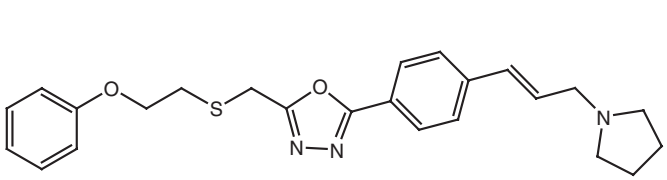
Isosteric replacements for the amide bond have been incorporated into simpler aryl amide compounds such as LY-2049255 (**22**) and **23**. LY-2049255 ($K_i = 1.9$ nM) uses the oxadiazole as a central core upon which linkers to a basic nitrogen atom are attached [83]. Though no alteration of unstimulated food intake was seen, **22** reduced MCH-stimulated food intake up to 6 h post-dose (82 nmol, i.c.v.). Aryl tetrazoles such as **23** were derived from a library synthesis, in which structural modifications indicated that the piperazine and tetrazole were both crucial for activity [84]. Substitution at the *meta*- or *para*-positions on the benzylic aryl group and absolute configuration were important for increased potency. Initial *in vivo* activity was demonstrated at 10 and 30 mpk (i.p.) in a fasted rat model at 1 h post-dose, however efficacy was only observed at 30 mpk after 2 h.



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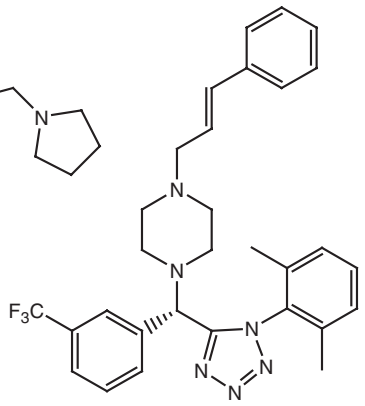

23

5. CONCLUSION

Seminal contributions to the field of MCH modulation as a treatment for obesity have been described herein. These studies have clearly demonstrated that MCH is an appropriate target for inducing weight reduction in rodents, and further studies in higher mammals remain to be disclosed. Recent patent literature has been replete with examples of structurally diverse MCH1-R antagonists. Though detailed reports of *in vivo* studies have yet to be made public, the voluminous patent activity indicates that small molecule MCH1-R antagonists remain under active investigation as potential therapies for obesity.

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Glycogen Synthase Kinase-3 (GSK-3): A Kinase with Exceptional Therapeutic Potential

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Contents

1. INTRODUCTION

The human genome analysis has shown that there are more than 500 kinases that, along with phosphatases, play an essential role in the regulation of enzymes and structural proteins. As our understanding of cellular signaling processes increases, kinases have emerged as attractive targets for disease therapy [1]. Kinase activity is regulated through a complex series of priming events leading to phosphorylation of specific protein substrates that generally activate downstream targets. Common approaches towards kinase regulation focus on small molecule inhibitors that effectively compete for the endogenous substrate adenosine triphosphate, ATP. The ATP binding site is highly conserved amongst kinases and particularly high homology exists within kinase sub-families, such that isoform selectivity is a major obstacle to developing a successful small molecule therapy. Current small-molecule kinase inhibitors capitalize on various structural attributes to achieve the desired affect. The Abelson tyrosine kinase (Abl) inhibitor Gleevec^{TM} buries the key kinase activation loop upon binding, thereby providing the necessary selectivity profile over the related Src kinases [2]. Both Iressa^{TM}, an inhibitor of the epidermal growth factor receptor, and a $_{P}$38 MAPK inhibitor for treating inflammation have been reported to achieve selectivity through interactions at the ATP-binding site [3,4].

Glycogen synthase kinase-3 (GSK-3) is a serine/threonine kinase that is ubiquitously expressed in mammalian tissues. As opposed to other kinases, GSK-3 is unusual in that it is constitutively active and it negatively regulates its downstream

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targets. GSK-3 has been implicated in a wide variety of disease states including obesity and type 2 diabetes mellitus [5], neurological disorders (e.g., Alzheimer's disease, bipolar disorder, neuronal cell death and stroke, depression) [6–8], inflammation [9,10], cardio-protection [11,12], cancer [13], skeletal muscle atrophy [14] and myotube hypertrophy [15], hair loss [16] and decreased sperm motility [17]. Comprehensive surveys on the chemistry [18,19], biology [19–28] and pharmacology [5–7,29–34] of GSK-3 inhibition have appeared.

2. BIOLOGY OF GSK-3

2.1. Glycogen synthase kinase-3 features and functions

GSK-3 exists in the cytosol and is identified with three known isoforms, GSK-3α, GSK-3β and GSK-3β2. The two major isoforms, GSK-3α and GSK-3β, are 51 and 47 kDa proteins, respectively, and exhibit high levels of homology (~85%) with essentially identical ATP-binding sites (93% identity). These are ubiquitous proteins, exhibiting little tissue specificity, though some differentiation in overall expression levels does exist. Endogenous and exogenous substrates show little preference towards the isoforms, though valproic acid derivatives have been observed to inhibit GSK-3β somewhat selectively over GSK3-α [35]. However, GSK-3α and GSK-3β do perform distinct regulatory functions; GSK-3β knock-out mice are not viable, suffering hepatic apoptosis (NF-κB/TNF-α pathway) as embryos, but no increase in stabilized β-catenin levels is observed via the GSK-3 moderated Wnt signaling pathway [36,37]. Also, small interfering RNA studies on somatic cells targeting both GSK-3α and GSK-3β have shown an increase in β-catenin levels but no whole-body work has been published to date [38]. A newly identified isoform, GSK-3β2, contains a 14 amino acid insert in the C-terminal region that apparently is a splicing variant of GSK-3β [39].

The functional activity of GSK-3 is described by an interesting series of phosphorylation events. The enzyme is constitutively activated through an intramolecular phosphorylation at Tyr-279 in GSK-3α and at Tyr-216 in GSK-3β [40]. This kinase generally phosphorylates substrates at a Ser/Thr residue located four amino acids C-terminal from a priming phospho-Ser/Thr site, and may perform more than one phosphorylation event given the proper sequence (-XX-S/T-XXX-S/T(P)-). However, not all substrates of GSK-3 require phosphorylative priming as proximal localization of substrate to the kinase through complex formation can facilitate kinase action [41]. The activity of GSK-3 may be modulated by phosphorylation on an N-terminal Ser residue (Ser-21 of GSK-3α and Ser-9 of GSK-3β), with this new phospho-Ser residue binding intramolecularly in the phospho-substrate binding site. The known priming enzymes and the interplay of various complexes on the activity of GSK-3 have been reviewed recently [6].

The active ATP binding site is defined by the confluence of the N- and C-terminal regions of the kinase into a hinge array, and an activating loop containing Tyr-216, an amino acid residue that imparts increased functional activity upon phosphorylation

[42]. There are several published crystal structures of GSK-3β: most notably, a structure of the catalytically active enzyme with a buffer sulphonate molecule mimicking phospho-Tyr-216 [43], one with a fragment of Axin bound [44], and one bound to a fragment of the endogenous protein FRATtide [45]; the latter two substrates are components of the Wnt signaling pathway that controls gene regulation. Four positively charged residues form a cationic pocket for the ATP phosphate, with the hinge region residues providing a "donor-acceptor" anchor for the adenosine moiety. Selective inhibition of GSK-3 will be a challenge because high levels of homology exist between the ATP-binding site of close family members such as the cyclin-dependent kinases (CDK-1, CDK-2, CDK-5), the mitogen-activated protein kinases (MAPK) and others (Aurora2, DYRK, CK1).

2.2. Lithium as proof of concept

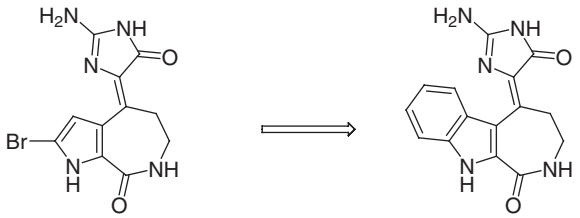
Lithium has long been the therapy of choice for bipolar disorder and manic syndromes though the exact mechanism of action has been difficult to discern [46]. Lithium is known to affect the function of a variety of enzymes, an effect attributed to lithium competing for essential magnesium binding sites [47]. Therapeutically efficacious doses of Li^+ (0.6–1.2 mM plasma levels) do approach its GSK-3 IC_{50} ($IC_{50} = 2$ mM). When the magnesium level is controlled at a relevant cellular level (~ 0.75 mM), however, the IC_{50} is estimated to be closer to 0.8 mM [22]. Numerous studies have established the link between lithium treatment and GSK-3 inhibition, and the positive effects on neurodegenerative endpoints such as tau phosphorylation, decreased β-amyloid production and neuronal apoptosis are well documented [32,48]. Inhibition of GSK-3 in male Wistar Kyoto rats with 1.2 or 2.4 g/kg Li_2CO_3 in chow for nine days increased the pool of stabilized β-catenin, suggesting a potential tumorigenic side-effect. An offsetting decrease in the production of β-catenin RNA, however, resulted in a negligible impact on total β-catenin levels [49].

Other *in vitro* lithium studies have shown effects that could be contraindicated for the treatment of chronic disease, though these side effects have not been documented in patients receiving chronic lithium treatment for psychotherapy. Effects on microtubule dynamics and axonal branching have been detected in cultured chick neurons exposed to 10 mM LiCl for 24 h [50], and treatment of pig airway epithelial cells with 10 mM LiCl for 24 h induced G2/M cell cycle arrest and increased the expression of cyclins D1 and B1 [51]. Interestingly, lithium treatment (20 mM for 48 h) of human cancer cell lines prevented the desired apoptotic events associated with treatment with etoposide and camptothecin by disrupting nuclear complexes of GSK-3/p53 and repressing the expression of the CD95 gene [52]. The concerns and studies addressing cell-cycling and/or cytotoxic outcomes from the inhibition of GSK-3 are many and this review can only provide a snapshot of the discussion to date. Interestingly, no clinical studies of chronic lithium treatment have been designed to read out on any primary endpoints of GSK-3 inhibition, such as plasma glucose, though the lack of target specificity could make interpretation of these clinical data challenging.

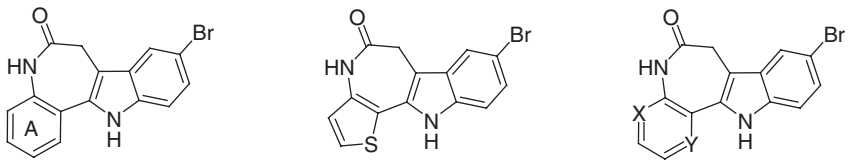
3. SMALL MOLECULE INHIBITORS OF GSK-3

3.1. Natural product derived GSK-3 inhibitors

Derivatives of the naturally occurring GSK-3 inhibitor hymenialdisine, **1**, such as indoloazepine **2**, were studied as anti-inflammatory agents through inhibition of the NF-κB pathway [53]. Compound **2**, which inhibited GSK-3 ($IC_{50} = 0.15\,\mu M$) in addition to CDK-1, MEK-1, CHK-1, and CHK-2 with $IC_{50} < 1\,\mu M$, was active in cellular models of inflammation and inhibited IL-2 ($IC_{50} = 2.4\,\mu M$) and TNF-α production ($IC_{50} = 8.2\,\mu M$), as well as NF-κB-DNA binding (49% @5 μM).



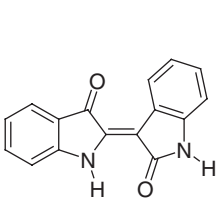
A structurally similar natural product kenpaullone, **3**, a potent GSK-3 inhibitor ($IC_{50} = 0.02\,\mu M$) with selectivity over CDK-1 (17-fold) and CDK-5 (37-fold), was the basis for a series of heterocyclic derivatives such as the thieno analog **4**, the 4-aza analog **5** and the 1-aza analog **6**. GSK-3 activity decreased slightly in **4** ($IC_{50} = 0.12\mu M$) with similar selectivity to **3**. Compound **5** was much less active versus GSK-3 ($IC_{50} = 6\,\mu M$) whereas compound **6** retained GSK3 activity ($IC_{50} = 0.018\,\mu M$) and the selectivity versus CDK-1 (111x) and CDK-5 (233x) was improved [54]. The decrease in binding affinity to the CDK's was speculated to arise from a local charge distribution change in ring A. 3D-QSAR CoMSIA models were also utilized to improve overall potency and kinase selectivity in this series [55].



3 **4** **5** X = N, Y = CH
6 X = CH, Y = N

A variety of indigoids, represented by indirubin **7** (GSK-3 $IC_{50} = 2.4\,\mu M$), were enzymatically produced from substituted indoles using cytochrome-P450 mutant enzymes [56]. When 5-methyoxyindole was incubated with the L240C/N297Q mutants, the crude cell extracts showed considerable GSK-3 and CDK-5 activity. Separation, characterization and biological evaluation of these crude mixtures

led to the identification of the more potent di-substituted indirubin, **8** (IC_{50}'s for GSK-3, CDK-5 and CDK-1 were 0.2 μM, 0.8 μM, and 0.4 μM, respectively).



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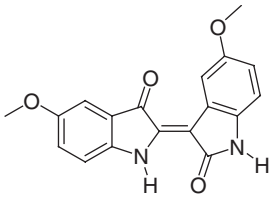
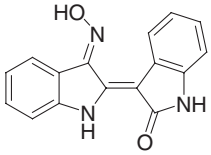


8

Molecular modeling and X-ray crystallographic structural data were used to guide the synthesis of a number of indirubin derivatives with improved GSK-3 potency and increased selectivity over CDK-1 and CDK-5 [57]. The 3'-oxime **9** showed increased GSK-3 potency with modest CDK selectivity (GSK-3 $IC_{50} = 0.022\,\mu M$; 8-fold vs. CDK-1, 5-fold vs. CDK-5). The increased activity was attributed to a more robust H-bonding network involving the oxime –OH and the protein sidechains. The addition of 6-Br and 5-NO_2 substituents in compound **10** further improved GSK-3 potency ($IC_{50} = 0.007\,\mu M$) and enhanced selectivity (1,700-fold vs. CDK-1; 21-fold vs. CDK-5) presumably through a repulsive interaction with a conserved Phe80 residue in the CDK's active site. The best selectivity and potency was achieved with acetoxime **11** (GSK-3 $IC_{50} = 0.006\,\mu M$; 1,800-fold vs. CDK-1; 5,100-fold vs. CDK-5).



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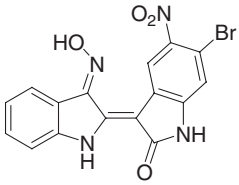

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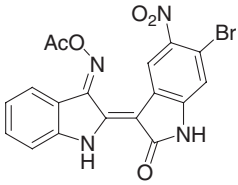


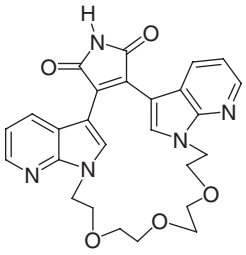
11

3.2. Maleimides

A variety of distinct small molecule ATP-competitive inhibitors of GSK-3 have been identified. Chemical classes including purines, pyrimidines, amino thiazoles, furo[2,3-*d*]pyrimidines, pyrazolopyridines, dihydropyrazolopyridines, and maleimides have been investigated with the maleimide family of inhibitors receiving the most attention.

The GSK-3 inhibitory activity of polyoxygenated macrocyclic maleimide **12** was discovered through an exploratory program targeting protein kinase C gamma inhibitors (PKC-γ) [58]. The cytotoxicity risk associated with the crown-ether construct prompted a search for a structural replacement and maleimide **13** (GSK-3

$IC_{50} = 11\,nM$) was identified through these efforts. Compound **13** was selective against a broad panel of 66 protein kinases ([ATP] = 100 μM) and demonstrated selectivity (60–70-fold) over CDK-2, PKCb-I, and RsK-3. This compound also demonstrated activation of glycogen synthase activity in human embryonic kidney (HEK293) cells ($EC_{50} = 0.330\,\mu M$).



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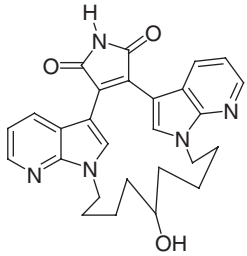
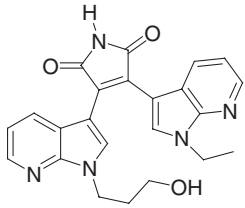


13

Acyclic classes of maleimides, such as the 3-(7-azaindolyl)-4-(aryl/heteroaryl)maleimides **14-16**, also have demonstrated potency and selectivity for GSK-3 [59,60]. Systematic optimization of the lead compound identified the potency-enhancing 3-hyroxypropyl side chain (**14**, $IC_{50} = 0.065\,\mu M$) while replacing the azaindole with a pyridyl ring, e.g., **15**, enhanced the selectivity profile against a broad panel of 70 protein kinases (>300-fold vs. PKC's, >100-fold vs. CDK's) and could improve the metabolic stability in human liver microsomes. Compound **16** was a potent inhibitor of GSK-3β ($IC_{50} = 0.02\,\mu M$) with excellent selectivity over other protein kinases, including the CDK's and PKC's and good microsomal stability ($t_{1/2}$ >100 min). Several analogs were also able to stimulate glycogen synthase activity in HEK293 cells as demonstrated by aryl-maleimide **14** ($EC_{50} = 0.62\,\mu M$).



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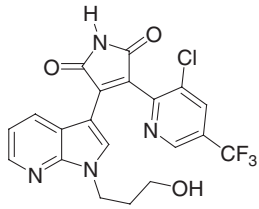

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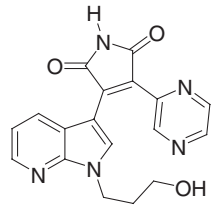
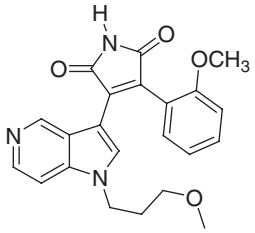


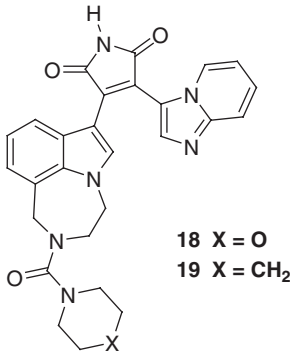
16

The structurally related 3-(1H-pyrrolo[3,2-c]pyridin-3-yl)-maleimide series was claimed as potent inhibitors of GSK-3 with IC_{50}'s in the low-nM range ($IC_{50} = 0.0018$–$0.020\,\mu M$) [61]. The introduction of a 3-methoxypropyl group on the pyrrole nitrogen paired with an *ortho*-substituted phenyl group at the maleimide 4-position afforded potent GSK-3 inhibition. One of the best derivatives was **17** with a GSK-3 $IC_{50} = 0.0018\,\mu M$ and >100-fold selectivity vs. PKC-α, PKCβII, and PKC-γ.



17

Further developments in the area of *bis*-aryl maleimide inhibitors of GSK-3 have delivered compounds with sufficient pharmacokinetic (PK) properties (**18**, $t_{1/2} = 2.8$ h, F = 23% in female rats) that oral efficacy could be demonstrated in an animal model of type II diabetes [62]. Compounds **18** and **19** inhibited GSK-3β ($IC_{50} = 0.0013$ and 0.0011 μM, respectively) and blocked GSK-3 dependent phosphorylation of the Tau protein in SY5Y cells (P-Tau $EC_{50} = 0.0026$ and 0.007 μM, respectively). Compound **18** exhibited selectivity for GSK-3 over a diverse kinase panel including CDK-2, CDK-4, CDK-5 and PKCβII (> 500-fold). Compounds **18** and **19** lowered plasma glucose 78% and 61%, respectively, in a Zucker diabetic fatty (ZDF) rat dose response study at 10 mg/kg/day dose. Also, an improved response to an oral glucose tolerance test was demonstrated in ZDF rats receiving compound **18** (0.1–3 mg, q.d.) after eight days of dosing.

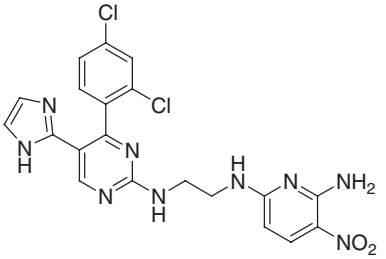


18 X = O
19 X = CH_2

3.3. Other chemical series

Substituted 2-aminopyrimidines, such as **20**, are potent and selective inhibitors of GSK-3 [63]. Compounds **20** and **21** (GSK-3 IC_{50}s = 0.001 and 0.01 μM, respectively) exhibited > 800-fold selectivity for GSK-3 in a panel including 20 other kinases, and compounds of this class were functionally active in models of diabetes and neuroprotection. Both compounds activated glycogen synthase in cells (EC_{50}s = 0.11 and 0.76 μM, respectively) and enhanced insulin-stimulated glucose uptake in skeletal muscle from ZDF rats. *In vivo*, an oral dose of compound **21** to fasted ZDF rats led to dose-dependent reductions in plasma glucose that were sustained for several hours. Compound **21** also reduced the magnitude of glucose

excursion in a glucose tolerance test in ZDF rats. Similar results on fasting plasma glucose and in glucose tolerance tests were observed in the *db/db* mouse model (10–30 mg/kg, p.o.). Another compound from this series was neuroprotective *in vitro* and *in vivo* [64], protecting hippocampal neurons and cortical neurons against glutamate toxicity and oxygen-glucose deprivation, respectively (efficacy observed at 0.1 μM compound concentration in both assays). *In vivo*, infarct size in rat brains was reduced following middle cerebral artery occlusion (MCAo); efficacious compound concentrations in brain were ~0.4 μM.



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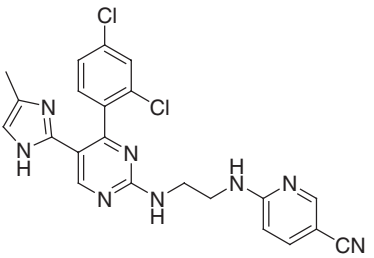
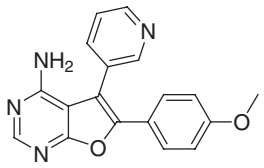


21

The furo[2,3-*d*]pyrimidine **22** was identified as a potent GSK-3 inhibitor during research targeting the VEGFR2 and TIE2 tyrosine kinases [65]. Modification of the core structure, including removal of the 5-pyridyl moiety and acylation of the amino group afforded compounds derived from the core **23**. Compound **23a** exhibited GSK-3 $IC_{50} = 0.032\,\mu M$, and was selective vs. CDK-2 and VEGFR2 (> 500- and 32-fold, respectively). Further modification of the selective lead **23a** yielded pyridyl analog **23b** (GSK-3 $IC_{50} = 0.005\,\mu M$), which also exhibited efficacy in a glycogen accumulation assay (L6 cells). Compound **23b** was selective when examined in a 20-kinase panel which included CDK-2; the R_1 = c-pentyl group was hypothesized to negatively affect binding with CDK-2.



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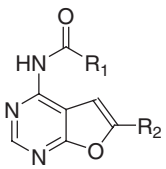
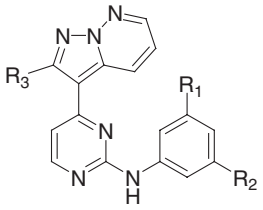


23

23a R_1 = c-pentyl
R_2 = 4-MeOPh
23b R_1 = c-pentyl
R_2 = 3-pyridyl

Compounds with the N-phenyl-4-(pyrazolo[1,5-b]pyridazin-3-yl)pyrimidin-2-amine core **24** were screening hits with potent GSK-3 and CDK activity (**24a**: GSK-3 $IC_{50} = 0.019\,\mu M$, CDK-2 $IC_{50} = 0.005\,\mu M$, CDK-4 $IC_{50} = 0.158\,\mu M$). Molecular modeling and extensive structure-activity studies were employed to substantially increase selectivity over the CDK's [66]. Substitution of the aniline provided > 30-fold selectivity against CDK-2/4 (**24b**: GSK-3 $IC_{50} < 0.010\,\mu M$).

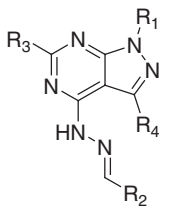
Synthesis of compounds with R_3 = aryl resulted in >1000-fold selectivity over CDK-2 (**24c**: GSK-3 $IC_{50} = 0.012\,\mu M$). The selectivity increase was hypothesized to result from unfavorable interactions between the R_3-phenyl group and Phe80 in CDK-2. Several compounds exhibited functional activity in rat muscle L6 cells, increasing glycogen deposition, for example **24c** ($EC_{50} = 2.1\,\mu M$, 76% maximal response compared to insulin). Furthermore, a representative compound **24d** showed oral exposure in mice at a dose of 10 mg/kg (Cmax = 0.56 g/mL).



24

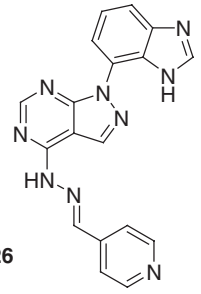
24a R_1, R_2, R_3 = H
24b R_1, R_2 = Cl, R_3 = H
24c $R_1 = CF_3$, R_2 = H, R_3 = Ph
24d $R_1 = CF_3$, R_2 = H, R_3 = 4-F-Ph

Pyrazolopyrimidine-derived hydrazones **25** were also reported as potent GSK-3 inhibitors [67,68]. Potency SAR was restrictive around the core, with the exception of the aromatic ring of the hydrazone. Initial experiments identified a *meta*-methoxy phenyl group at R_1 as the preferred group, which was attributed to a combination of steric and electronic effects that influenced the biaryl ring system conformation. Pyridyl and phenyl were tolerated at R_2, with para-substitution on the ring providing a useful means of modulating the physicochemical properties of the compounds. Compound **25a** exhibited GSK-3 $IC_{50} = 0.006\,\mu M$. A structural analysis of the binding mode of these compounds suggested that a potential intramolecular hydrogen bond could be accessed *via* introduction of a benzimidazole unit at R_1 leading to compound **26**, with $IC_{50} = 0.003\,\mu M$ and $EC_{50} = 0.11\,\mu M$ in a cellular assay measuring glycogen synthesis (L6 cells). Variations in cellular potency were correlated to membrane permeability in MDCK cells.



25

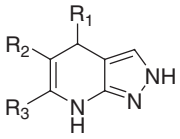
25a R_1 = 3-OMePh,
R_2 = 4-pyridyl,
R_3, R_4 = H



26

A series of dihydropyrazolopyridines **27** with IC_{50}'s as potent as <0.001 μM versus GSK-3 was reported in an extension of prior work on this series [69]. Pre ferred R_1 groups included mono- and bicyclic aromatic rings; the benzoxadiazole group of compound **28** (GSK-3 $IC_{50} = 0.003\,\mu M$) was present in multiple exemplified

compounds. Electron-withdrawing groups, including –CN, –COR, $–SO_2R$ were preferred at R_2. Groups at R_3 included alkyl-linked amines and amides, as well as ketones.



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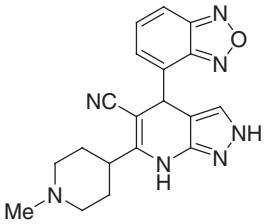
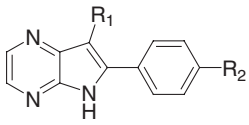


28

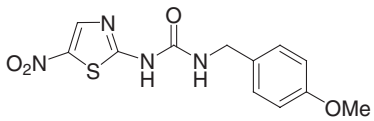
5H-Pyrrolo-[2,3b]-pyrazines **29** were described as potent, non-selective GSK-3 and CDK inhibitors [70]. A particular example, Aloisine A (**29a**), had similar potency versus CDK-1, CDK-2, CDK-5, and GSK-3 (GSK-3 $IC_{50} = 0.65\,\mu M$, CDK-1 $IC_{50} = 0.15\,\mu M$, CDK-5 $IC_{50} = 0.20\,\mu M$), with weak activity against CDK-4 and 18 other kinases tested. Compound **29a** demonstrated antiproliferative effects, blocking cell cycle transitions between G0/G1 and G2/M. N-Methylation of the pyrrole nitrogen led to significant reductions in activity, which was in accord with X-ray crystallographic data of compound **29b** bound to CDK-2, where the 4-N and 5-NH formed hydrogen bonds to the backbone oxygen and nitrogen atoms of Leu 83.



29

29a R_1 = n-Bu, R_2 = H
29b R_1 = i-Pr, R_2 = Cl

Aminothiazoles such as **30** were potent GSK-3 inhibitors ($K_i = 0.038\,\mu M$) with selectivity versus 26 other kinases [71]. In cells, **30** inhibited tau phosphorylation ($EC_{50} = 2.7\,\mu M$) and protected against cell death mediated by the PI3K/PKB survival pathway. β-Amyloid neuronal death in hippocampal slices was significantly reduced in the presence of **30**. In the forced swim test, a measure of anti-depressant-like activity, rats treated with **30** (8.8 mg/kg, i.p.) showed significantly reduced immobility times versus control animals. The effects were not a result of non-specific increased locomotor activity, as both spontaneous and amphetamine-induced activity was decreased following treatment with the compound [72].



30

4. CONCLUSION

Our better understanding of the fundamental roles that GSK-3 plays in a myriad of physiological processes has driven the research devoted to identifying selective inhibitors as therapeutic agents. There are many critical areas of unmet medical need that involve this kinase at some level. The central role played by this kinase in regulating basic developmental processes, however, underscores the need to address target safety around long-term inhibition of GSK-3. Small molecule inhibitors with vastly improved pharmacokinetic properties and kinase selectivity have been developed in the past few years such that pre-clinical studies addressing chronic inhibition of GSK-3 can be undertaken. Exploratory toxicology studies will be an integral component of a program focused on delivering a therapy based on GSK-3 inhibition.

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Inhibitors of Dipeptidyl Peptidase 4

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Contents

1. INTRODUCTION

Dipeptidyl peptidase 4 (EC 3.4.14.5, DPP-IV, DPP4, CD26) is a ubiquitous serine protease that modulates the biological activities of numerous peptides, including glucagon-like peptide-1 (GLP-1). GLP-1 plays an important role in the control of post-prandial glucose levels by potentiating glucose-stimulated insulin release and inhibiting the release of glucagon. Other actions of GLP-1 include delaying gastric emptying, inducing satiety and increasing *beta* cell mass. GLP-1 has shown efficacy in diabetics, but suffers from a very short physiological half-life ($t_{1/2} \sim 2$ min) due to DPP4-mediated cleavage of the active peptide (7-36 amide or 7-37) to an inactive form (9-36 amide or 9-37). Intense research in the pharmaceutical industry aims to discover and develop stable GLP-1 analogs, exogenous agonists of the GLP-1 receptor or small-molecule inhibitors of DPP4. This research has been buoyed recently by positive clinical trial data on GLP-1 analogs and DPP4 inhibitors. The field of DPP4 inhibition has been reviewed extensively [1–12]. This review attempts to provide an update to the previous ARMC article on DPP4 inhibitors [13] covering the primary literature from 2001 through the end of March 2005. It is not the intent of the authors to provide another review of the pharmacology of DPP4, but to concentrate on the medicinal chemistry in the field.

1.1. Function of DPP4

DPP4 functions as a serine protease and cleaves the amino-terminal dipeptide from oligopeptides with a proline or alanine at the penultimate position. Peptides with residues other than Pro or Ala at the penultimate position may also be low-affinity substrates for DPP4. In contrast, DPP4 is not selective with respect to the N-terminal

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residue [14] and shows little discrimination of various prime-side residues [15,16]. A number of biologically important peptides are substrates for DPP4 *in vitro* [17,18].

1.2. Structure of DPP4

DPP4 is a 110-kDa glycoprotein expressed on the cell surface and widely distributed throughout the body. Cleavage of the extracellular portion of DPP4 from the 22-residue transmembrane section results in a soluble, circulating form of approximately 100 kDa. Functional DPP4 is a homodimer, although an active heterodimer with fibroblast activation protein has been observed [19]. The consensus sequence for DPP4 is G-W-S-Y-G and the catalytic triad is made up of Ser630, Asp708 and His740. It has been shown that the glycosylation state of the enzyme is not important for enzyme activity, dimerization, and adenosine deaminase binding [20].

Several groups have reported crystal structures of human DPP4 [15,21–24], and one group has reported the structure of porcine DPP4 [25]. These structures show the dimeric nature of the enzyme and reveal that the catalytic site is located in a cavity between the α/β hydrolase domain and an eight-bladed propeller domain. Also revealed is the oxyanion hole, which is composed of the backbone NH of Tyr631 and the OH of Tyr47. A co-complex of DPP4 and the inhibitor Val-pyrrolidide demonstrates that two glutamates in the active site play an important role in substrate binding by forming a salt bridge with the N-terminus of a peptide substrate. The pyrrolidine of the inhibitor effectively fills a hydrophobic pocket that will only accommodate small residues. This pocket engenders DPP4's selectivity for proline at P1. This work also revealed that two openings in the enzyme may provide access to and egress from the catalytic site for some substrates and products [21]. The importance of Tyr547 in the stabilization of the intermediate oxyanion was confirmed through site-directed mutagenesis [26]. Most authors agree that peptides enter the larger side opening to access the active site [15]. It has been postulated that the dipeptide product is expelled through the narrow β-propeller opening [21,24]. The co-complex of DPP4 and a compound related to NVP-DPP728 [23] confirms that cyanopyrrolidine inhibitors form an imidate with the active site serine, consistent with a model proposed earlier [27]. Two groups have observed the trapping of tetrahedral intermediates in co-complexes of peptides with DPP4 [15,24].

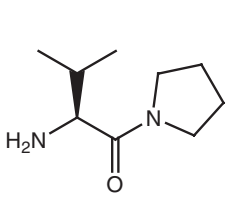
1.3. Therapeutic significance

Relative to wild-type controls, DPP4-deficient mice are resistant to the development of obesity and hyperinsulinemia when fed a high-fat diet [28]. DPP4 knockout mice also show elevated GLP-1 levels and improved metabolic control. Relative to DPP4 positive controls, DPP4-deficient Fischer rats show improved glucose tolerance following an oral glucose challenge due to enhanced insulin release mediated by high levels of active GLP-1 [29,30]. In these studies, the authors note that fasting and post-challenge glucose levels in both strains are similar, supporting previous assertions that hypoglycemia is unlikely during treatment with DPP4 inhibitors.

The use of GLP-1 and its analogs in the treatment of diabetes has been reviewed recently [31,32]. It has been shown that DPP4 inhibition prevents the degradation of endogenous GLP-1 and glucose-dependent insulinotropic polypeptide (GIP) in dogs, thereby preserving the insulinotropic effects of these peptides [33]. In the same study, it was noted that total incretin secretion was reduced, suggesting that feedback mechanisms restrict the secretion of incretins when levels of active peptide are elevated. It has been demonstrated that agonism of the GLP-1 receptor results in growth and differentiation of pancreatic islet *beta* cells [34–36]. If realized in humans, such an effect may result in preservation or restoration of β-cell function in diabetics. In human clinical trials, infusion of GLP-1 led to such beneficial effects as decreases in post-prandial glucose excursions, increases in post-prandial insulin, reductions in HbA_{1c}, weight loss, enhanced insulin sensitivity and improved β-cell function [37,38]. Administration of the GLP-1 analogs exendin-4, CJC-1131 and NN2211 resulted in similar beneficial effects [31,32]. Notably, DPP4 inhibition has been shown to augment the insulin secretion effects of not only GLP-1 and GIP, but also pituitary adenylate cyclase-activating polypeptide (PACAP) and gastrin-releasing peptide (GRP) [39].

2. PRECLINICAL DPP4 INHIBITORS

Early DPP4 inhibitors closely mimicked DPP4 substrates, as exemplified by valine-pyrrolidide (Val-Pyr, **1**), P32/98 (**2**) and FE 999011 (**3**). A large body of data has been reported for these compounds and provided early biological validation for the use of DPP4 inhibitors as an approach to the treatment of diabetes.



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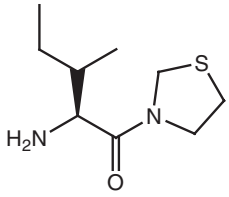

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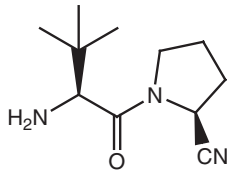


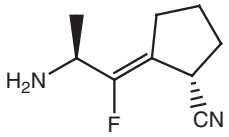
3

Treatment of six-week-old *db*/*db* mice with Val-Pyr resulted in increased endogenous GLP-1 levels, potentiated insulin secretion and improved glucose tolerance; however, while the effects on GLP-1 and insulin were maintained in mice at 23 weeks of age, the improved glucose control was lost [40]. Studies in rats demonstrated that combining Val-Pyr with metformin leads to reduced food intake and body weight gain, improved glucose tolerance and increases in active plasma GLP-1 and that these effects are absent or less significant when using either drug as monotherapy [41,42]. In related work, treatment of rats with metformin or pioglitazone resulted in reduced serum DPP4 activity. Since the authors found that these agents are not inhibitors of DPP4 *in vitro*, they suggested that the effect resulted from reduced DPP4 secretion [43].

Double incretin receptor knockout (DIRKO) mice are genetically altered to lack both the GLP-1 receptor and the GIP receptor. A study in these animals with

Val-Pyr and a structurally unrelated inhibitor, SYR106124, showed that while these inhibitors provide improved glucose tolerance and increased insulin levels in wild-type and single incretin receptor knock out mice, these effects were lost in the DIRKO mice. This result points to the essential nature of the incretin receptors in the actions of DPP4 inhibitors [44].

While inhibitors such as **4** ($K_i = 6.03\,\mu M$) and **5** ($IC_{50} = 12\,\mu M$) are related to the cyanopyrrolidine DPP4 inhibitors through the use of the fluoroolefin amide isostere, these compounds are only weak inhibitors of the enzyme [45–47].



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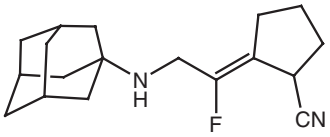
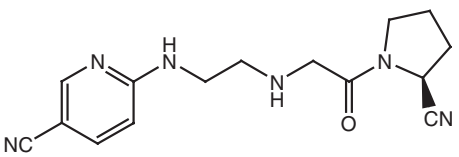


5

Several recent papers have examined the effects of long-term treatment with P32/98 (**2**) in rodent models of diabetes. A three-month treatment regimen provided sustained improvements in glucose tolerance, increased β-cell responsiveness and improved peripheral insulin sensitivity in Zucker *fa/fa* rats [48,49]. The same investigators have shown that 7 weeks of treatment with **2** enhances β-cell survival and islet neogenesis in a streptozotocin-induced diabetes model [50]. A study designed to compare the effects of **2** with those of rosiglitazone and to the effects of the combination of the two agents found that the DPP4 inhibitor provided improved glucose tolerance in both prediabetic and diabetic animals. While rosiglitazone resulted in increased body weight, **2** was body-weight neutral. However, neither agent was very effective at improving the diabetic condition of older ZDF rats [51]. Studies have shown that the metabolism of **2** is dominated by oxidation of the sulfur atom and glucuronidation of the primary amine [52].

In rodent models of diabetes, chronic treatment with FE 999011 (**3**) provided improved glucose tolerance, postponed the progression to hyperglycemia by 21 days, reduced hypertrigylyceridemia and prevented a rise in circulating free fatty acids [53].

Rodent studies using NVP-DPP728 (**6**, $IC_{50} = 7\,nM$) [54] and the structurally related K579 (**7**, $IC_{50} = 5\,nM$) have demonstrated similar pharmacological effects as those seen with the inhibitors discussed above. In a comparative study, **7** appeared to provide better control of DPP4 activity and glucose excursions than did **6** [55]. Combination of **7** with glibenclamide further enhanced the glucose control without significant hypoglycemia [56].



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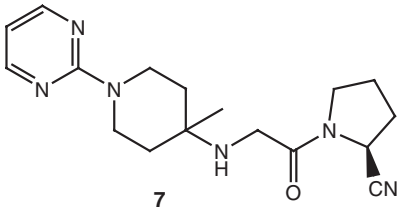
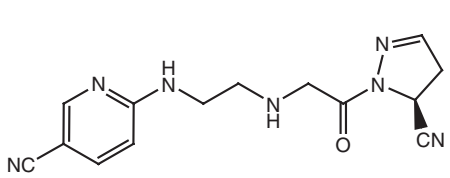


7

The 2-CN pyrrolidine present in **6** can be substituted by a cyanopyrazoline, but this results in a less potent compound (**8**, $IC_{50} = 360\,nM$) [57]. A pyrazolidine heterocycle has also been examined (**9**, $IC_{50} = 1.56\,\mu M$) [58].



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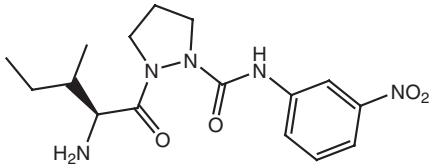
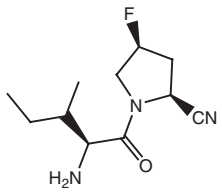


9

Several groups have examined substituted pyrrolidines in an effort to improve potency or stability of the inhibitors. Attempted incorporation of hydroxy or methoxy substituents at various positions on the ring led to reduced potency, but fluorination at the 4-position gave increased potency as in compound **10** ($IC_{50} = 0.6\,nM$). This compound also displayed increased plasma drug concentrations relative to the unsubstituted inhibitor [59]. In an examination of pyrrolidines cyclopropanated at either the 3,4 or 4,5 positions, it was found that while introduction of the cyclopropane on the face of the pyrrolidine *trans* to the cyano group led to compounds with micromolar IC_{50}s, the *cis*-3,4-methano and *cis*-4,5-methano moieties were well tolerated. One goal of this work was to reduce the intramolecular amine-nitrile cyclization that plagues many cyanopyrrolidine DPP4 inhibitors. Bulky substituents on the amino acid and the cyclopropane moiety provided impressive improvements in solution stability. Compound **11** ($IC_{50} = 1.5\,nM$) has a half-life of 5 hours, while compound **12** ($K_i = 8\,nM$) has one of 27 hours and compound **13** ($K_i = 7\,nM$), 42 hours. Compound **13** reduced glucose excursions following an oral glucose tolerance test (OGTT) in Zucker *fa/fa* rats [60].



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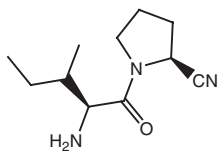

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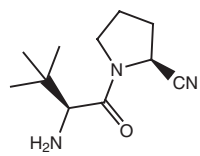

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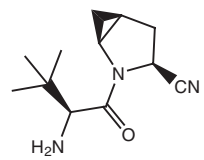
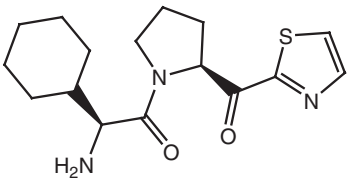


13

Ketopyrrolidines and ketoazetidines, which replace the cyano group with a heteroaryl ketone, have also been examined as DPP4 inhibitors. Heteroaryl ketones have been used extensively as reversible serine protease inhibitors and act by providing an electrophilic carbonyl that can form a tetrahedral species with the active site serine. An examination of rings from four to six atoms revealed that only the piperidine derivatives were not inhibitors of the enzyme. 2-Thiazolyl and 2-benzothiazolyl substituents provided sufficient activation of the carbonyl to give low nanomolar inhibitors such as **14** ($IC_{50} = 30$–$42\,nM$). These compounds suffer from an internal cyclization followed by oxidation to give dihydroketopyrazines such as **15** [61].



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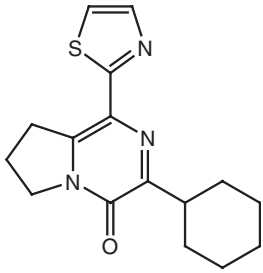
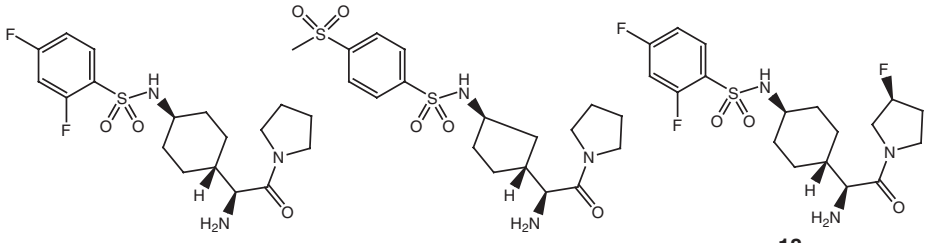
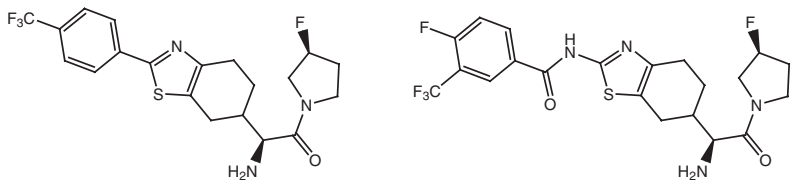


15

Substituted cycloalkylglycine thiazolidides and pyrrolidides are potent DPP4 inhibitors. Compound **16** ($IC_{50} = 88$ nM) demonstrated good PK in both the rat and dog with bioavailabilities of 36% and 100%, respectively [62]. An extensive examination of the SAR surrounding cyclopentyl and cyclohexylglycine derived pyrrolidides and thiazolidides has been reported. The cyclopentylglycine derivatives were found to be more potent than their cyclohexyl counterparts. While the thiazolidides provided greater potency, these compounds suffered from reduced metabolic stability. Compound **17** ($IC_{50} = 13$ nM) was found to be a potent inhibitor selective for DPP4 over QPP and PEP [63]. In a series of mono or disubstituted pyrrolidides with fluorine at the 3 and 4 positions, the monofluorinated compounds were more potent than the difluoro analogs. Compound **18** ($IC_{50} = 48$ nM) was bioavailable in rat and dog and gave a 42% reduction in glucose excursion following an OGTT in lean mice [64]. This compound undergoes metabolic activation and subsequent conjugation with biological nucleophiles. This is believed to occur through oxidation and defluorination events, which produce an enal that acts as a Michael acceptor [65]. Compounds **19** ($IC_{50} = 6$ nM) and **20** ($IC_{50} = 6$ nM) are potent inhibitors of DPP4 that also incorporate the monofluorinated pyrrolidine [66].



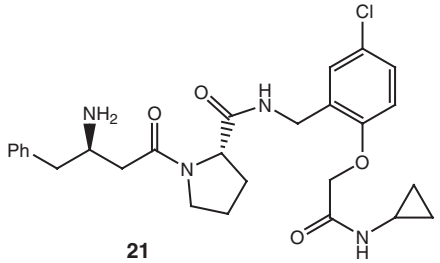
16 17 18



19 20

Starting from high-throughput screening (HTS) hit **21** ($IC_{50} = 1.9\,\mu$M), a series of β-homophenylalanine thiazolidides was developed [67]. Substitution of fluorine at the

2-position of the phenyl ring was found to provide an approximately 3-fold improvement in potency. The most potent compound reported in this series was **22** ($IC_{50} = 119$ nM). This work was extended to a series of proline and thiazolidine amides such as **23** ($IC_{50} = 0.48$ nM). While very potent, these analogs demonstrate poor PK properties [68]. Investigation of the SAR in a series of related piperazines represented by **24** ($IC_{50} = 19$ nM) revealed that the R-benzyl group was important for potency [69]. These analogs also suffer from short metabolic half-lives due to oxidation of the piperazine ring and poor pharmacokinetics. These liabilities were addressed through the discovery of MK-0431, which will be discussed in the section on clinical DPP4 inhibitors.



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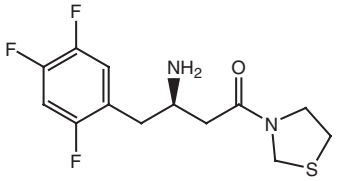

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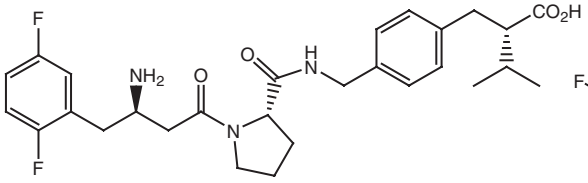

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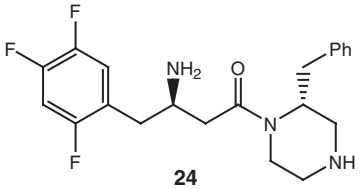
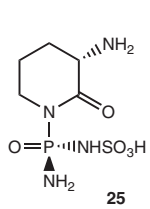


24

Sulphostin (**25**) is a natural product with an IC_{50} of 6.0 ng/mL, which corresponds to approximately 20 nM [70,71]. In an examination of the structure-activity relationships for analogs of sulphostin, it was found that the carbonyl and C-3 amino group of the parent structure were important for maintaining potency, as was the absolute configuration at phosphorus. Heterocycle ring sizes of 5–7 atoms were well tolerated. The sulfonic acid moiety could be removed while maintaining potency, but deletion of this group negatively impacts the stability of these analogs. Compound **26** is an 11 nM inhibitor of DPP4 [72].



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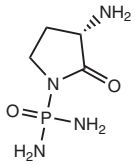

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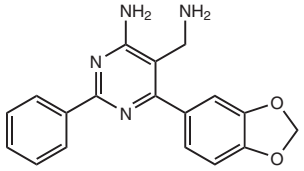

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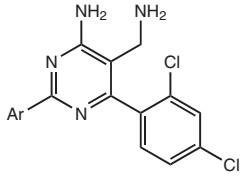


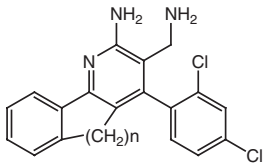
28: Ar = phenyl
29: Ar = 3,5-dimethoxyphenyl

Starting from HTS hit **27** ($IC_{50} = 10\ \mu M$), the potency in a series of aminomethylpyrimidines was improved 100,000 fold through modification of the two aryl

substituents [73]. *Ortho* and *para* substituents on the C-6 phenyl ring were tolerated, whereas *meta* substitution generally led to loss of potency. A breakthrough was realized when the 2,4-dichlorophenyl derivative **28** ($IC_{50} = 10\,nM$) was prepared. Optimization of **28** through modification of the C-2 phenyl group led to compound **29**, reported to be a 100-picomolar inhibitor. An X-ray crystal structure of **29** in DPP4 reveals that the dichlorophenyl group effectively fills S1, the pyrimidine ring forms a cation-π interaction with Arg125, the aminomethyl group interacts with Tyr662 and the two active-site glutamates, and the anilino nitrogen forms an additional H-bond with the backbone carbonyl of Glu205.

Analogs of these compounds where the pyrimidine is replaced with a pyridine (e.g. **30**) were explored. It was found that potency is improved by reducing the torsion angle between the phenyl ring and the pyridine core. Optimization in this series led to compound **34** [74].

Cmpd	n	Torsion (calc.)	IC_{50} (μM)
30	0		0.92
31	3	32°	0.24
32	2	22°	0.045
33	1	0°	0.039
34			0.007



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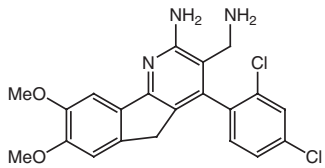

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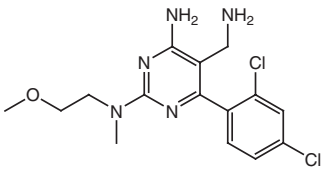


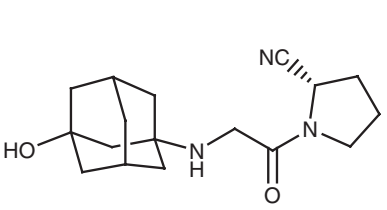
35

Compound **28** is an inhibitor of CYP450 3A4 with an IC_{50} of 5.4 μM. This compound also caused phospholipidosis in cultured fibroblasts. It was anticipated that by reducing the lipophilicity of these compounds, improved properties would be realized. While replacing the 2-phenyl group with small groups such as Me, OMe and NH_2 led to significant decreases in potency, groups such as 4-thiomorpholinyl and N-hydroxyethyl, N-methylamino were well tolerated. Compound **35** has an IC_{50} of 9 nM versus DPP4, does not induce phospholipidosis and has an IC_{50} of 30 μM versus CYP450 3A4 [75].

3. DPP4 INHIBITORS IN CLINICAL DEVELOPMENT

Small-molecule DPP4 inhibitors have advanced into clinical trials. First-generation inhibitors P32/98 (**2**) [76] and NVP-DPP728 (**6**) [77] were important tools to validate the concept that DPP4 inhibition is an effective method to improve glucose control in diabetic patients through an increase in active GLP-1 levels. In a 4-week study evaluating ninety-three type 2 diabetic patients, NVP-DPP728 (**6**), at doses of 100 mg three times daily and 150 mg twice daily, demonstrated meaningful reductions of plasma glucose, insulin, and the glycohemoglobin, HbA_{1c} [78].

The early agents, however, have been replaced by a second generation of DPP4 inhibitors, which have improved potency, selectivity, and pharmacokinetics over their pioneering predecessors. These include LAF237 (**36**, vildagliptin), MK-0431 (**37**, Sitagliptin), BMS-477118 (**38**, saxagliptin), and GSK23A (**39**). Other DPP4 inhibitors that are known to have entered clinical trials are P93/01 and SYR322, though the chemical structures of these compounds have not been disclosed [79,80].



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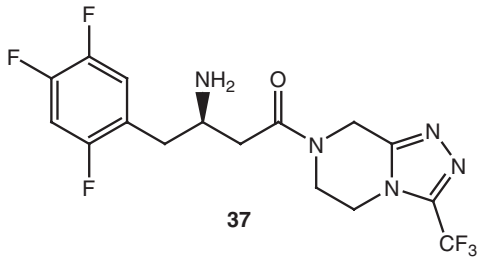

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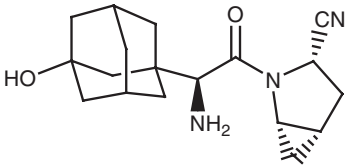

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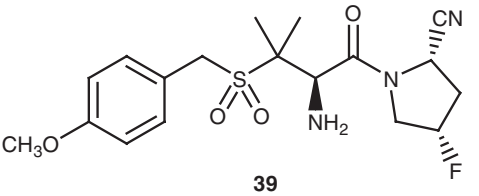
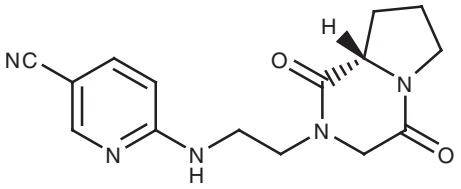


39

The chemical architecture of the DPP4 inhibitors that have been advanced into clinical trials is interesting both for their common structural features and those features that make them unique. A cyanopyrrolidine amide is a frequently repeating motif in small-molecule DPP4 inhibitors. In each of the compounds possessing this functionality, the cyano group undergoes nucleophilic addition by the catalytic serine, resulting in covalent modification of the DPP4 enzyme. Introductions to the pyrrolidine moiety of fluorine substitution (e.g., **39**) or a fused ring (e.g., **38**) are reported to provide improved *in vivo* and/or stability properties. An α-amino acid fragment is also common among DPP4 inhibitors, and it has been revealed from crystallography studies that the basic, protonated amino group interacts with a pair of Glu residues in the DPP4 active site. Like the modifications on the pyrrolidine ring, the installation of a quaternary center adjacent to this essential amino group serves the purpose of inhibiting the internal cyclization reaction, which gives rise to a bicyclic amidine that undergoes further hydrolysis to a diketopiperazine. In the case of NVP-DPP728, the observed by-product of this decomposition pathway is **40** [81].



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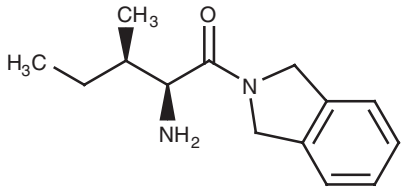
LAF237 (**36**) is a 4 nM inhibitor of DPP4 with > 10,000-fold selectivity over post-proline converting enzyme (PPCE) and DPP-II [81]. The identification of the 1-amino-3-hydroxyadamantane ring system was the result of careful and systematic SAR studies on the P-2 site, wherein a loss of enzymatic potency was observed with both carbamate and ester derivatives of the 3-hydroxy group. As expected, the steric encumbrance of the adamantane retards the rate of intramolecular cyclization by about 30-fold. Compound **36** demonstrated oral efficacy in a standard OGTT model using obese Zucker *fa/fa* rats.

In a 12-week, placebo-controlled phase II trial, **36** was effective at improving glycemic control when given as monotherapy to drug-naïve patients [82]. The mean change in HbA_{1c} was 0.6% from a baseline of 8.0. The most common adverse event was hypoglycaemia, which was observed in 7 patients (10%) and was considered mild.

In a double-blinded trial, 107 patients with type 2 diabetes being treated with metformin, patients were randomized to receive **36** or placebo [83]. After 12 weeks of combination treatment, patients completing the 12-week therapy study were eligible to extend treatment to 52 weeks. In the 56 patients randomized to **36** (50 mg once daily), HbA_{1c} levels after 12 weeks were reduced by 0.6% from an average baseline of 7.7%. In contrast, HbA_{1c} levels were unchanged in patients ($n = 107$) receiving placebo. In the 42 patients that progressed to the extended study, HbA_{1c} levels in the **36**-treated groups were unchanged from 12 weeks to 52 weeks. Patients in the metformin-plus-placebo group ($n = 29$) showed a gradual increase in HbA_{1c} over the 40-week extension, resulting in a between-group average difference of –1.1% HbA_{1c} levels. These 52-week data on **36** in combination with metformin provide compelling evidence that DPP4 inhibition represents a robust method for longer-term glycemic control.

The discovery of MK-0431 (**37**, Sitagliptin), and the incorporation of a β-amino acid moiety, represented a notable departure from the characteristic α-amino acid fragment featured in most reported DPP4 inhibitors [84]. Indeed, X-ray evidence suggests a binding orientation of the amide carbonyl that is opposite to its α-amino acid progenitors, with the β-amino group retaining the predicted interactions to the Glu 205/206 pair. Compound **37** is a non-covalent, 18 nM inhibitor of DPP4 that possesses excellent selectivity (2000 to 5000-fold) over related peptidases DPP2, DPP8, and DPP9. The desired animal pharmacokinetics of **37** came from modifications of piperazine amides such as **24**, which undergo extensive metabolism yet are also potent DPP4 inhibitors [69]. Interestingly, and perhaps generally pertinent to the development of safe and effective DPP4 inhibitors for chronic administration, DPP8 and DPP9 were specifically highlighted for cross-reactivity as it has been reported that the DPP8/9 selective inhibitor **41** is associated with "multi-organ pathology and mortality" when administered to rats for 2 weeks at a dose of 100 mg/kg/day. Moreover, these effects were observed in both wild-type and DPP4-deficient mice, suggesting that the toxicities were independent of DPP4 inhibition [85]. The preclinical oral efficacy of **37** was demonstrated in an OGTT model using C57BL/6N male mice.



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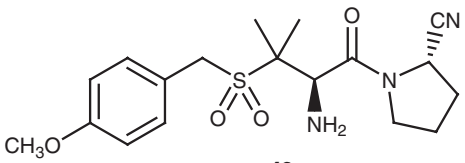
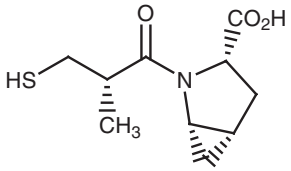
A randomized, placebo-controlled OGTT in fifty-six type 2 diabetics was conducted to assess the glucose-lowering activity and safety/tolerability of **37** following a single oral dose of 25- or 200-mg. As compared to placebo, incremental glucose AUC was reduced by approximately 22% and 26%, for the 25- and 200-mg doses respectively [86]. Dose-responsive plasma increases in insulin, c-peptide, and GLP-1, and reductions in glucagon were also noted, leading to the conclusion that pharmacologic proof-of-concept had been achieved.

GSK23A (**39**) is a penicillinamine-based inhibitor of DPP4 with a K_i value of 53 nM [87]. The compound contains a 4-fluoro substituent on the cyanopyrrolidine ring, which confers unique biochemical and physical properties versus its des-fluoro analog **42**. For example, **39** has a half-time to onset of DPP4 inhibition of 120 min, as measured in human plasma, compared to <20 min for compound **42**. In addition, **39** has a half-time for internal cyclization (37 °C, pH 7.2) of 1733 hr versus 360 hr for **42**. In a standard OGTT in *ob/ob* mice **39** showed an expected lowering of plasma glucose with an increase in both GLP-1 and insulin. By contrast, in the *db/db* mouse model, serum levels of glucose were unchanged following 8 weeks of treatment with **39**, presumably due to the severe insulin resistance of these animals.

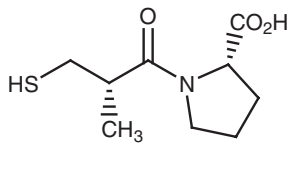


42

BMS-477118 (**38**) is a methanoproline-based DPP4 inhibitor [88,89]. The *cis*-fused, 4,5-methano bridge on the pyrrolidine ring appears to have been borrowed from the existing ACE inhibitor literature, wherein it has been shown that captopril analogs such as **43** having a fused cyclopropane ring retain the full ACE inhibitory potency of captopril (**44**) itself [90]. This modification is highlighted as a structural tool to enhance the chemical stability of the compound by retarding the rate of the internal cyclization reaction. BMS-477118 is a potent inhibitor of DPP4 with a reported IC_{50} value of 0.45 nM. It is reported to be selective over the related peptidases DPP2, DPP8, DPP9, and fibroblast-activating protein (FAP). In preclinical OGTT studies using both Zucker *fa/fa* rats and *ob/ob* mice, BMS-477118 displays the phenotypic profiles characteristics of DPP4 inhibition including transient lowering of glucose with concomitant increases in plasma insulin.



43



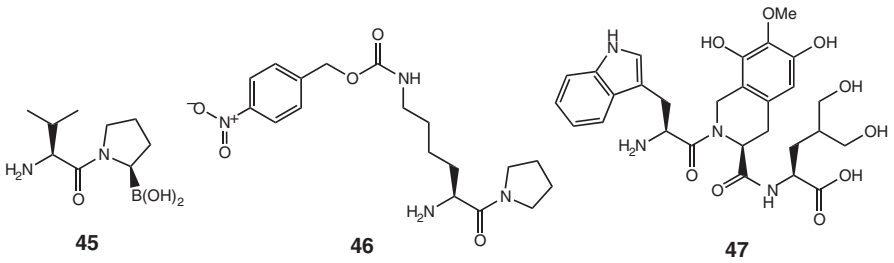
44

It is widely accepted that the level of glycohemoglobin, HbA_{1c}, is the best measure of long-term glycemic control and should be a primary endpoint for assessing the effectiveness of diabetes therapy [91]. A key topic that remains unanswered is the relationship between *in vivo* DPP4 inhibition over time and its effects on both HbA_{1c} and safety, though the early reports are highly encouraging that sustained DPP4 inhibition is both efficacious and well tolerated. Based on the large volume of primary journal literature, patent literature, and reports from scientific meetings, it is fair to speculate that there are a number of other small-molecule DPP4 inhibitors that are undergoing preclinical and clinical evaluation.

4. ALTERNATIVE INDICATIONS FOR DPP4 INHIBITORS

Numerous studies suggest DPP4 inhibition for pharmacological uses other than the restoration of glycemic control and type 2 diabetes. The common underlying hypotheses of these studies are divided into two categories that describe fundamental properties of DPP4. CD26, a membrane-associated peptidase that has DPP4 activity has been extensively studied in relation to its role in regulating T-cell physiology. As such, DPP4 activity has been studied in the context of T-cell activation and immune function [92]. Activated T-cells are known to have increased cell-surface expression of DPP4 [93]. Furthermore, cytokines such as RANTES, SDF-1α, MCP-2, and TNF-α have all been characterized as substrates for DPP4, so it is a reasonable hypothesis that DPP4 plays an immunomodulatory role [94]. However, recent studies have raised questions on the dependence of DPP4's proteolytic activity to T-cell activation and other functions such as proliferation and cytokine release. In a study using compounds with varying selectivity profiles for DPP4 and related peptidases, such as QPP, DPP8, and DPP9, it was found that both a DPP4 and a QPP inhibitor had no effects in *in vitro* assays measuring the immune responses of T-cell proliferation and IL-2 release [95]. By contrast, less selective compounds, such as Val-boro-Pro (**45**) and Lys[Z(NO_2)]-pyrrolidide (**46**), which also show inhibitory activity against DPP8 and DPP9, were effective in these assays. The authors conclude that the T-cell-mediated effects previously assigned to the inhibition of DPP4 might actually be a consequence of DPP8 and/or DPP9 activity. In addition, the uncompetitive DPP4 inhibitor, TMC-2A (**47**), [96] has been shown to suppress paw swelling in a rat adjuvant model for arthritis [97]. The authors of this study also note that DPP4 inhibition, *per se*, does not affect T-cell function, and that mice with mutated DPP4 lacking enzymatic activity still show a normal immune response. It is suggested that the binding of TMC-2A (**47**) may

affect the function of other proteins that associate with CD26, specifically the PTPase activity of CD45 [98]. Compound **45** is also known as talabostat or PT-100 and is in clinical trials for hematological malignancies and hematopoiesis [99]. The efficacy of **45** in these therapeutic indications may be derived from the compound's inhibition of FAP [100]. Numerous other reports have focused on the involvement of DPP4 and/or use of DPP4 inhibitors in various inflammatory and autoimmune diseases and pathologies, such as Crohn's disease, [92] and organ transplantation [101].



In addition to its identity with the membrane-associated protein CD26, DPP4 is ubiquitously distributed, and many important biomolecules other than GLP-1, including hormones, neuropeptides, chemokines, and cytokines, have been characterized as DPP4 substrates [3]. Among these substrates for DPP4, the peptide hormone GLP-2 has received considerable attention recently for its activity as an intestinal growth factor [102]. This activity has led to the hypothesis that a DPP4 inhibitor could show intestinotrophic effects that may be useful in the treatment of inflammatory bowel disease (IBD). Indeed GLP-2 itself has shown efficacy in a rodent model of IBD [103].

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Recent Advances in Therapeutic Approaches toType 2 Diabetes

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Contents

1. INTRODUCTION

Type 2 diabetes (T2D) affects an increasing proportion of populations of both the developed and developing parts of the world. According to the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) 17 million Americans – 6.2% of the U.S. population – have diabetes, and more than one third of these are undiagnosed. Another 16 million have insulin resistance or pre-diabetes. Worldwide figures are even more staggering: in 2000 the World Health Organization (WHO) reported a worldwide incidence of 154.4 million diabetes patients. Hence, intense efforts towards the discovery and development of more efficacious and safer diabetes therapies are underway in academic and industrial research organizations.

Since the appearance of the last review of diabetes in Annual Reports in Medicinal Chemistry in 2000, sales of troglitazone, the first peroxisome proliferator of activated

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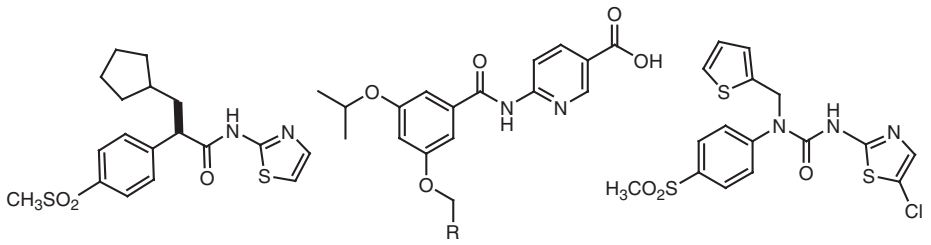
receptor gamma (PPAR-γ) agonist on the market, were halted due to hepatotoxicity in a small number of patients. Two more potent new PPAR-γ agonists, rosiglitazone and pioglitazone were introduced and appear to be free of the hepatic liability associated with troglitazone. One other new molecular entity (NME) is Starlix, an ATP-sensitive pancreatic potassium channel inhibitor. However, there have been many significant developments in the discovery and development of novel molecular entities that are in various phases of clinical and preclinical development. These recent developments (post 2000) discussed in this chapter, can be broadly classified into 1) enhancers of insulin release, 2) enhancers of insulin action, 3) inhibitors of hepatic glucose production, 4) inhibitors of glucose absorption from the gut.

2. ENHANCERS OF INSULIN RELEASE

2.1. Glucokinase activators

Glucokinase (GK) or hexokinase IV is one of the four hexokinases that phosphorylate glucose and plays a key role in whole-body glucose homeostasis through its action in β-cells and hepatocytes. The rationale for GK activators was derived from the study of GK mutations as manifested in maturity onset of diabetes of the young Type II (MODY II) and persistent hyperinsulinemic hypoglycemia of infancy (PHHI) in humans and associated gene manipulation studies in mice. Recently, a potent GK activator RO0281675 **1**, which increased the enzymatic activity of recombinant human GK in a dose-dependent and stereospecific manner [1], was identified. At a concentration of 3 μM, **1** increased the V_{max} of GK by a factor of about 1.5 and decreased the substrate concentration at 0.5 ([S]0.5) for glucose from 8.6 mM to 2.0 mM. In numerous *in vivo* studies GK activators were shown to cause glucose-dependent insulin release in the pancreas, and also to increase glucose utilization in the liver. Since this initial report, additional examples of GK activators, **2** and **3** [2–4] were disclosed.



1

2 a R = isopropyl
2 b R = -CH_2-3-thiophene

3

2.2. Potassium channel openers

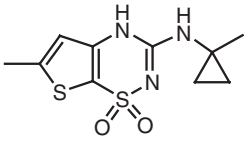
Unlike the conventional sulfonylureas, which stimulate insulin secretion by blocking ATP sensitive potassium channels, NN414 **4**, is postulated to selectively open the

pancreatic β-cell potassium channel, SUR1/Kir6.2 and consequently suppress overstimulation of insulin secretion resulting in an improvement in the insulin response to glucose challenge. In ZDF (*fa*/*fa*) rats, **4** was shown to reduce fasting blood glucose levels and improve glucose tolerance in a 21 day study at a dose of 1.5 mg/kg, bid dosing [5]. NN414, **4** was advanced to Phase II clinical trials, however, further development was halted due to a reported elavation of liver enzymes in treated patients.

2.3. GLP-1 agonists and dipeptidyl peptidase IV (DPPIV) inhibitors

Glucagon-like peptide 1 (GLP-1) is a 36 amino acid peptide secreted by the gut in response to nutrients that exert control over glucose levels by promoting insulin secretion, reducing glucagon levels, and slowing the rate of gastric emptying. GLP-1 is rapidly degraded by the endopeptidase dipeptidyl peptidase IV and the neuroendopeptidase NEP24.11 and thus has a short half-life. Approaches that are underway to potentiate GLP-1 activities include the preparation of stable GLP-1 analogs, and use of inhibitors of DPPIV, which slow degradation of the active form of GLP-1 and prevent the formation of the GLP-1 antagonist GLP1 [9–36].

Exenatide **5**, a GLP-1 analog, exhibits several antidiabetic actions and is being developed as an injectable therapy. In Phase II clinical trials, exenatide **5** caused statistically significant reductions in post-prandial glucose and glucagon concentrations and reductions in the rate of gastric emptying [6]. These results, plus data from two pivotal studies of **5** in combination with sulfonyl ureas and metformin, formed the basis of an NDA submission to the FDA, which was approved in April 2005. Three additional stable and potent GLP-1 analogs, liragutide, (NN-2211, Novo Nordisk) [7], BIM-51077, (Beaufour-Ipsen), and CJC-1131 [8], (ConjuChem) are in phase II or III clinical testing. The latter compound consists of a GLP-1 analogue coupled to a reactive malimide through a linker designed to covalently bind plasma albumin and has a circulating life of 2 weeks.



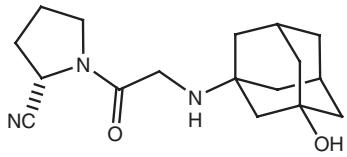
4

H-His-Gly-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Leu-Ser-Lys-Gln$^-$
Met-Glu-Glu-Glu-Ala-Val-Arg-Leu-Phe-Ile-Glu-Trp-Leu-Lys$^-$
Asn-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser-NH_2

5

Inhibitors of DPPIV are under investigation as orally active mediators of GLP-1 levels. NVP-LAF237, **6** a potent DPPIV inhibitor, was shown to increase active GLP-1 levels, and improve glucose tolerance in rodents. Chronic treatment with **6** had no effect on weight gain in mice and rats and delayed gastric emptying in cynomolgus monkeys [9]. In humans, **6** improved hyperglycemia in T2D patients at 100 mg TID, in a 4 week study. The issues that remain to be addressed include breadth and specificity of action of **6**, the durability of its effect and effects in combination with other drugs. Another DPPIV inhibitor that reached phase III clinical testing, MK431, **7** ($IC_{50} = 18$ nM), has excellent selectivity over other

proline-selective peptidases, oral bioavailability in preclinical species, and *in vivo* efficacy in animal models [10]. There are a number of other DPPIV inhibitors in clinical development.



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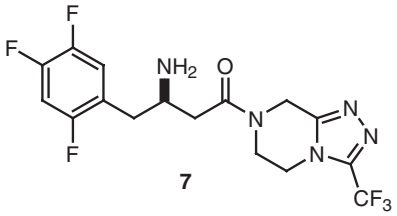

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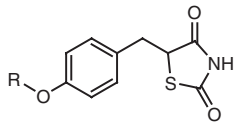
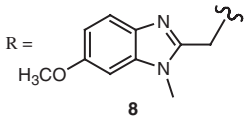
3. ENHANCERS OF INSULIN ACTION

3.1. Ligands for peroxisome-proliferator activated receptors (PPARs)

Investigation of the family of nuclear receptors PPAR- α, β, and γ remains a highly active area of research in the diabetes field and was recently reviewed [11]. Findings that several PPAR agonists cause cancer in mice and rats, prompted a recently imposed FDA requirement for two-year carcinogenicity evaluation of all PPAR modulators prior to dosing in patients longer than six months [12]. This is expected to delay the clinical study of several compounds. Most advanced are the PPAR-γ agonists balaglitazone **8** (Dr. Reddy's Laboratories/ Novo Nordisk, Phase II) rivoglitazone **9** (Sankyo, Phase II), FK614 (Fujisawa, Phase II), and R483 (Roche/Chugai, Phase II), according to company press releases or company website information.

PPAR-γ agonists promote adipocyte differentiation and consequently cause weight gain. In attempts to minimize this side effect, there is interest in developing dual acting PPAR- α and -γ co-activators which are expected to simultaneously promote fatty acid oxidation and improvements in insulin sensitivity. A leading entry was MK-0767 **10** (Merck, Phase III) until its suspension from development due to the formation of a rare hepatic tumor during long term safety studies. In April 2004, BMS and Merck entered into a collaborative agreement to develop muraglitazar **11** [13] and NDA approval is anticipated in mid 2005. Tesaglitazar **12** (Astra Zeneca) is in Phase III clinical development, and three other candidates with dual PPAR- α– and γ– co-activators are in Phase II clinical development [14].





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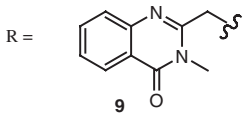

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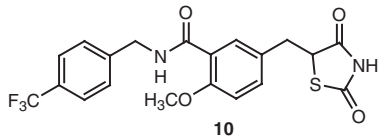

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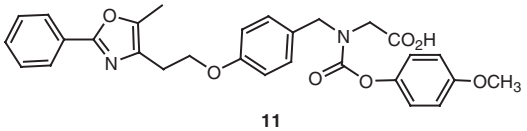

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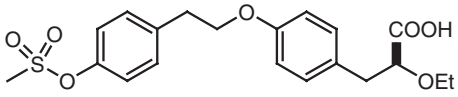
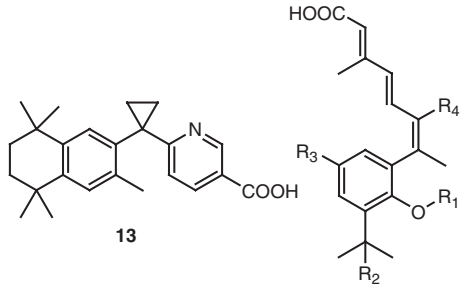


12

3.2. Retinoid X receptor (RXR) modulators

RXR is a nuclear receptor that plays a critical role in the activation of many genes by formation of functional heterodimers with other nuclear hormone receptors including the PPARs and LXR in the presence of small molecule ligands. The potent RXR modulator LG100268, **13**, activates the RXR:PPAR-γ heterodimer as efficiently as the PPAR-γ agonist (BRL-49653 - rosiglitazone) in *in vitro* assays. Compound **13**, was found to improve glucose tolerance in Zucker female *fa*/*fa* rats and also restrained weight gain in a 6 week study relative to the PPAR-γ agonist BRL-49653. However, **13** was found to raise triglyceride (TG) levels 2 h post-dose, and to lower TSH levels 24 h post-dose in Sprague Dawley rats following a single administration of 10 or 30 mg/kg, po. Because of this undesired activity, the team further optimized the molecule and identified, LG101506, **14**, a partial agonist that is selective for the RXR:PPAR-γ heterodimer. This compound did not increase TG levels and had no effect on TSH levels, while improving insulin sensitivity without weight gain [15]. However, **14**, had poor PK properties (low AUC, short Tmax, and low Cmax). Recently, compounds **15, 16,** and **17** with improved PK properties have been reported [16].



13

$R_1 = CH_2CHF_2$, $R_2 = CH_3$, R_3 = t-butyl, R_4 = H
14

$R_1 = CH_2CHF_2$, $R_2 = CH_3$, R_3 = t-butyl, R_4 = F
15

$R_1 = CH_2CHF_2$, R_2 = H, R_3 =2-fluorophenyl, R_4 = H
16

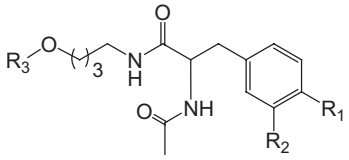
$R_1 = CH_2CH_3$, R_2 = H, $R_3 = CF_2CF_3$, R_4 = H
17

3.3. Protein tyrosine phosphatase 1B (PTP1B) inhibitors

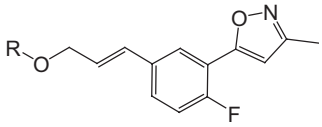
PTP1B negatively regulates insulin receptor (IR) and insulin receptor substrate-1 (IRS-1) phosphorylation. Mice that lack the PTP1B gene have increased insulin sensitivity with resistance to weight gain on a high-fat diet and are otherwise normal. This unique combination of desired attributes has driven an intense search for PTP1B inhibitors for treatment of both T2D and obesity. The discovery of effective inhibitors of PTP1B has proven challenging. This is due to both the selectivity requirements over other protein tyrosine phosphatases, particularly T-cell protein tyrosine phosphatase (TC-PTP) with which it shares high sequence homology near the catalytic site, and the need for potent antagonists to incorporate polar phosphate mimics, thus limiting cell penetration.

Abbott workers identified a peripheral site in the x-ray crystal structure of a PTP1B-inhibitor complex located near the catalytic site where the substrate phosphotyrosine residues bind. Using an SAR by NMR approach, they identified unique binders to each site. They then linked these fragments to obtain the potent inhibitor **18**, containing two free carboxylic acid groups. This compound has a K_i of 18 nM against PTP1B and 65 nM against TC-PTP. Further optimization to improve transport properties led to the discovery of the monocarboxylate **19**. Compound **19**, showed modest potency against PTP1B (PTP1B K_i 9 uM), good selectivity over TC-PTP (K_i 182 uM) and was active in a cell line in which PTP1B was over expressed [17,18]. Additional work led to the identification of **20**, that has improved cell permeability [19]. Other interesting antagonists include the deoxybenzoin bis-fluorophosphonate inhibitor, **21** (PTP1B $IC_{50} = 120$ nM) which was found to have 13% oral bioavailability in rats. In Zucker *fa/fa* rats, a single oral dose of 30 mg/kg caused a reduction of glucose AUC by 50% in an oral glucose tolerance test [20].

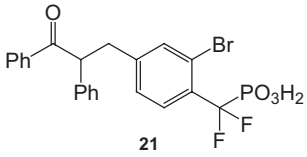
A novel approach has been reported that used a PTP1B anti-sense oligonucleotide (ASO) to target transcription of PTP1B mRNA. *In vivo* studies with the PTP1B ASO showed that a 25 mg/kg ip dose either once or twice per week in *ob/ob* and *db/db* mice normalized plasma glucose levels, postprandial glucose excursions and reduced HbA1c. Efficacy was observed despite the finding that PTP1B protein and mRNA were reduced in liver and fat, but not in skeletal muscle [21]. This PTP1B ASO entered Phase II in September, 2003 [22].



18 R_1 = N(COCOOH)(2-carboxy-phenyl), R_2 = CH_2CH_3
R_3 = (2-carbomethoxy-3-hydroxy)phenyl
19 R_1 = OCH_2COOH; R_2 = OH,
R_3 = (2-carbomethoxy-3-hydroxy)phenyl



20 R = (2-carbomethoxy-3-hydroxy)phenyl



21

4. INHIBITORS OF HEPATIC GLUCOSE PRODUCTION (HPG) [23]

4.1. Inhibitors of pyruvate dehydrogenase kinase (PDHK)

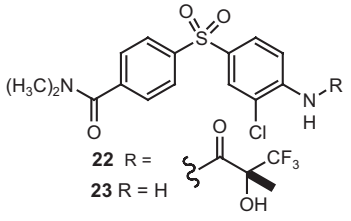
Increasing the activity of pyruvate dehydrogenase (PDH) by inhibiting PDHK, is expected to decrease blood glucose by increasing glucose oxidation in peripheral tissues and by decreasing the supply of the gluconeogenic precursors, lactate and alanine to the liver. Dichloroacetate (DCA), a known inhibitor of PDHK was shown to reduce plasma glucose levels both in animal models of diabetes and in patients. Administration of DCA for seven days to T2D patients decreased plasma glucose,

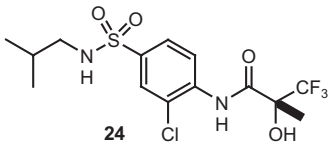
and caused marked decreases in lactate and alanine levels. However, DCA was not suitable as a therapeutic agent due to its low potency, lack of specificity, poor PK, and toxicity. AZD7545, **22** is a potent rat PDHK inhibitor ($IC_{50} = 0.021\,\mu M$) that increased PDH activity with an EC_{50} value of $0.105\,\mu M$ in rat hepatocytes. When given to male obese Zucker *fa/fa* rats, 10 mg/kg of **22**, given orally b.i.d. was also found to markedly improve the 24h glucose profile by eliminating postprandial elevations in glucose [24]. The development of **22** was stopped in view of the formation of the aniline metabolite, **23** and further work to identify compounds without this liability is in progress. Interestingly, Novartis researchers did not observe lowered glucose levels with their PDHK inhibitors, for example **24**, in rodent models of type 2 diabetes [25,26], but did observe significantly diminished blood lactate levels.

4.2. Liver-selective glucocorticoid receptor antagonists

The correlation between elevated hepatic glucose output and fasting hyperglycemia in type 2 diabetic patients is well established. Also, the link between elevated glucocorticoids (GCs) and glucose control suggested the desirability of exploring glucocorticoid receptor antagonism as a potential therapy for T2D. However, the critical role played by GCs in the hypothalamic pituitary axis (HPA) and potential toxicity due to systemic GC antagonism, suggests that liver-selective glucocorticoid receptor (GR) antagonists would be required to safely treat T2D patients.

To this end a novel strategy to design liver-selective GC receptor antagonists was employed in which a bile-acid conjugation (BCA) was prepared by linking RU-486, a potent GC antagonist ($IC_{50} = 1.1\,nM$) with cholic acid via a two carbon linker to give **25** (A-348441). Cholic acid is known to enter the liver and intestine via bile acid transporters and thus this approach could potentially minimize the systemic exposure to RU-486. The x-ray structure of GR ligand binding domain with RU-486 was used as a starting point for modeling studies in the design of **25**. This complex was found to retain potent GC antagonist activity ($IC_{50} = 9\,nM$), and blocked GR mediated gene expression in primary hepatocytes (IC_{50}'s: **25** - $0.12\,\mu M$; RU486 - $0.21\,\mu M$). The conjugate **25** was also evaluated in various rodent models of type 2 diabetes and found to have desirable effects on glucose homeostasis and dyslypidemia [27,22].





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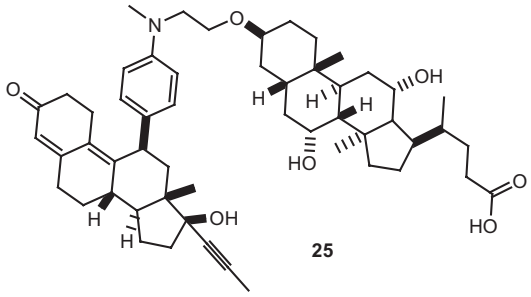
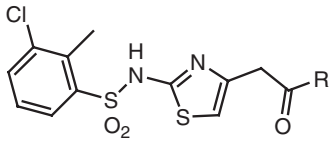


25

4.3. 11-β-hydroxysteroid dehydrogenase-1 (11-β-HSD-1) inhibitors

In humans, the circulating levels and activity of cortisol and cortisone are tightly regulated. The enzyme 11-β-HSD-1 catalyzes the conversion of cortisone to cortisol, using NADPH as co-factor, while the reverse reaction is catalyzed by 11-β-HSD-2. Cortisol is the ligand for glucocorticoid receptors and modulates numerous biological functions, including the HPA axis. Studies using transgenic mice lacking either 11-β-HSD-1 or 11-β-HSD-2 indicated the desirability of selective inhibition of 11-β-HSD-1 to reduce hepatic glucose production, and improve glucose homeostasis. Numerous steroid based inhibitors have been discovered including glycyrrhetinic acid and carbenoxolone [28,29]. Recently, 2-aminothiazole based rat- and human-selective 11-β-HSD-1 inhibitors, **26** and **27** respectively, were disclosed [30,31]. Compound **27**, was found to lower circulating glucose levels by 50–88% and insulin by 52–65% of control in *ob*/*ob* and KK-Ay mice after dosing at 200 mg/kg b.i.d for 4 days. BVT.3498 entered into Phase II clinical trials for T2D according to a press release in March, 2003, and is thought to belong to this class of 11-β-HSD-1 inhibitors. Since then, no updates have been reported.

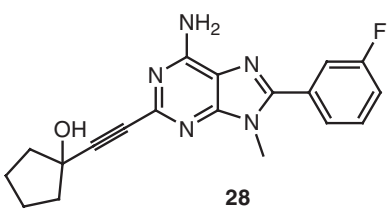


26 R = Diethylamino
27 R= 4-Methyl-1-piperazinyl

4.4. Adenosine A2B receptor antagonists

Adenosine is an autocoid produced in many tissues to mediate various functions through four receptor subtypes, A1, A2A, A2B and A3. Current literature reports suggest that adenosine A2B receptor antagonists would reduce hepatic glucose production and enhance glucose uptake in muscle. In human skeletal muscle cells, adenosine A2B and A2A but not A1 receptors were detected [32], while in rat skeletal muscle cells, A2A and A2B receptors but not A1 or A3 receptors were found [33]. Earlier reports ruled out a role for A2A receptors as modulators of muscle insulin sensitivity [34]. Using specific adenosine receptor agonists and antagonists, further evidence suggesting involvement of adenosine acting through A2B receptors in promoting hepatic glucose production has been provided. The potent A2B receptor antagonist **28** (A2B CHO-cAMP 100 nM) was effective in lowering glucose levels in KK-Ay mice, at a 10 mg/kg oral dose [35,36]. BWA1433, **29**, is a potent but non-selective A2B receptor antagonist that is efficacious in improving glucose clearance as measured through an ip glucose tolerance test (ipGTT) in Zucker *fa*/*fa* (obese phenotype) rats. In hyperinsulinemic euglycemic clamp studies, **29** increased whole body glucose uptake in obese Zucker *fa*/*fa* rats [37]. Based on muscle tissue A2B receptor distribution and the clamp study results,

the authors conclude the effects of **29** are primarily mediated through adenosine A2B receptor antagonism in muscle [38]. Thus, adenosine A2B receptor antagonists are potentially useful in treating T2D through their action in both liver and muscle.



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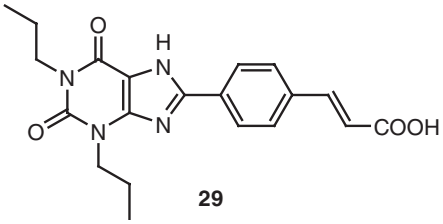

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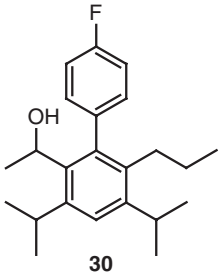
4.5. Glucagon receptor antagonists

Glucagon is a key hormone that acts as a counter regulator of the actions of insulin and as a consequence, it contributes to insulin resistance in T2D. Glucagon is secreted by α-cells of the pancreas and it promotes hyperglycemia by increasing glycogenolysis and gluconeogenesis in liver. In T2D patients, circulating glucagon levels are normal or slightly elevated suggesting that elevated fasting glucagon levels that fail to appropriately decrease postprandially, contribute to hyperglycemia. Mice lacking glucagon receptors were found to have normal glucose levels, and improved insulin sensitivity. Treatment of *ob/ob* mice or streptotozocin (STZ) induced diabetic rats with a glucagon monoclonal antibody (Glu-mAB) normalized or slightly lowered glucose levels. Recently, similar observations were made using a specific glucagon receptor antisense oligonucleotide (GR-ASO) [39]. In healthy humans, Bay 27-9955, **30** a small molecule competitive glucagon receptor antagonist with moderate potency ($IC_{50} = 110$ nM) demonstrated efficacy relative to placebo in glucagon challenge experiments [40,41]. These results fueled further interest in this target and interesting new orally bioavailable antagonists such as **31** are appearing [42].



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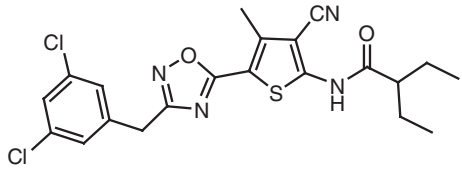

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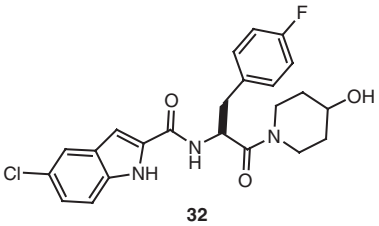
4.6. Glycogen phosphorylase inhibitors

Glycogen phosphorylase is a dimeric enzyme which plays a key role in the breakdown of glycogen to glucose-1-phosphate, and its activity is modulated by signals

that promote glycogen breakdown as well as its storage. The three isoforms of GP, brain, liver and muscle share about 80% homology. Inhibition of liver GP in T2D is considered to be desirable in view of its rate-limiting role in glycogenolysis and indirect inhibitory role in gluconeogenesis pathways. The activity of GP is known to be modulated by the affinity of ligands binding to six different binding sites, thus offering multiple opportunities for its modulation. The most interesting of these is the allosteric site which spans the GP dimer interface characterized by the identification of the inhibitor CP-320626, **32** [43]. Compound **32** was found to be efficacious at 10 mg/kg po dose in *ob*/*ob* mice [44]. A compound from this class has been studied clinically, and preliminary results have confirmed its glucose lowering potential [45]. Related compounds have recently been shown to reduce cholesterol in several species through inhibition of lanosterol demethylase [46]. Other inhibitors of GP include the allosteric inhibitor **33** [47] and the competitive inhibitor, **34** [48].

4.7. Glucose-6-phosphatase inhibitors

Glucose-6-phosphatase (G6Pase) catalyzes the terminal step in gluconeogenesis and glycogenolysis by converting glucose-6-phosphate to glucose and inorganic phosphate. G6 Pase is a multicomponent enzyme, located in the endoplasmic reticulum, and has a wide tissue distribution. In T2D animal models, the G6Pase activity, GTPase protein content and mRNA levels are elevated. The potential use of G6Pase inhibitors in diabetes treatment has been reviewed [49].



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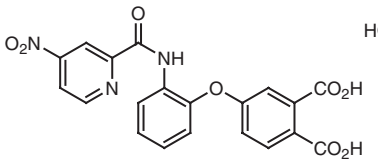

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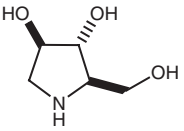

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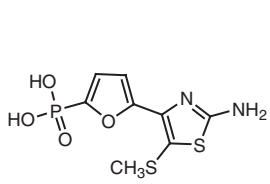
4.8. Fructose-1,6-bisphosphatase inhibitors

Fructose-1,6-bisphosphatase (FBPase) catalyzes the conversion of fructose-1,6-bisphosphate to fructose-6-phosphate (F6P) and inorganic phosphate. FBPase is allosterically regulated by AMP and indirectly by glucagon and insulin. FBPase is a homotetramer and exists in active (R) and less active (T) states. Aminothiazole phosphinic acid **35** ($IC_{50} = 15$ nM**)** and prodrug **36** (CS-917) represent the most potent inhibitors reported to date. The aminothiazole, **36** was shown reduce glucose levels relative to controls in *db*/*db* mice and Zucker diabetic fatty rats [50–52]. Thus, inhibitors of FBPase may provide therapeutic benefit for T2D patients, by lowering hepatic glucose production. Clinical trials of **36** were recently stopped due to

observations of lactic acidosis in two patients, according to a company's March, 2005, press release.

4.9. Glycogen synthase kinase-3 inhibitors

Glycogen synthase kinase (GSK-3) is a serine/threonine kinase that phosphorylates glycogen synthetase and inhibits its activity. Thus, inhibition of GSK3 is expected to activate glycogen synthase and promote glucose uptake into muscle. Human GSK-3 exists in two isoforms, α and β, encoded by two distinct genes, located on chromosomes 19 and 3 respectively. GSK-3 has wide tissue distribution and has multiple key biological functions. Although selective GSK-3 inhibitors with desired enzyme and tissue distribution may be beneficial in several indications, identification of sufficiently selective inhibitors has been challenging since most inhibitors are ATP site binders. Among the reported GSK-3 inhibitors, CHIR98014, **37** and CHIR98023, **38** increased glucose uptake in human skeletal muscle cell culture [53].



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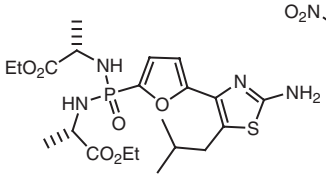

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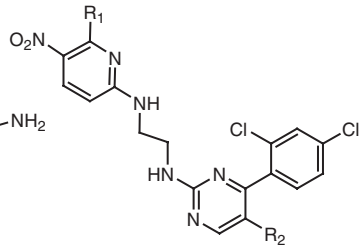


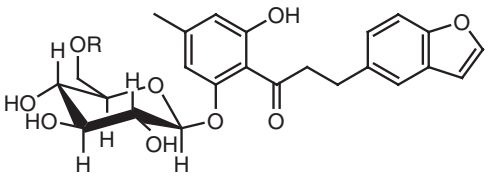
37 $R_1 = NH_2$, R_2 = N1-imidazole
38 $R_1 = H$, R_2 = 2-(1H)-imidazole

5. INHIBITORS OF GLUCOSE UPTAKE

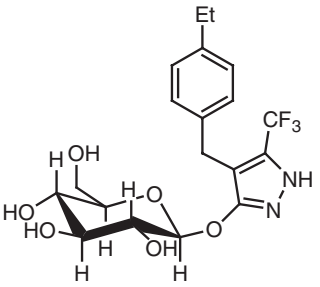
5.1. Sodium-glucose transporter (SGLT) inhibitors

Both intestinal absorption and renal re-absorption of glucose are mediated by SGLTs. Three isoforms, SGLT-1, SGLT-2 and SGLT-3, have been reported to date. Phlorizin, a specific inhibitor of SGLTs is the earliest of the reported inhibitors to show efficacy in *in vivo* models of T2D. Based on these observations, stable analogs of phlorizin, T-1095A, **39** and the pro-drug T-1095, **40**, were evaluated in various T2D animal models. These studies suggested that **39** inhibited renal SGLTs. Thus, at 100 mg/kg po, **40** effectively suppressed renal reabsorption of glucose resulting in increased glucose excretion in urine in rats and mice. The compound was found to improve glucose homeostasis in yellow KK-mice and STZ-induced diabetic rats [54]. Recently, **40** was reported to be in phase II clinical trials. Among others, novel pyrazole-O-glucosides were also found to be potent inhibitors of SGLTs *in vivo*, as measured by development of glucosuria. For example, **41**,

induced 63 mg of urinary glucose excretion, at a 3 mg/kg iv dose, while at the same dose T-1095A induced 300 mg of urinary glucose excretion, in Wistar rats [55].



39 R = H
40 R = $COOCH_3$



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6. SUMMARY AND OUTLOOK

T2D and associated morbidities are prevalent in an increasing proportion of populations of both the developed and the developing parts of the world. Major current therapies for T2D include sulfonylureas, metformin, and TZDs. Each of these therapies has limitations with regard to their efficacy or side-effect profile. Among the targets discussed in this chapter, the most advanced are those based on GLP-1 agonist activity, i.e., Exenatide, which recently received FDA approval and DPPIV inhibitors. Both strategies are directed to potentiate the actions of GLP-1 on insulin secretion and have shown promise in Phase II/III clinical trials. These agents may avoid complications related to hypoglycemia and also may limit the potential for weight gain, thus complementing existing therapies. Glucokinase activators offer a potential new avenue for glucose regulation through a dual effect of improved glucose utilization in the liver and glucose-dependent insulin secretion by the pancreas. They could offer an advantage over the current therapies where sulfonylureas and other insulin secretagogs are used. New dual activating PPAR agonists may also offer new therapies with decreased weight gain relative to the PPAR-γ agonists currently marketed. We await clinical results on several other approaches to managing glucose homeostasis with the knowledge that a number of promising new drugs have failed in late stage clinical trials.

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The TRPV1 Vanilloid Receptor: A Target for Therapeutic Intervention

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Contents

1. INTRODUCTION

Capsaicin (**1**), the active component of hot chili peppers, and related irritant compounds exert their pharmacological effect via activation of an excitatory ion channel expressed on nociceptors [1]. The cellular target for **1** was cloned and characterized from a cDNA from rat sensory neurons in 1997 and named the VR1 receptor [2]. Additional members of the family were subsequently cloned in several laboratories leading to multiple names for the same receptor. This prompted the adoption of the transient receptor potential (TRP) nomenclature whereby the VR1 receptor is now known as the TRPV1 receptor [3]. This family of receptors consist of a large class of ion channels characterized by their permeability to monovalent cations and calcium ions, exhibiting a common structure made up of subunits with six membrane spanning domains [4,5]. The cloning of the rat receptor was quickly followed by the cloning of the human isoform [6] together with the characterization of TRPV1 ''knockout'' mice by two groups [7,8]. Knockout studies demonstrated that the receptor plays a key role as an integrator of noxious and chemical stimuli that produce pain. The receptor may be activated by heat, low pH, additional vanilloids, including the ultrapotent daphnane diterpenoid resiniferatoxin (RTX) (**2**), and a range of endogenous mediators encompassing products of the lipoxygenase pathway, bradykinin, and the endocannabinoid anandamide [1,9,10]. Thus numerous activators, often associated with tissue injury or inflammation, appear to operate by reducing the heat threshold of the receptor.

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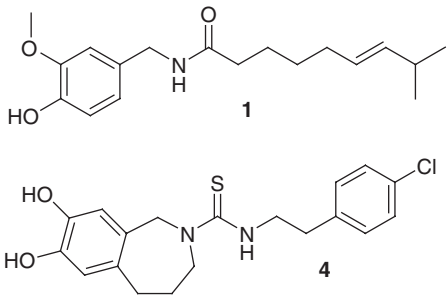
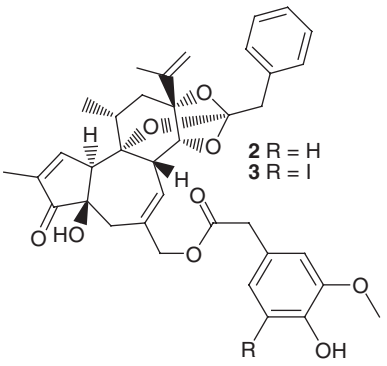
2. THERAPEUTIC POTENTIAL

Capsaicin and related agonists activate TRPV1 and are irritants upon application but ultimately lead to receptor desensitization and a concomitant reduction in sensitivity to painful stimuli. These agents have found therapeutic applications as topical analgesics (**1**) and for the treatment of urge incontinence (**2**) [11]. In contrast to the long history of TRPV1 agonists, the first small molecule antagonist, capsazepine (**4**) was only described in 1994 [12].





Although **4** behaves as a weak competitive antagonist that leads to anti-hyperalgesic effects against both capsaicin challenge and other inflammatory stimuli, it lacks selectivity for TRPV1 and also exhibits differential selectivity across species [13–15]. Studies using capsazepine for purposes other than agonist blockade must be interpreted with caution. In addition to its activity at TRPV1 (IC_{50} vs. capsaicin 100 nM: 74 nM at the rat receptor and 365 nM at the human [16]), capsazepine is a nonspecific blocker of voltage dependent calcium channels ($IC_{50} \sim 8\,\mu M$) [17], of nicotinic cholinergic receptors [18], and of I_h (IC_{50} 8 μM), the pacemaker current [19]. The synthesis of iodo-resiniferatoxin (**3**) [20] provided a more selective, high potency TRPV1 antagonist. This compound appears to lack agonist effects *in vitro*; however some stimulatory effects have been observed *in vivo*, which argue for caution when interpreting the apparent antagonist effects. Selective TRPV1 antagonists offer a potential treatment for a range of painful conditions without the initial irritation associated with TRPVI agonists. Following the cloning of the TRPV1 receptor the search for small molecule antagonists has attracted the attention of several pharmaceutical companies, which has in turn led to the identification of numerous structures that are now being evaluated to determine their broader therapeutic utility [9,21,22]. A number of excellent reviews have been published in this area covering work done prior to 2004, consequently this chapter will primarily focus on work published in the last year [9,15,21].

3. EVALUATION OF TRPV1 ANTAGONISTS IN ANIMAL MODELS

3.1. Phenotype of the knockout

The phenotype of the knockout mouse provides some specific guidance as to the potential therapeutic applications of a TRPV1 antagonist, and has been independently characterized by two research groups [7,8]. As expected, TRPV1 knockout mice have no nocifensive or hypothermic responses to capsaicin. The major consensus finding is that absence of the TRPV1 channel creates significant deficits in inflammatory (carrageenan or complete Freund's adjuvant (CFA)) or chemically evoked (mustard oil) thermal hyperalgesia, without alteration in the extent of inflammatory edema (carrageenan model). These deficits do not extend to nerve-ligation induced thermal hyperalgesia. Findings with regard to acute thermal sensory deficits are somewhat contradictory in the two reports. Caterina *et al.* described reduced pain responses above 48–50 °C, whereas Davis *et al.* did not observe acute thermosensory deficits up to 52.5 °C. These observations are noteworthy, given the considerably lower thermal activation threshold of TRPV1 *in vitro*, ~43 °C, and point to redundancy in thermosensory mechanisms. Responses to acute mechanical stimuli, such as tail pinch, are no different in the knockout. Spontaneous pain behaviors to noxious chemical stimulation are unaltered in the paw formalin test. Tactile allodynia of any etiology, other than capsaicin application, is not altered. No published reports address paw pressure responses after inflammation or nerve ligation, arthritis pain, or acetic acid-evoked writhing responses in the knockout.

Further studies of the knockout [23] have focused on the role of TRPV1 in normal urinary bladder function. TRPV1 appears to be indirectly involved in transduction of bladder stretch. TRPV1-/- mice have increased bladder capacity, and deficient reflex voiding responses to bladder distension under anesthesia. While the TRPV1 receptor itself is not activated by cell membrane distortion, TRPV1-/- mouse bladder mucosa releases less ATP when stimulated *in vitro* by pressure or hypotonicity, suggesting that activation of TRPV1 is required for stretch-evoked ATP release from urothelial cells.

3.2. Pharmacology of antagonists in *in vivo* models

The therapeutic effects that have been described in the literature include the exploration of analgesia (antihyperalgesia), cough suppression, and treatment of inflammation.

3.2.1. Hypothermia

Core body temperature and metabolic responses to systemic capsaicin are subtle in humans [24]. In contrast, in rodents, the systemic administration of capsaicin causes

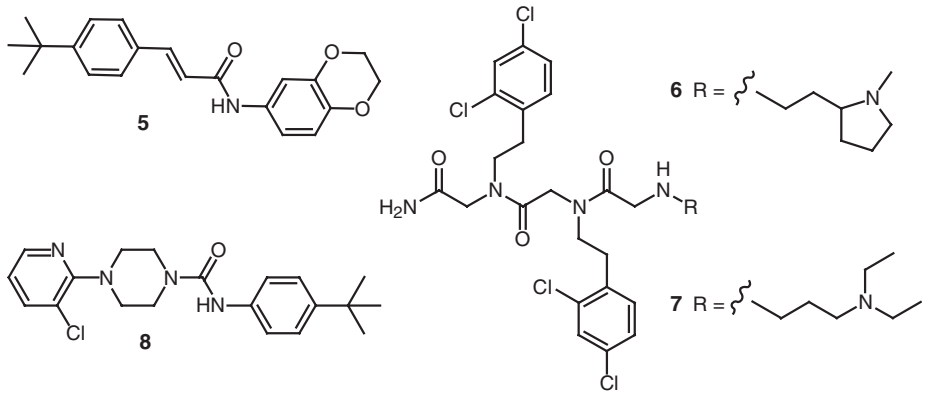
a drop in core body temperature of several degrees, accompanied by peripheral vasodilatation [25]. Pretreatment with a TRPV1 antagonist blocks this response, and provides a simple assay for the efficacy of a test compound [16,26].

3.2.2. Pain

Agonist Effects: Animals receiving a topical ocular application of capsaicin solution wipe the affected eye vigorously [27]. The ability of systemic dosing with an antagonist to reduce this behavior provides a convincing demonstration of occupancy of TRPV1 receptors at the tissue level. AMG 9810 (**5**) reduced but did not entirely abolish eye wipes in a dose dependent fashion. Injection of capsaicin into a hindpaw provokes several phases of behavior: an initial period of paw flinching [28], followed by a brief period of thermal sensitivity, followed by tactile allodynia and thermal desensitization. Alternative vanilloid agonists, such as N-arachidonoyl dopamine, provide a longer period of thermal hyperalgesia without desensitization [16]. Intrathecal capsaicin injection provokes intense agitation which can be prevented by systemic dosing with antagonist [20]. While the poor solubility of capsaicin in CNS-compatible vehicles is a methodological problem, this assay offers a direct means of evaluating the CNS penetration of a test compound.

Acid-evoked Responses: In the rodent acetic acid writhing (abdominal constriction) test, stereotyped behaviors after i.p. injection of acetic acid solution [29,30] are dose dependently reduced by a variety of known analgesics. Capsazepine, iodoresiniferatoxin and a number of more recent compounds have shown efficacy to reduce these behaviors after systemic administration (~85%) [31–33]. Blockade of similarly evoked phenylbenzoquinone responses appears equivocal [33,34].

Acute Thermal Pain: Hot Plate, Tail Flick. A study of two TRPV1 antagonist peptoids, DD161515 (**6**) and DD191515 (**7**) [28] demonstrated prolonged hot plate escape latencies at 52 °C. Other TRPV1 antagonists have not shown promising efficacy in acute thermal pain [35].



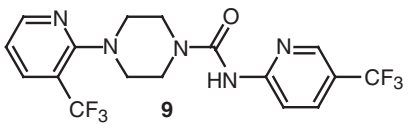
Nerve Injury Models: Upregulation of TRPV1 message or protein has been described in nerve injury models [36–38]. While an electrophysiological study suggests

that nociceptive pathways are less damped by **3** in nerve injury studies compared to controls [39], one group has reported positive findings with a TRPV1 antagonist in nerve-injury evoked pain modalities. Using the partial sciatic nerve injury model [40], both **3** and BCTC (**8**) showed effects on paw withdrawal thresholds to pressure in the range of 50–100 grams. Whereas **8** was effective in all species studied (rat, guinea pig, mouse), **4** was effective only in the guinea pig [41]. Furthermore, **8** partially reversed tactile allodynia in this model in the rat, a finding that is unexpected based on the phenotype of the knockout [42].

Inflammatory Pain Models: Upregulation of TRPV1 has been described in a number of clinical and experimental inflammatory states [43–46], and nocifensive reponses to capsaicin are augmented in inflammatory states [44,47–51]. Consistent with expectations based on the knockout, hyperalgesic thermal thresholds after mustard oil-evoked irritation were normalized by treatment with **6** and **7**, though allodynic responses were unchanged [28]. Carrageenan-induced inflammatory thermal hyperalgesia was also reduced by **4** [49,52], and in electrophysiological studies in this model, exaggerated spinal cord dorsal horn neuron responses were reduced by **4** as well [52]. An early report, however, found no effect of **4** in a kneejoint arthritis model [35].

Species differences have been reported in the efficacy of **4** in paw inflammation models, with positive effects in the guinea pig, but not in the rat [42] (but see [49,52]). In contrast, **8** was highly effective in the rat [41]. This group suggested that the significant activity of **8,** compared to **4,** against heat and low pH stimuli at the rat receptor *in vitro,* is relevant to its improved rat *in vivo* efficacy [53] (but see [54]). A more recently described compound, **5** is also reported to block low pH stimuli at the rat receptor, albeit less potently than **8**. This compound fully reversed thermal hyperalgesia due to CFA in the rat, while hyperalgesic paw pressure thresholds were partly normalized [27].

Bone Pain: In a malignant tumor model of bone destruction pain in the mouse, the selective TRPV1 antagonist (**9**) reduced both movement-related and spontaneous pain behaviors, without affecting disease progression. Mice lacking the TRPV1 gene had reduced movement-related and ongoing pain responses that were comparable to those of wild-type mice treated with the compound [55].



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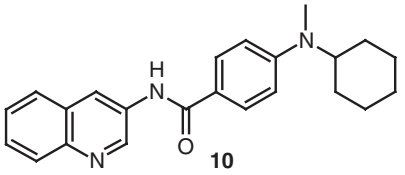

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3.2.3. Cough

Increased expression of TRPV1 has been recently shown in the airways of patients with chronic cough of multiple etiologies [44]. A functional role in airway irritability appears likely, since the presence of chronic cough was associated with ~4x greater

bronchial mucosal TRPV1 expression, and ~30-fold greater sensitivity to cough provoked by inhaled capsaicin. Both **4** and iodo-resiniferatoxin prevent capsaicin- or citric acid-evoked coughing in normal guinea pigs [44,57] in a dose-dependent manner. The prevention has pharmacological specificity; neither compound prevents hypertonic saline-evoked cough [56,57].

3.2.4. Disease modification

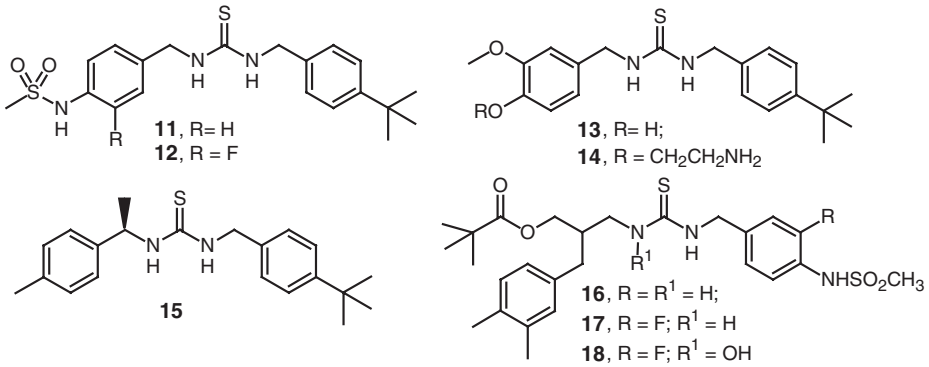
Two antagonists, capsazepine and (**10**), were studied for their ability to reduce inflammation related changes in DSS induced colitis. Positive signs of disease modification for both compounds were improved colon weight, reduction in diarrhea, and reduction in histological inflammation. No indices of visceral pain were studied [43].

4. MEDICINAL CHEMISTRY

Over 60 patents and papers describing new TRPV1 antagonists were published in 2004. Recent patent activity clearly indicates that many pharmaceutical companies have drug discovery efforts in the TRPV1 antagonists area. In the following section, unless otherwise noted, the reported activities represent the inhibition of capsaicin induced calcium flux in HEK293 or CHO cell lines over-expressing human or rat TRPV1.

4.1. Capsazepine based antagonists

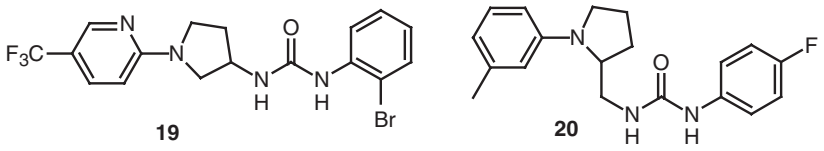
Capsazepine was the first reported small molecule inhibitor of TRPV1, although at the time of its discovery the channel had not been fully characterized [58]. As a result, there have been a number of compounds reported which are structurally related to capsazapine. Extensive SAR studies on this type of antagonist have been carried out over the last two years. MK056 (**11**) ($K_i = 110$ nM) and SC0030 (**12**) ($K_i = 37$ nM) were early examples of conformationally flexible capsazepine based antagonists, as well as the partial agonist **13**, and the closely related agonist SDZ249482 (**14**). The latter is currently in clinical development as a topical analgesic [59–62]. SC0030 was recently reported to be effective in 2-phenyl-1,4-benzoquinone (PBQ) induced writhing in mice ($ED_{50} = 0.1$ mpk i.p.) [34]. Previous SAR studies suggested that introduction of the sulfonamide was critical for achieving functional antagonism in thioureas of this type (many of which exhibit functional agonism). The recently reported α-methylbenzyl compound, **15** ($K_i = 400$ nM), was shown to be a full antagonist in rat dorsal root ganglia (DRG) in the absence of a sulfonamide group. Thus conformational considerations may also play a role in both the affinity and function of these ligands.



Branched thioureas have also been reported by the same research group with early studies identifying compounds **16** ($IC_{50} = 67\,nM$) and **17** ($IC_{50} = 8\,nM$) as potent antagonists of TRPV1 with excellent efficacy in the acetic acid writhing model in mice [29]. Interestingly, hydroxythiourea analogs, including **18** ($K_i = 94\,nM$) were reported to have 100 fold greater efficacy in the same model [30]. Several additional reports describing SAR and modeling studies of this class of inhibitors have also appeared [62–65]. In these studies, antagonist potency was highly dependent on the chain lengths of the methylene linkers.

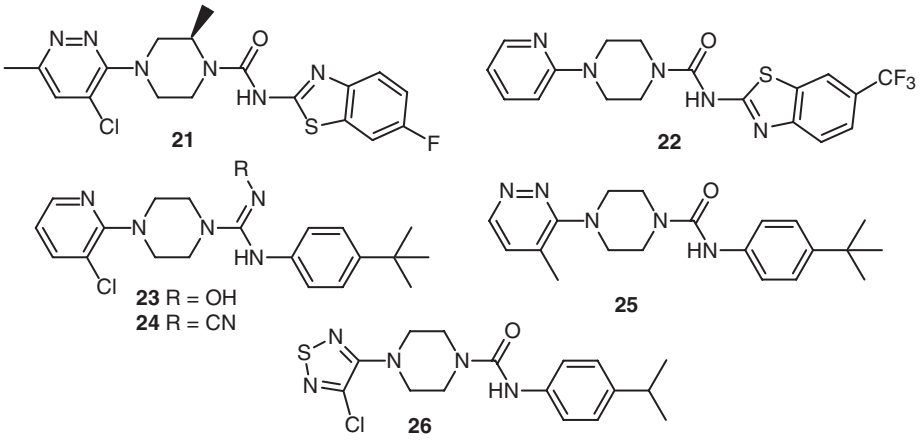
4.2. Piperazine ureas

A number of companies have reported TRPV1 antagonists containing the 4-aryl-piperazine urea structure. The earliest examples of this chemotype are exemplified by BCTC (**8**) ($IC_{50} = 34\,nM$). These structures appear to have been independently discovered by several groups [66–68], possibly an outcome of widely distributed purchased libraries populating corporate compound collections. Anecdotally, the two ureas, **19** and **20,** were disclosed almost simultaneously and they retain substantial structural homology with the piperazine ureas [69,70].



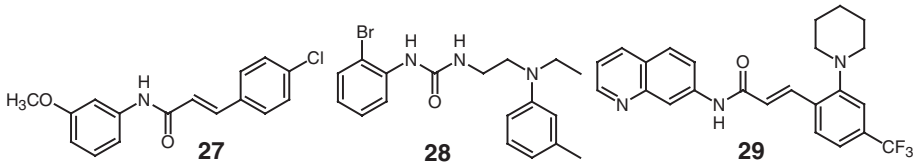
Pharmacological evaluation of **8** in rat showed it to be effective in models of inflammatory and neuropathic hyperalgesia, despite its rapid metabolism [41,72]. Separately, a close analog of **8**, compound **9** ($IC_{50} = 65\,nM$), was reported to have significantly improved bioavailability and clearance [16,74], and **9** was also shown to be efficacious in capsaicin evoked allodynia when administered orally ($ED_{50} = 16\,\mu M/Kg$). A related compound, **21** ($IC_{50} = 103\,nM$), from the same group had equivalent activity at TRPV1 but significantly improved clearance; no

efficacy data was reported [73]. More recently, several reports have divulged closely related analogues that retain the central piperazine urea core with variations in the flanking aryl moieties [75–81], exemplified by compounds **22** ($IC_{50} = 50\,nM$), **23** ($IC_{50} = 58\,nM$), **24**, **25** ($IC_{50} = 47\,nM$), and **26** ($IC_{50} = 374\,nM$). Notably, the prototypical urea isosteres, hydroxyl-guanidine (**23**) and cyano-guanidine (**24**) appear to be tolerated.



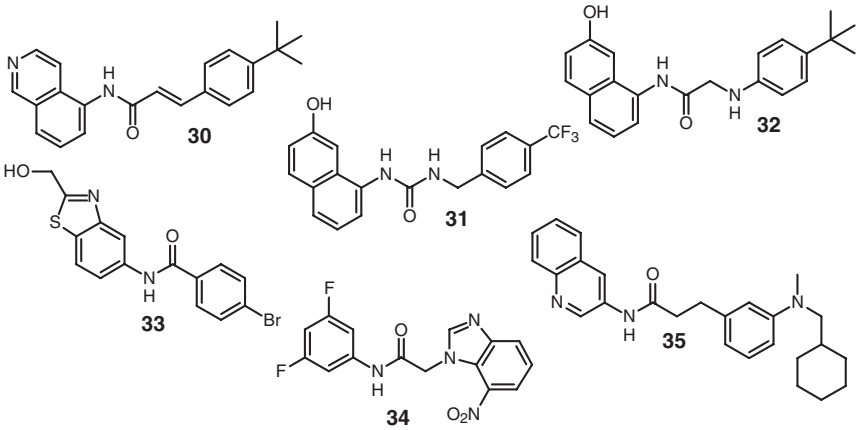
4.3. Aryl amides and ureas

Aryl amides and aryl ureas containing some of the key elements of capsaicin and capsazepine have been identified by a number of research groups. Two of the early examples are the cinnamide SB-366791 (**27**) ($pK_b = 7.7$) and the urea **28** [71,82]. Extensive profiling of **27** was recently reported [83] and the compound was found to be highly selective for TRPV1 and lacked any significant activity at I_h and calcium channels. Noteworthy is a report that an alkyl quaternary salt of **28** only blocked capsaicin invoked calcium influx when applied to the extracellular surface of cells in patch clamp experiments. This provides strong evidence that the capsaicin binding domain of TRPV1 is extracellular [84]. At least four other groups have described related analogues. For example, SAR and PK studies on cinnamide **27** have just been reported. Thus **29** was found to exhibit superior oral bioavailability and clearance compared to **28** ($IC_{50} = 0.42\,nM$) [85].



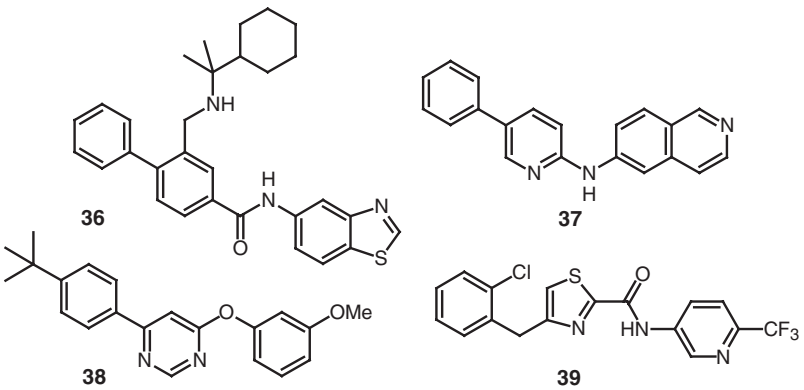
The SAR for similar quinoline (**30**, $IC_{50} = 5\,nM$) and naphthalene-based (**31**, $IC_{50} = 4\,nM$) ureas and cinnamates was also reported [86,87]. Additionally, a large

number of patent applications containing related structures have also issued [88–98]; representatives of these structures are highlighted below and include **32** ($IC_{50} = 60\,nM$), **33** ($IC_{50} = 10\,nM$) and **34** ($IC_{50} = 50\,nM$). Dihydrocinnamates were also described, including compound **35** ($K_i = 81\,nM$) which displayed oral activity in the rat carrageenen induced thermal hyperalgesia model ($ED_{50} = 0.28$ mpk p.o.) [97,98].

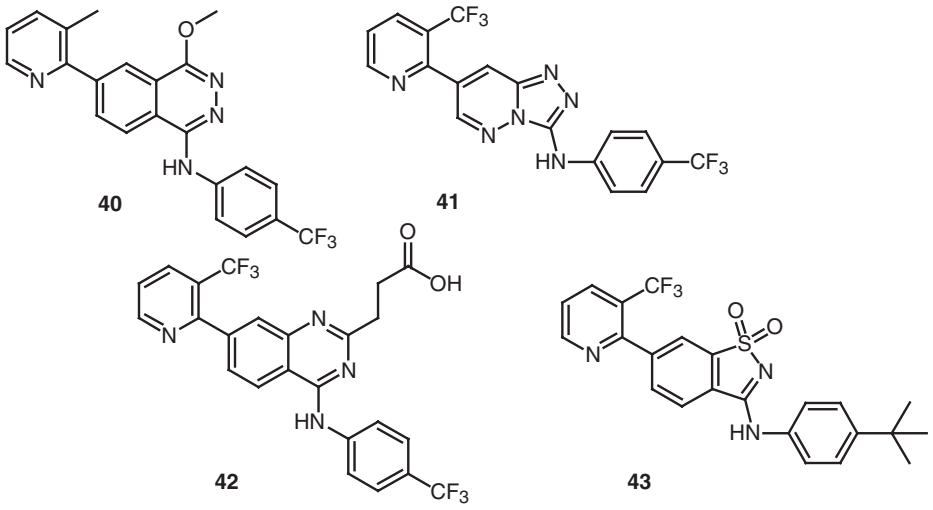


4.4. Miscellaneous structures

In addition to the familiar chemotypes described above, a range of new structures have emerged in the patent literature [99–104] during the past eighteen months. Many of the new structures lack the ubiquitous olefin or urea conformational restraint and instead contain a range of heterocyclic replacements. Examples include the amide **36** [99] which was shown to be orally efficacious in a capsaicin induced eye irritation model in mice at 30 mpk. Similar structural features are exemplified by **37** [100] **38** and **39** [101,102], although in these cases, no specific biological data was reported.



Two groups have described the related structures **40** and **41** [105,106], and (**42** and **43**) [107–109]. These compounds may be considered as analogues of the heteroaryl amides, above, in which the flanking aryl moieties are retained but situated on either side of a bicyclic heterocycle spacer as opposed to a heteroaryl amide.



5. CONCLUSION

The identification of the molecular target for capsaicin and other pungent natural products as the TRPV1 receptor has generated significant interest in this target as an opportunity for therapeutic intervention in a range of disorders. The level of interest amongst major pharmaceutical companies is attested to by the significant volume of patent activity during the past year. With potent and selective TRPV1 antagonists now available, and at least one company acknowledging a clinical presence [22], these agents are now poised to be carefully and critically evaluated in a clinical setting.

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Leukotriene Biosynthesis Inhibitors

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Contents

1. INTRODUCTION

The leukotrienes (LTs) are potent lipid mediators, derived from arachidonic acid (AA), that historically have been implicated in a variety of inflammatory diseases including asthma and allergy [1,2]. In the last 20 years, much research has been aimed at discovering selective inhibitors of the various enzymes involved in the biosynthetic pathway leading from AA to the LTs (including 5-lipoxygenase (5-LO), 5-lipoxygenase activating protein (FLAP), LTA_4 hydrolase and LTC_4 synthase) since such inhibition holds promise for therapeutic intervention in diseases characterized by LT mediated inflammation. To date, however, zileuton is the only inhibitor of an enzyme in this pathway, 5-LO, to be approved as a therapeutic agent for the treatment of asthma and this occurred in late 1996. More recently, the involvement of LTs in the inflammatory component of various cancers [3,4] and atherosclerosis [5–7] has been studied and the link to these clinical indications has rekindled interest in the discovery of inhibitors of LT synthesis.

A growing body of literature has accompanied this renewed interest in the LT pathway. Since the subject was last reviewed in this forum in 1997 [8], comprehensive reviews on the biochemistry [9,10], pharmacology [11,12], and inhibitors of

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5-LO and FLAP [2,13–15] have been published. Reviews on the biochemistry [16,17] and inhibitors [18] of LTA_4 hydrolase have appeared. Although there have been no reports of new LTC_4 synthase inhibitors, the biochemistry of this enzyme has been reviewed [19,20]. In addition, a number of reviews have summarized recent developments in the chemistry and biology of dual 5-LO/cyclooxygenase (COX) inhibitors [21–25].

2. NEW BIOLOGY AND PHARMACOLOGY OF THE LEUKOTRIENE PATHWAY

The LTs are generated by the initial conversion of AA to LTA_4 by 5-LO and FLAP which is further converted by LTA_4 hydrolase to LTB_4 or by LTC_4 synthase to yield the cysteinyl-LTs, LTC_4, LTD_4 and LTE_4. Receptors for LTB_4 (BLT1) and LTD_4 (cystLT1) have been previously described [26,27]. Mice deficient in BLT1 have been generated and the use of these mice in models of allergic pulmonary inflammation has demonstrated the role of this receptor in T cell recruitment [28]. Recent developments in the molecular biology of the pathway include the cloning of a second low affinity form of the LTB_4 receptor (BLT2) and of a second cysLT receptor (cysLT2), both of which have distinctive agonist and antagonist binding properties and patterns of tissue expression compared the previously identified receptors [29].

2.1. Inflammation

Further characterization of the 5-LO deficient mice has provided additional evidence for the role of the LT pathway in inflammation. Mice lacking 5-LO show a reduction in carageenan-induced lung inflammation [30], resistance to acute pancreatitis induced by cerulein [31], and a reduction in tissue leukocyte infiltration and injury caused by endotoxemia [32]. Unexpectedly, 5-LO deficiency worsened allergic encephalomyelitis [33].

2.2. Atherosclerosis

One of the most important developments in the LT area relates to atherogenesis where several different studies have implicated LTs in disease progression [29]. 5-LO, FLAP and LTA_4 hydrolase are expressed in human atherosclerotic lesions [34]. LTB_4 stimulates the expression of genes related to atherogenesis in rat basophilic leukemia cells [35]. Using mouse genetic models of atherogenesis (apo-$E^{-/-}$ or $LDLR^{-/-}$), the treatment with LTB_4 antagonists [36], the deletion of 5-LO [37] and deletion of the BLT1 receptor [35] were found to reduce disease severity. The 5-LO gene was identified as part of the gene cluster on chromosome 6 conferring resistance to atherogenesis in the mouse strain CAST [37]. A congenic strain,

containing the athero-resistant chromosome 6 region, was found to express 5-LO at reduced levels [37]. The deduced sequence for 5-LO from the atherosclerosis resistant mice contained mutations that affected enzyme activity when introduced into the human enzyme [38]. A polymorphism in the 5-LO gene promoter has recently been identified in relation to increased atherosclerosis [39].

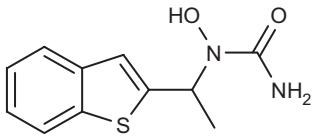
2.3. Cardiovascular diseases

LTs have also been implicated in myocardial infarction (MI) and stroke. Both increased neutrophilic LTB_4 production and a polymorphism in the gene encoding for FLAP (ALOX5AP) have been associated with a greater risk of MI and stroke [40]. In mouse, 5-LO deficiency was found to reduce injury in models of renal ischemia [41] and splanchnic artery occlusion [42] but not in cerebral ischemia [43].

3. CLINICALLY STUDIED INHIBITORS

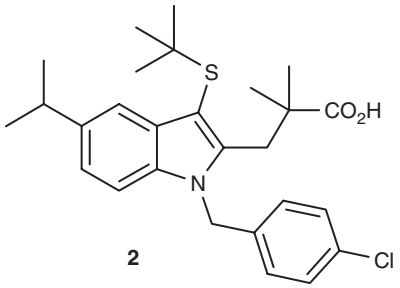
3.1. 5-LO and FLAP inhibitors

Zileuton (**1**) is the only marketed 5-LO inhibitor and is approved for the treatment of asthma [44]. The treatment of mild asthmatics with zileuton (600 mg qid, 2 weeks) resulted in a 96% increase in plasma thromboxane B_2 from baseline levels and a corresponding 62% increase in spontaneous platelet aggregation, suggesting a shunting of arachidonic acid metabolism to the cyclooxygenase pathway [45]. In a small clinical trial, zileuton provided a magnitude of prophylaxis in exercise-induced asthma (as measured by FEV_1) equivalent in magnitude but considerably shorter in duration than salmeterol, montelukast and zafirlukast [46]. Zileuton inhibited bronchoalveolar lavage (BAL) fluid eosinophil counts by 68% upon antigen challenge in a sub-population of allergic asthmatics who exhibited a significant increase in BAL leukotrienes and inflammatory cytokines, but not in those patients where leukotriene levels were unchanged upon antigen challenge [47]. Zileuton provided minimal efficacy [48] or no effect [49] in aspirin-induced respiratory reactions. A pilot clinical study for the treatment of acne demonstrated that zileuton (600 mg qid, 3 months) afforded a 71% mean reduction in inflammatory lesions, a 65% reduction of sebum lipids and a 59% decrease in the acne severity index [50]. The synthesis of sebum lipids upon zileuton treatment can be normalized after 2 weeks with inhibition levels similar to isotretinoin treatment [51]. Zileuton also exhibited efficacy in a pilot study of atopic dermatitis [52]. The weak, reversible inhibition of CYP1A2 has been identified as the mechanism whereby zileuton elicits clinically relevant drug interactions resulting in the decreased clearance of CYP1A2 substrates such as (R)-warfarin and propranolol [53].



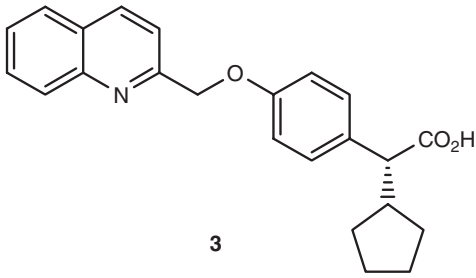
1

A number of FLAP inhibitors have demonstrated clinical efficacy in the treatment of asthma [1]; however, no FLAP inhibitor has achieved regulatory approval for this or any other indication. A new activity has been described for the clinically tested FLAP inhibitor MK-886 (**2**). MK-886 is an inhibitor ($IC_{50} = 3.2\,\mu M$) of inducible rat microsomal prostaglandin E synthase-1 (mPGES-1) and, consistent with this activity, inhibits PGE_2 production in IL-1β stimulated chondrocyte lysates from osteoarthritis patients [54–56]. This inhibitory potency is >100-fold less than its FLAP binding affinity, suggesting that the inhibition of mPGES-1 is not involved in the observed clinical efficacy of MK-886.



2

The FLAP inhibitor BAY X 1005 (**3**) was tested in a pilot clinical COPD study (500 mg bid, 14 days) and produced modest reductions in sputum levels of LTB_4 (48%) and myeloperoxidase (34%) although no change in total chemotactic activity was observed [57].



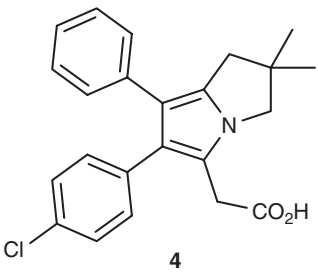
3

In a phase IIa trial, DG 031 (BAY X 1005, 250 mg qd, bid or tid for 4 weeks), a FLAP inhibitor previously in the clinic for asthma and the first to be tested clinically for cardiovascular indications, suppressed the production of LTB_4 and

reduced levels of biomarkers (MPO and sICAM-1) that may be linked to arterial inflammation and heart attack risk [58].

3.2. Dual 5-LO/COX inhibitors

Licofelone (ML-3000, **4**) is the most clinically advanced dual 5-LO/COX inhibitor [59,60]. Licofelone was shown to be a balanced inhibitor of both 5-LO and COX when assayed for calcium ionophore stimulated eicosanoid formation in cell based assays, including polymorphonuclear leukocytes (PMNL). Licofelone selectively inhibits COX-1 over COX-2 (ratio IC_{50} COX-1/IC_{50} COX-2 = 0.43) in stimulated bovine aortic coronary endothelial cells [61]. Although licofelone exhibited inhibition of TxB_2 production in calcium ionophore stimulated human whole blood (indicative of COX inhibition), LTC_4 levels were unaffected [62]. Licofelone inhibits IL-1β stimulated matrix metalloprotease-13 production and expression in human osteoarthritis chondrocytes, suggesting another mechanism unrelated to the inhibition of 5-LO/COX by which it may modulate the inflammatory process [63]. Licofelone (200 mg bid) demonstrated clinical efficacy similar to naproxen (500 mg bid, 12 weeks or 52 weeks) or the COX-2 selective inhibitor celecoxib (**5**) (200 mg qd, 12 weeks) in treating osteoarthritis pain [60,64–66]. A number of publications and meeting abstracts have reported the superior gastric/duodenal tolerability and safety profile of licofelone (200 mg bid) relative to naproxen (500 mg bid), both in normal volunteers [60,67] and in osteoarthritis patients [60,64,65]. However, in contrast to COX-2 selective inhibitors, licofelone retains its favorable GI tolerability profile when co-administered with low-dose aspirin (81 mg qd) [68].



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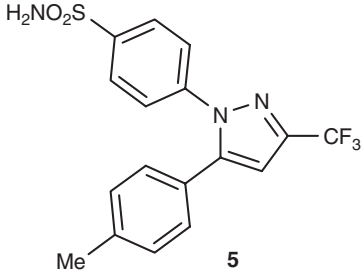

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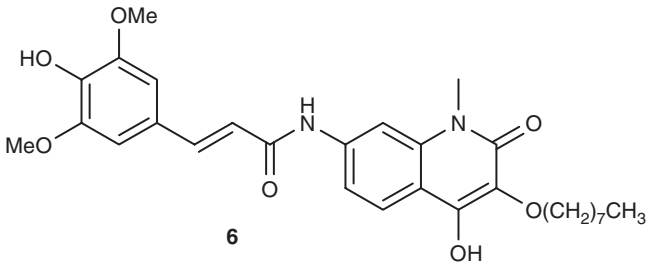
4. NEW INHIBITORS

4.1. 5-LO and FLAP inhibitors

A recent QSAR analysis of the published data for several distinct series of 5-LO inhibitors suggests that hydrophobicity strongly correlates with inhibitory potency. Furthermore, it is the log P parameter that is of greater importance than electronic

parameters in establishing this correlation [69]. The quinolinone phenol TA-270 (**6**) is a 10-fold more potent inhibitor of 5-LO than zileuton in RBL-1 cells [70] and is equally efficacious as pranlukast in inhibiting the early and late-phase bronchoconstriction responses in an ovalbumin-challenged guinea pig model [71]. A series of non-redox 5-LO inhibitors derived from the dihydroquinolinone tetrahydropyran ZD-2138 (**7**) has been described [72–76]. Studies conducted to address the metabolic and toxicological liabilities of the lead imidazole CJ-12,918 (**8**) culminated in the discovery of the optimized imidazolylphenyl carboxamide CJ-13,610 (**9**). CJ-13,610 inhibited ($IC_{50} = 70\,nM$) the Ca^{2+}-ionophore-induced formation of 5-LO products in human PMNL and, although no data have been published, CJ-13,610 has been evaluated clinically in a 6 week, phase II COPD trial [74]. Several patents have reported new series of 5-LO and FLAP inhibitors, including thiazolyl coumarins [77], benzoxazoles [78], benzofuranyl hydroxyureas [79], and diphenyl cycloalkanes [80], exemplified by **10**–**13**, respectively.



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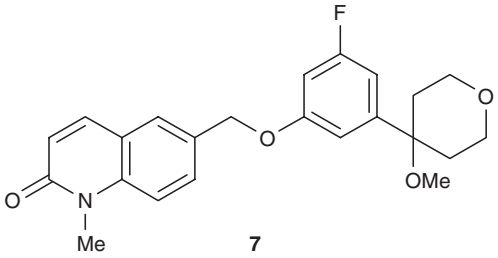

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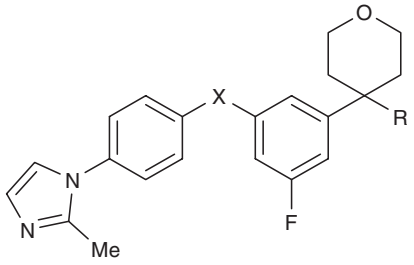
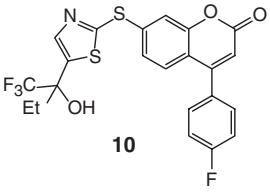


8 X = CH_2O, R = OMe
9 X = S, R = $CONH_2$



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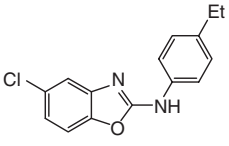

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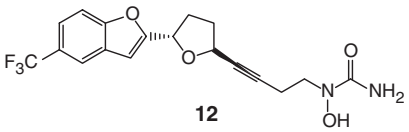

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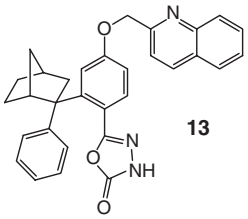

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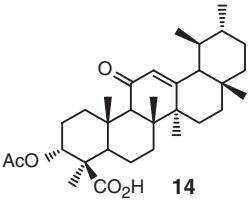

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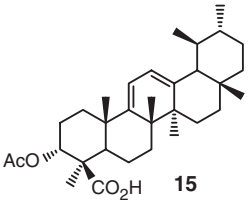

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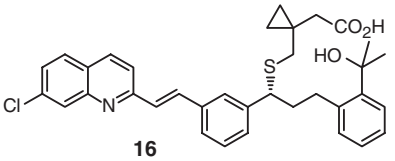
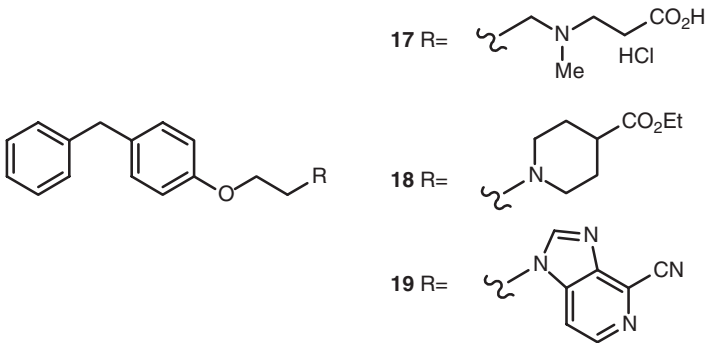


16

The non-redox, non-competitive 5-LO inhibitor AKBA (**14**), isolated from frankincense, has demonstrated clinical efficacy in the treatment of colitis and bronchial asthma. The boswellic acid analog **15**, an artifact observed upon isolation and purification of **14**, is a 4-fold more potent 5-LO inhibitor than **14** in intact PMNs [81]. A new activity has been reported for the marketed LTD_4 receptor antagonist montelukast (**16**). Montelukast inhibits 5-LO in a non-competitive manner with an IC_{50} of 2.5 μM in a rat mast cell model and at concentrations greater than 1 μM in a human PMNL assay [82]. This inhibition may have potential clinical relevance since the therapeutic dose of montelukast that is normally recommended in asthma treatment (10 mg) provides maximal plasma concentrations of approximately 0.6 μM.

4.2. LTA_4 hydrolase inhibitors

The LTA_4 hydrolase inhibitors SC-57461A (**17**) and SC-56938 (**18**) have been identified as clinical candidates based on their potent inhibition of ionophore-induced LTB_4 production in human whole blood ($IC_{50} = 49$ nM and 820 nM, respectively) and oral activity in a variety of *in vivo* rat and murine models of inflammation [83–86]. Replacement of the amino propanoic acid or piperidine with heterocycles led to potent analogs such as **19** [87].



4.3. Dual 5-LO/COX inhibitors

A multitude of recombinant enzyme, microsomal, intact cell, and whole blood assays have been used to characterize dual inhibitors of the 5-LO and COX enzymes. As a consequence, inter-compound comparison of inhibitory potency and selectivity is difficult. The summary data presented in Table 1 should be viewed with

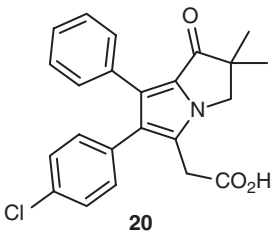
Table 1. Inhibitory Potencies of Dual 5-LO/COX Inhibitors[a]

Compound	5-LO IC_{50} (μM)	COX-1 IC_{50} (μM)	COX-2 IC_{50} (μM)	Reference
4	0.21 c	0.16 c	0.37 c	[61]
20	2.3 c	0.046 c	2.1 c	[88]
21	10 c	0.7 c	0.005 c	[61]
22	0.3 w	26.1 c	0.045 c	[90]
23	0.74 w	25.7 c	0.1 c	[90]
25[b]	4.7 c	12.1 c	-	[91]
27	0.07 c	0.18 c	0.002 c	[92]
28	0.05 w	5.1 e	>100 e	[95]
29[b]	0.017 c	0.37 c	-	[96]
31[b]	0.15 e	0.06 e	-	[97]
	0.25 c	0.34 c		
32[b]	1[c]	0.1[d]	-	[98]
33	2.5 c	27 c	0.011 c	[100]
34	0.6 c	>10 c	1.2 c	[101]
35	0.15 c	>50 e	0.83 e	[102]
36	0.37 c	65.3 c	1.89 c	[103]
37	1–1.5 c	0.3–3 c	>30 c	[104]

[a]Assay type: e – enzyme; c – intact cell; w – whole blood.
[b]Not specified whether COX-1 or COX-2 inhibition.
[c]105% inhibition of LTB_4 production at 1 μM.
[d]99% inhibition of PGE_2 production at 0.1 μM.

this in mind since it is beyond the scope of this review to describe the variety of experimental conditions employed in these assays.

Several new dual 5-LO/COX inhibitors based on the licofelone structure (**4**) have been described. Oxidized analogs, such as **20**, are 45-fold more potent inhibitors of COX-1 than COX-2 while maintaining a balanced 5-LO/COX-2 inhibitory profile [88]. Licofelone analogs, such as **21**, that lack the acetic acid side chain and that bear the methyl sulfone moiety characteristic of COX-2 selective inhibitors, are moderate 5-LO inhibitors that exhibit the expected selectivity for inhibition of COX-2 [61].



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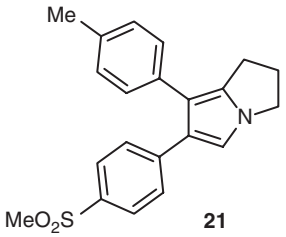
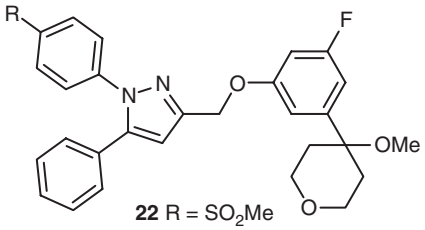


21

Three classes of dual inhibitors have been described that combine the two key pharmacophores associated with known 5-LO and COX inhibitors. One of these classes is exemplified by compounds **22** and **23**, dual inhibitors that combine the pyrrazole triaryl motif of the selective COX-2 inhibitor celecoxib with the tetrahydropyranylphenyl pharmacophore found in the non-redox 5-LO inhibitor ZD-2138 (**7**). They display balanced 5-LO/COX-2 inhibition and are as efficacious as zileuton and rofecoxib in a rat model of AA-induced ear edema [89,90].

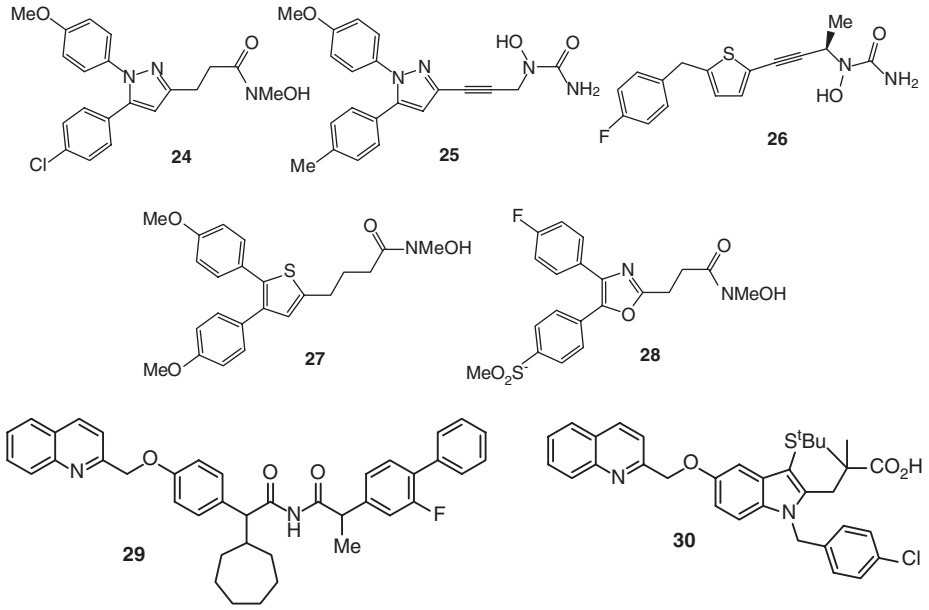


22 R = SO_2Me
23 R = SO_2NH_2

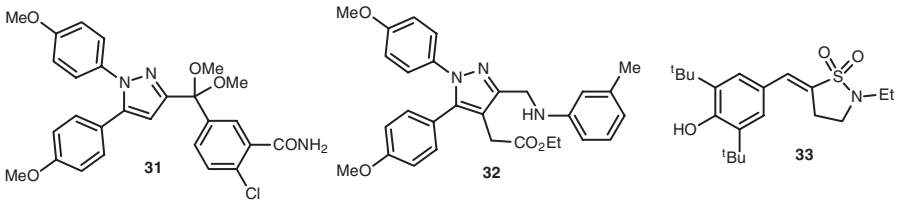
A second class combines the triaryl COX inhibitor pharmacophore with the N-hydroxy urea or hydroxamic acid pharmacophores that are present in redox 5-LO inhibitors. These compounds are structurally related to tepoxalin (**24**), an early dual 5-LO/COX inhibitor [22]. Compound **25**, which bears an acetylenic N-hydroxy urea moiety reminiscent of the redox 5-LO inhibitor ABT-761 (**26**), is a dual inhibitor that exhibits a relatively short-lived inhibition of COX and 5-LO in a canine blood *ex vivo* assay [91]. Replacement of the tepoxalin pyrazole with either a thiophene or oxazole gives the dual inhibitors S-19812 (**27**) [92–94] and **28** [95]. S-19812 is efficacious in rat models of carrageenan-induced hyperalgesia ($ED_{50} = 8.3\,mg/kg$, therapeutic) and adjuvant-induced arthritis

(ED_{50} = 11 mg/kg), and is gastric sparing up to 800 mg/kg compared to indomethacin which induces lesions at 5 mg/kg [94].

A third class is represented by the dual inhibitor **29** which combines the quinolinylmethoxyaryl moiety found in a number of FLAP inhibitors, such as MK-0591 (**30**) and Bay X 1005 (**3**), and the biaryl pharmacophore of the NSAID flurbiprofen [96].

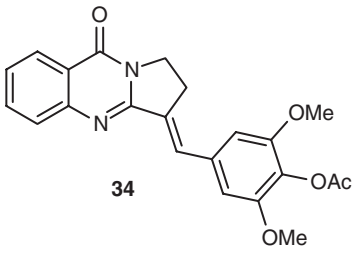


ER-34122 (**31**) and **32** are examples of pyrazole based triaryls modeled on celecoxib and tepoxalin that exhibit dual inhibition profiles [97,98]. ER-34122 is 3- to 10-fold less potent than indomethacin in inhibiting carrageenan-induced rat paw edema [97]. S-2474 (**33**) is a dual inhibitor that incorporates a γ-sultam moiety and the 2,6-di-*tert*-butylphenol pharmacophore characteristic of antioxidant radical scavengers [99]. S-2474 exhibits a selectivity for COX-2 inhibition (IC_{50} COX-1/IC_{50} COX-2 = 2500) similar to that of celecoxib, and is efficacious in rat models of carrageenan-induced paw edema (ED_{50} = 3.5 mg/kg) and adjuvant-induced arthritis (ED_{50} = 0.76 mg/kg) [100].



A number of natural products exhibit dual 5-LO/COX inhibitory activity. The pyrroloquinazoline alkaloids **34** and **35** are dual inhibitors that are COX-2 selective.

34, an acetylated analog of isaindigotone, inhibits both carrageenan-induced paw edema ($ED_{50} = 27.2\,mg/kg$) and phenyl-*p*-benzoquinone-induced writhing ($ED_{50} = 2.6\,mg/kg$) in murine models [101]. Tryptanthrin (**35**), isolated from woad, inhibits LTB_4 release from stimulated neutrophils with potency equal to that of zileuton [102]. The dual 5-LO/COX-2 inhibitory activities of the lignan deoxypodophyllotoxin (**36**) may underlie its use in traditional medicine as an antipyretic and analgesic [103]. In addition to antidepressive and antibacterial activities, a dual 5-LO/COX-1 inhibition has been observed with hyperforin (**37**) one of the main active constituents of St. John's wort [104].



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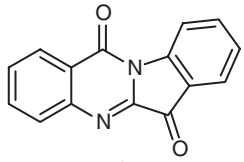

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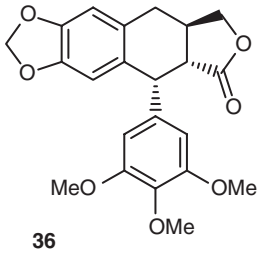

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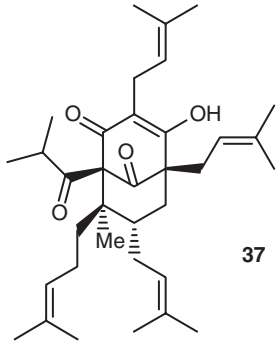

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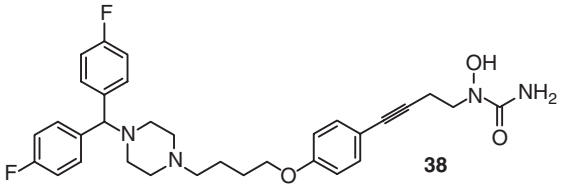
4.4. Other dual inhibitors

The dual 5-LO inhibitor/H_1 histamine receptor antagonist UCB-62045 (**38**), combining the pharmacophores of zileuton and cetirizine, inhibited histamine-induced bronchoconstriction and *ex vivo* calcium ionophore-induced LTB_4 production in a guinea pig model [105]. E3040 (**39**) is a dual 5-LO/thromboxane A_2 synthase inhibitor that exhibits balanced inhibition in human blood cells [106] and inhibited LPS-induced large intestine vascular permeability in a rat model [107]. The dual 5-LO/thromboxane A_2 synthase inhibitor F-1322 (SOA-132, **40**) inhibited antigen-induced late phase asthmatic response and prevented airway and BAL fluid eosinophilia upon oral administration to guinea pigs [108]. LDP-392 (**41**), a dual 5-LO inhibitor/PAF receptor antagonist, is 7-fold less potent than zileuton in a human whole blood assay for LTB_4 inhibition and inhibited ($ED_{50} = 2.5\,mg/kg$) AA-induced ear edema in rats [109].



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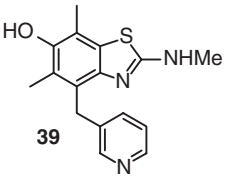

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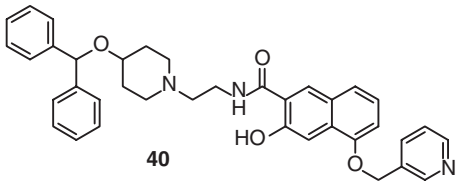

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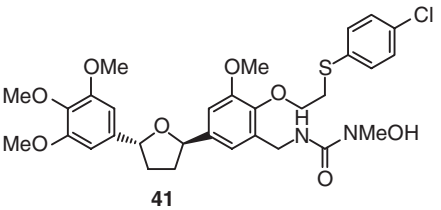

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5. CONCLUSIONS

The importance of the enzymes involved in LT biosynthesis in asthma is well established from the data obtained with both 5-LO and FLAP inhibitors. Emerging data from many independent studies now indicate a role for LT in the pathogenesis of atherosclerosis and potentially other cardiovascular diseases. At the same time, new approaches are being developed to inhibit the LT pathway with the LTA_4 hydrolase inhibitors and dual 5-LO inhibitors of different types. Some of these new molecules are currently in clinical trials and should contribute to further define the therapeutic application of modulating the LT pathway.

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CXCR3 Antagonists

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Contents

1. INTRODUCTION

Chemokine receptors and their ligands play an important role in mediating leukocyte trafficking [1,2]. Chemokines are proteins of approximately 10 kD that are secreted at the site of inflammation and bind to specific G-protein coupled receptors (GPCRs) expressed on the surface of T cells and other leukocytes [3]. The secreted chemokines form a concentration gradient by binding to glycosaminoglycans on the surface of cells adjacent to the inflamed tissue, including the endothelial cells that line the blood vessels. As T cells approach the site of inflammation, they slow down in a process that is mediated by selectins. This allows the chemokine receptors expressed on the surface of T cells to come in contact with their ligands on the surface of the vascular endothelial cells. Chemokine receptor activation triggers integrin-mediated arrest of T cells on the surface of the endothelial wall and their extravasation guided by the chemokine gradient.

CXCR3 is a chemokine receptor primarily expressed on activated CD4+ and CD8+ T cells with a Th1 phenotype [4], although it is also expressed on B cells [5], natural killer (NK) cells [6], malignant T cells [7] and astrocytes [8]. The ligands for CXCR3, Mig (CXCL9), IP-10 (CXCL10), and ITAC (CXCL11), are induced primarily by IFN-γ and are produced by macrophages as well as other cell types in inflamed tissue [9–15].

Disease tissue samples taken from patients suffering from a variety of autoimmune diseases show that CXCR3 is expressed at high levels on infiltrating T cells. At the same time, the ligands for CXCR3 are upregulated in these disease tissue

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samples. In inflammatory bowel disease patients, there is an increased number of $CXCR3^+$ cells that are found in the lamina propria and submucosa of colon tissue [16], as well as an increase in the numbers of IP-10 secreting cells [17]. In rheumatoid arthritis (RA) patients, researchers have reported that as many as 97% of the infiltrating cells in synovial fluid express CXCR3 [4] and protein levels of IP-10 and Mig are elevated as much as 50 to 100 fold relative to normal individuals [18]. In psoriasis patients, IP-10 [19] and Mig [20] levels are increased in psoriatic plaques and CXCR3 expressing cells infiltrate into the dermis and basal layer of the epidermis of psoriatic lesions [21].

In multiple sclerosis (MS) patients, 80–86% of $CD4^+$ T cells and 92–97% of $CD8^+$ T cells in cerebrospinal fluid have been reported to express CXCR3 [22–24]. Also, CXCR3-expressing T cells and the ligands Mig and IP-10 are found in brain lesions of MS patients [24–26]. In addition, elevated levels of Mig and IP-10, were found in the CSF of MS patients experiencing acute attacks [22,23]. Moreover, in an adoptive transfer model of experimental autoimmune encephalomyelitis (EAE), mice treated with a neutralizing antibody to IP-10 show decreased signs of disease severity [27]. However, conflicting results were observed with the IP-10 knock-out mice in an active immunization model of EAE [28], as well as with rats treated with a neutralizing antibody to IP-10 [29].

In patients undergoing transplant rejection, increased levels of the CXCR3 ligands and a large number of infiltrating T cells that express CXCR3 are found in biopsies from organs undergoing rejection [30–38]. Furthermore, cardiac allograft experiments with CXCR3 knock-out mice show increased allograft tolerance when compared to similar experiments performed with wild-type mice [39]. Likewise, transplant experiments with antibodies to the CXCR3 ligands, Mig and IP-10, enhance allograft survival [40,41]. The significance of the role that CXCR3 mediated cellular recruitment plays in transplant has now been demonstrated in a broad variety of *in vivo* models, including cardiac, lung, small bowel and islet transplant models [39,42–44]. Moreover, cardiac allografts taken from mice lacking IP-10 show prolonged allograft survival time in wild-type mice [40]. No enhancement in allograft survival time is observed when a cardiac allograft from a wild-type mice is transplanted into an IP-10-deficient mice, indicating the importance that the CXCR3 ligand plays in promoting allograft rejection.

It is thought that blockade of CXCR3 will prevent inflammatory cells from reaching sites of inflammation and thus should alleviate the disease. In this article we will provide a literature overview regarding potential therapeutic applications for a CXCR3 antagonist and examine the recent reports of CXCR3 antagonism, including blockade of the CXCR3 receptor by antibodies, peptides, and small molecules.

2. ANTIBODIES TO CXCR3

Antibodies that block ligand binding to CXCR3 have been shown to be beneficial in animal models of disease. In a murine model of acute cardiac allograft rejection, animals treated with a monoclonal antibody against CXCR3 tolerated the allograft for three weeks while animals treated with a control antibody rejected the allograft

within one week [39]. This beneficial effect was observed when the anti-CXCR3 antibody was administered from the time of the transplant surgery as well as when the antibody was given beginning on day four following the transplant surgery (at a time when transplant rejection should be starting). CXCR3 knock-out mice tolerated the cardiac allografts for >8 weeks, nearly three times longer than the mice treated with anti-CXCR3 antibody [39]. Since receptor occupancy studies and pharmacokinetic data for the antibody were not provided, it is not possible to determine whether the differences in efficacy were due to insufficient receptor coverage by the antibody or to developmental changes in the effector cell population as a result of the genetic knockout. In a murine model of chronic lung allograft rejection, animals treated with a selective neutralizing polyclonal antiserum to CXCR3 showed markedly reduced symptoms of transplant rejection [45]. In these studies, the authors demonstrate that treatment with anti-CXCR3 antiserum prevents leukocytic infiltration of the allograft, resulting in a reduction in the histopathological markers of tissue destruction. These results indicate that agents that antagonize CXCR3 can be of therapeutic benefit in the organ transplant rejection setting.

Several research groups have discussed in the patent literature the potential utilities of CXCR3 antibodies for treatment of human diseases [46–48]. Investigators have presented data suggesting that immunization of SJL mice with an oligopeptide containing amino acid sequences from the first and fourth extracellular domains of CXCR3 prevents development of disease symptoms when the mice are subsequently immunized with an encephalitogenic peptide ($PLP_{139–151}$) [47]. Furthermore, investigators have also claimed that treatment of SJL mice with a monoclonal antibody generated against the first 37 amino acids of human CXCR3 reduces incidence of disease in response to immunization with the same encephalitogenic peptide [48]. These data suggest that CXCR3-directed antibodies could potentially be useful in the treatment of multiple sclerosis.

Therapeutic antibodies for the treatment of human disease must be, in large part, of human derivation to prevent potentially life-threatening complications that result when the human immune system reacts to foreign antibody sequences. There are several methods currently in use for generating human antibodies in mice or for "humanizing" antibodies. No such antibodies for CXCR3 have been reported in the peer-reviewed literature. However, data for a human anti-CXCR3 monoclonal antibody were reported at the American Transplant Congress Meeting in 2004 [49]. This antibody, named 5H7, has a half-life of approximately 30 hours in monkeys. In a monkey model of acute renal allograft rejection, animals treated with 5H7 (at 4 mg/kg/day) showed prolonged allograft survival (up to 21 days, $N = 4$) compared to control animals (7–8 days).

3. MODIFIED LIGANDS AS ANTAGONISTS

The ligands for CXCR3 have been demonstrated to undergo proteolytic processing. IP-10 is carboxy C-terminally processed by furin [50], gelatinase B (MMP-9) [51] and by neutrophil collagenase (MMP-8) [51], yielding a form that lacks the last four amino acids but retains full agonistic properties *in vitro* [50]. Mig is also

C-terminally processed by gelatinase B and neutrophil collagenase [51] but not by furin [50], yielding a form that lacks 9–14 of the last amino acids and is predicted to be largely inactive *in vitro* [52]. C-terminal processing of Mig may therefore represent a means of controlling the immune response naturally by regulating the activity of Mig. ITAC is not C-terminally processed by these three proteases.

All three CXCR3 ligands have been shown to undergo amino N-terminal proteolytic processing by dipeptidyl peptidase IV (also termed CD26) [53]. The resulting chemokines lack the first two amino acids and have greatly reduced agonistic activities *in vitro*. IP-10 and ITAC retain significant capacity for binding to CXCR3 and are able to inhibit calcium mobilization induced by intact CXCR3 ligands. Local application of the N-terminally processed forms of Mig and IP-10 inhibited IL-8-induced angiogenesis *in vivo* with efficacy equivalent to that observed with the intact ligands. N-terminally processed Mig, IP-10 and ITAC may thus act as natural antagonists of CXCR3 and may be useful in the treatment of disorders involving angiogenesis. The potential utility of truncated forms of Mig, IP-10 and ITAC for the treatment of a variety of diseases has been discussed in the patent literature [54]. No information is available on pharmacokinetic properties of the truncated CXCR3 ligands or on their *in vivo* efficacy following parenteral administration, thus it is not possible to gauge the feasibility of this approach for developing a therapeutic agent.

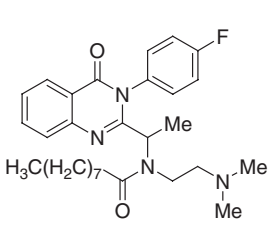
Although chemokines are produced as monomeric proteins, they oligomerize to form dimers and/or tetramers in solution. *In vivo*, chemokines additionally form complexes with glycosaminoglycans (GAGs). These interactions have been demonstrated to be crucial for the *in vivo* activity of several chemokines, although they have limited effect on the *in vitro* potency [55]. A disclosure in the patent literature elaborates on this finding [56], showing that a GAG-deficient mutant form of ITAC (CXCL11-3B3, which has reduced binding to heparin due to mutation of three basic residues in the C-terminus of ITAC) inhibits cellular recruitment to wild-type ITAC *in vivo*. CXCL11-3B3 was also shown to inhibit delayed-type hypersensitivity response. Although no information on the pharmacokinetic properties of CXCL11-3B3 is disclosed, it is encouraging that parenteral treatment is efficacious in an *in vivo* model.

The sequences of several variants of IP-10, intended to act as agonists or antagonists of CXCR3 have also been discussed in the recent patent literature [57]. No additional data on the *in vitro* or *in vivo* activity of any of these IP-10 variants are provided.

4. SMALL-MOLECULE CXCR3 ANTAGONISTS

4.1. Quinazolinones and 8-azaquinazolinones

There is very limited information in peer-reviewed journals regarding small-molecule antagonists of CXCR3; however, several classes of antagonists have been reported in the patent literature and at scientific meetings. Among the CXCR3 antagonists reported are a series of quinazolinones and 8-azaquinazolinones typified by **1–3** [58,59].



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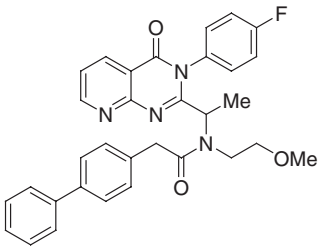

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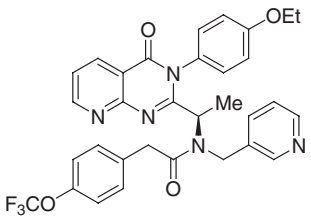
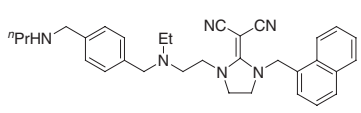


3

The most advanced of this family of compounds is AMG 487 (**3**), which was evaluated in a phase 2a psoriasis trial [60]. AMG 487 has been reported to inhibit binding of ^{125}I-IP-10 and ^{125}I-ITAC to activated human T lymphocytes with IC_{50} values of 7.7 and 8.2 nM, respectively [61]. In the presence of 50% human plasma, AMG 487 inhibits ^{125}I-IP-10 binding with an IC_{50} value of 46 nM. Furthermore, AMG 487 also inhibits CXCR3-mediated *in vitro* cell migration to Mig, IP-10 and ITAC with IC_{50} values of 36 nM, 8 nM and 15 nM, respectively. In phase 1 clinical trials AMG 487 achieved good oral exposure and was well tolerated [62]. The pyridyl *N*-oxide metabolite of AMG 487, which is observed in humans after dosing with AMG 487 has also been described as a CXCR3 antagonist [63,64].

4.2. Imidazolidines

Several reports have recently become available describing a series of imidazolidine derivatives, exemplified by **4–10**, as CXCR3 antagonists [65–67]. The inhibitory activities of the example compounds were determined in a CXCR3/^{125}I-IP-10 binding assay and reported as percent inhibition of receptor-radioligand binding at a single antagonist concentration of 10 μM. The source of CXCR3 was a membrane preparation from CXCR3-tranfected L1/2 cells. Examples **4–10** exhibited >99% binding inhibition at 10 μM.



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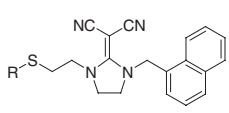

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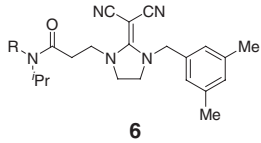

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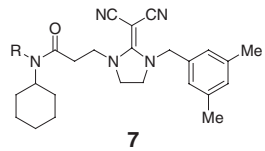

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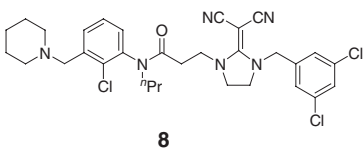

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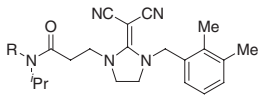

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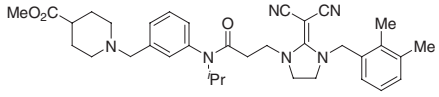

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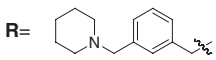
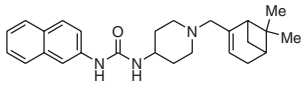


4.3. Ureas

A family of 1-aryl-3-piperidinyl ureas, exemplified by **11–15**, was identified as CXCR3 antagonists [68]. In a separate report, quaternary piperidinium urea analogs, exemplified by **16**, were revealed as modulators of CXCR3 [69].

In both reports, the antagonist activities for the example compounds were determined in a chemokine-mediated calcium mobilization FLIPR^{TM} assay with hCXCR3-transfected Chinese hamster ovary cells. Inhibitory activities *vs.* recombinant human chemokines Mig, IP-10 and ITAC were measured. Example compounds exhibited $>50\%$ binding inhibition at $5\,\mu M$. It was reported that the most active compounds had IC_{50} values $\leqslant 1\,\mu M$; however, neither IC_{50} values for specific compounds nor a qualitative ranking of the antagonists were revealed. The inhibitors were reported to exhibit at least five times greater binding selectivity for CXCR3 *vs.*, for example, the chemokine receptor CCR3.



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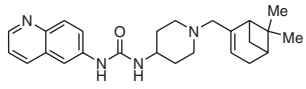

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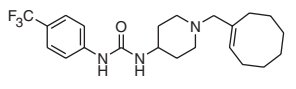

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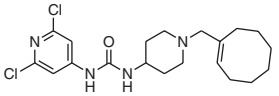

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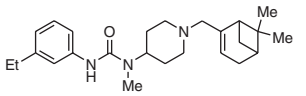

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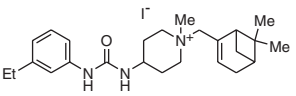

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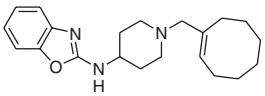
4.4. 4-aminopiperidines

N-piperidinyl benz[*d*]oxazoles, exemplified by **17** and **22**, *N*-piperidinyl benz[*d*]thiazoles, exemplified by **18**, *N*-piperidinyl benz[*d*]imidazoles, exemplified by **19**, *N*-piperidinyl quinoxalines, exemplified by **20**, and *N*-piperidinyl quinoline, exemplified by **21**, have been recently reported as CXCR3 antagonists [70]. The CXCR3 inhibitory activities for the example compounds were determined in a calcium mobilization FLIPR^{TM} assay with hCXCR3-transfected Chinese hamster ovary cells as described above for the urea inhibitors. As with the urea inhibitors of CXCR3, the most active compounds were claimed to give IC_{50} values $\leqslant 1\,\mu M$. No IC_{50} values for specific compounds were reported and it was claimed that all of the antagonists exhibited at least five times greater binding selectivity for CXCR3 *vs.* the CCR3 chemokine receptor.



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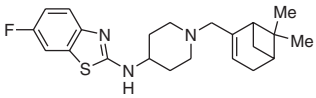

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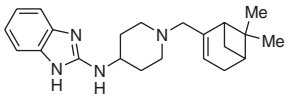

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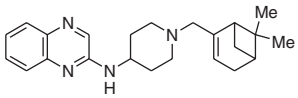

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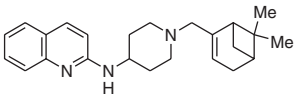

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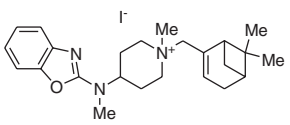

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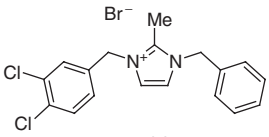
4.5. Imidazoliums

Imidazolium derivatives have been reported as inhibitors of CXCR3 [71]. The majority of the inhibitors fall into one of five general structure classes: non-symmetrical 1,3-dibenzyl-2-methyl-3*H*-imidazoliums exemplified by **23**; 1-phenacyl-3-phenethyl-3*H*-imidazoliums exemplified by **24**; non-symmetrical and symmetrical 1,3-bisphenacyl-3*H*-imidazoliums exemplified by **25** and **26** respectively; and 1,3-bis-phenacyl-3*H*-benzoimidazolium **27**. The inhibitory activity of the antagonists was determined using an IP-10-mediated calcium mobilization FLIPR^{TM} assay with rat basophilic leukemia 2H3 cells expressing CXCR3. However, specific IC_{50} values for the antagonists were not revealed, nor was a qualitative ranking of the antagonists' inhibitory activity disclosed.



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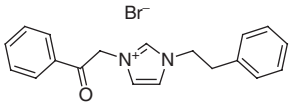

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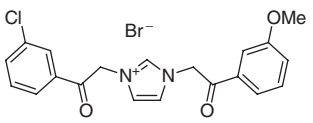

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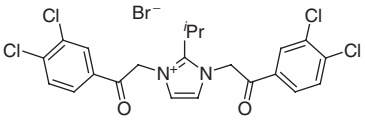

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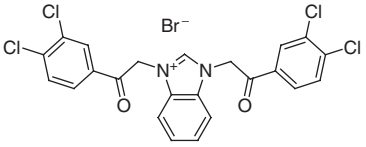

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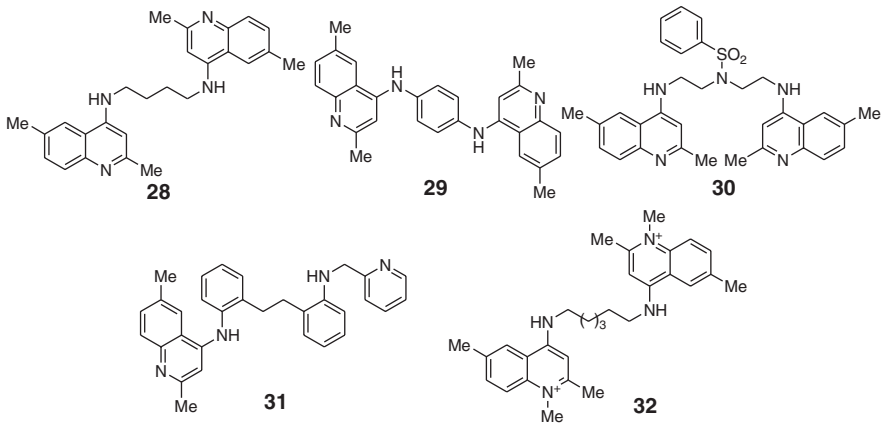
4.6. Aminoquinolines

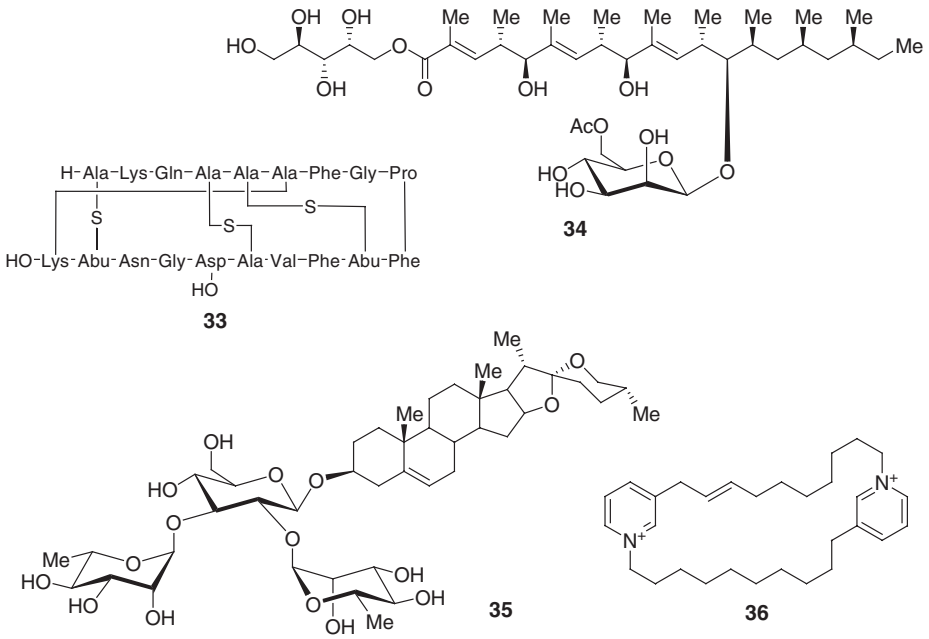
Aminoquinoline compounds exemplified by structures **28–32** were recently disclosed as CXCR3 antagonists [72]. Both symmetric and asymmetric aminoquinoline dimers were disclosed. Most examples feature the 2,6-dimethylquinolin-4-amino ring system, although many compounds possess various functional groups. The aminoquinoline units are generally connected at the 4-amino group by alkyl or aryl linkers, as shown by **28–32**, and a number of example compounds are joined by sulfonamides such as **30**. A group of 1-*N*-methyl-quinolinium salts was described, for example compound **32**. Mono-2,6-dimethyl-quinolin-4-amino compounds, exemplified by **31**, in which the quinoline is tethered to a variety of functional groups, were also identified as CXCR3 antagonists.

The example compounds were evaluated for their efficacy in blocking activation of CXCR3 by IP-10 in an *in vitro* time-resolved fluorometric assay utilizing a plasma membrane preparation from CXCR3-expressing HEK293 cells. Several analogs exhibited IC_{50} values less than 1 μM. Binding IC_{50} values for specific compounds were not revealed.



4.7. Natural product antagonists

Several natural products derived from microbial, plant and marine sources were recently described [73]. The natural products were identified in a high throughput filter binding assay using ^{125}I IP-10 and recombinant CXCR3 expressed in RBL cells and the IC_{50} values determined using the same binding assay. The screen identified the cyclic peptide duramycin **33** ($IC_{50} = 0.1\,\mu M$) as CXCR3 antagonists, as well as three roselipins, exemplified by **34** ($IC_{50} = 14.6\,\mu M$), three diosgenin glycosides, exemplified by **35** ($IC_{50} = 0.47\,\mu M$) and a 3-alkyl pyridinium alkaloid **35** ($IC_{50} = 0.69\,\mu M$).



5. CONCLUSION

There is great interest in the pharmaceutical industry for agents that can modulate CXCR3 because of the growing evidence that CXCR3-mediated cellular recruitment is implicated in a variety of inflammatory and autoimmune disorders with unmet medical needs. The next few years hold a lot of promise as some of the early chemical series mature into development compounds.

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PDE7 Inhibitors: Chemistry and Potential Therapeutic Utilites

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Contents

1. INTRODUCTION

Phosphodiesterases (PDEs) catalyze the hydrolysis of the key intracellular signaling 3'-5'cyclic nucleotides cAMP and cGMP, resulting in the formation of their respective inactive nucleotide 5'monophosphates AMP and GMP [1–3]. cAMP and cGMP serve as second messengers in a number of cellular signaling pathways and their specific modulation control a variety of physiological functions [4]. Elevation of intracellular levels of these cyclic nucleotides, by inhibition of PDEs, activates a specific protein phosphorylation pathway [5]. Selective PDE5 and PDE3 inhibitors have been marketed and a PDE4 inhibitor (Roflumilast) is in pre-registration for COPD and asthma. Among the 11 PDEs isoenzymes identified so far, PDE7 is a cAMP-specific enzyme insensitive to Rolipram (PDE4 inhibitor) [6]. Considering the functional role of PDE7 reported in T-cells [7] and recent findings on PDE7 mRNA tissue distribution [8,9], there is currently great interest in designing selective and potent PDE7 inhibitors to uncover the physiological role of PDE7 subtypes in several diseases.

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2. BIOLOGY OF PHOSPHODIESTERASE VII

2.1. PDE7 overview: subtypes and distribution

In human [8,10,11], mouse [10,11] and rat tissues [12,13], the cAMP-specific PDE7 family consists of two major subtypes, PDE7A and PDE7B, that are 70% homologous in the catalytic region. PDE7A [14] occurs as three splice variants in human [15] and as two for the mouse and rat. PDE7B exists as a single isoenzyme in human and mouse whereas in rat three splice variants of PDE7B can be found [13].

Human and mouse PDE7A1 (*K*m 0.2 μM) are predominantly expressed in the immune system [9,14–17]. In contrast, PDE7A2 is mainly expressed in skeletal muscle, kidney and heart tissue (*K*m 0.2 μM) [11,15,18–21]. In human, PDE7A3 mRNA is detected in activated CD4+ lymphocytes [20], ovaries and testes [22]. Human (*K*m 0.13–0.2 μM), mouse PDE7B (*K*m 0.03–0.1 μM), rat PDE7A1 and PDE7A2 (*K*m 0.2 μM) are mostly expressed in the brain [8,10–12,23]. Debate regarding the expression of human PDE7B in lymphoid tissue [10,24] and mouse PDE7B expression in the pancreas [10,11] is still ongoing. Low levels of rat PDE7B3 (*K*m 0.05 μM) are restricted to heart, lung and skeletal muscle, whereas rat PDE7B2 (*K*m 0.07 μM) is restricted to spermatocytes [13].

PDE7 tissue distribution in mice is very similar to that in humans and hence, suggests the usefulness of mouse studies to explore the role of the enzyme's relevance to human pathophysiology [15]. Since the tissue-specific expression pattern of PDE7A and PDE7B splice variants differ, the discovery of specific inhibitors might allow control of cAMP cellular function and therefore, regulate the physiology of the corresponding organs.

2.2. PDE7 and immunological response

The PDE7 enzyme has been shown to be predominant over PDE3 and PDE4 in CD4+ and CD8+ T cells [25]. Moreover, the PDE7A1 isoform, expressed in T cells, has been proposed to be essential for T lymphocyte activation and proliferation, since blocking its expression by a PDE7A antisense oligonucleotide correlated with an increase in cAMP and decrease in proliferation and IL-2 production [7]. As an elevation of cAMP has been associated with immunosuppressive and anti-inflammatory effects [6,26], PDE7 inhibition could be useful in the treatment of T cell-mediated diseases. Specifically, the similar pattern of PDE7A1 expression in T cells in mice and human [11,15] reinforces the potentially prominent role of PDE7A1 in regulating T cell related diseases compared to PDE7A2, which is not expressed in the immune system [15].

Disruption of the PDE7A gene in mice (PDE7A-/-) showed neither a deficiency in T cell proliferation nor changes in Th1- and Th2-cytokine production driven by CD3 and CD28 co-stimulation [27], strongly supporting a controversial but non-essential role of PDE7A for T lymphocytes activation. This discrepancy could be attributed to the different level of activation of those cells or to a possible regulation

of a specific cAMP pool by PDE7 that is not crucial for TCR-mediated activation but may alter others T cell functions [27]. Thus, a specific PDE7-dependant cAMP subcellular localization could be responsible for the lack of efficacy in mediating secondary T cell survival and immune response [4]. Additionally, a non-specific targeting of the PDE7 sequence by naked oligonucleotides [7] or potential toxicity could also explain these differences [28]. Another study described the constitutive expression of PDE7A in normal and malignant human B cells [24]. Interestingly, PDE7A expression is up-regulated by non-specific PDE inhibition implicating a possible compensatory feedback loop to augment the cell's ability to catabolize the increased levels of cAMP. IC242, a selective PDE7 inhibitor, was also able to up-regulate PDE7A but failed to increase cAMP levels. This result suggests, similarly to T cells [4], that this enzyme may contribute to maintain low cAMP levels in a localized subcellular compartment. Although PDE7A protein has been shown to be expressed in human B cells [27] and PDE7 mRNA has been detected in purified CD19+ lymphocytes [29], its role in Ab production remains to be assessed.

The role of PDE7B in lymphoid cells is controversial but its involvement in immune diseases could be supported by virtue of its expression in human thymus tissue and bone marrow [10,23].

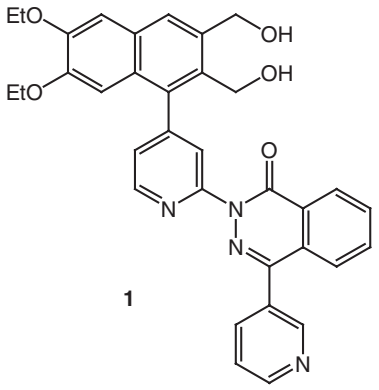
2.3. PDE7 as a potential target for airway diseases

Immune cells are amongst the key players in the development of airway inflammatory diseases. The PDE4 inhibitors currently in development suffer from typical side effects like nausea and vomiting [30]. Accordingly, a new cAMP-PDE-specific isoform-based treatment could increase specificity and consequently reduce side effects. PDE7A1 is highly homologous between human, mouse [15], and porcine cells [16] and is ubiquitously distributed among human pro-inflammatory, immune, and constitutive cells of interest for pulmonary diseases [9,16,31]. In addition, $CD4^+$ and $CD8^+$ T lymphocytes levels are increased in the airways of patients with asthma and COPD where they may play a critical role in the pathogenesis of these diseases [9]. PDE7 inhibition could represent an alternative/additive treatment to PDE4 inhibitors in airway diseases. PDE7B1 and to a lesser extent, PDE7B3 are also expressed in the lung of rats [13] but no specific involvement in any respiratory diseases has been described. Finally, the expressions of PDE7A in B cells, which produce IgE, PDE7A1 in lung [17], PDE7B in human fetal lung, thymus, bone marrow, neutrophil [23], and epithelial cells [16], reinforce the potential of PDE7 inhibitors in the treatment of respiratory diseases.

2.4. Toward elucidating the role of PDE7 using chemical tools

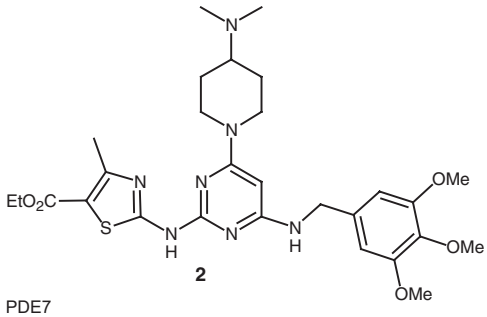
Recently, it was reported that T-2585 (**1**) (PDE4 $IC_{50} = 0.00013\,\mu M$, PDE7 $IC_{50} = 1.7\,\mu M$) is a regulator of T-cell functions in a dose range at which the drug

inhibits PDE7A activity, whereas the selective PDE4 inhibitor, RP 73401 (PDE4 $IC_{50} = 0.00031\,\mu M$, PDE7 $IC_{50} = 10\,\mu M$), only weakly suppressed T cell responses at $10\,\mu M$. This comparison between a dual PDE7/PDE4 inhibitor, (**1**) and a selective PDE4 inhibitor, RP 73401, indirectly highlights the potential role of specific PDE7-linked inhibition in human peripheral T cell function by suppressing PBMC derived CD4 + T cell proliferation, IL-5, IL-2, and IL-4 secretion and CD25 expression [26]. However, considering the low inhibitory activity of (**1**) for PDE7 compared to PDE4 and other studies wherein the specific PDE4 inhibitor, Rolipram, has been shown to regulate T cell functions [25], the benefit of dual PDE4 and PDE7 enzyme inhibition versus the comparative efficacy of highly potent and selective PDE7 inhibitors with different chemotypes, requires further examination.

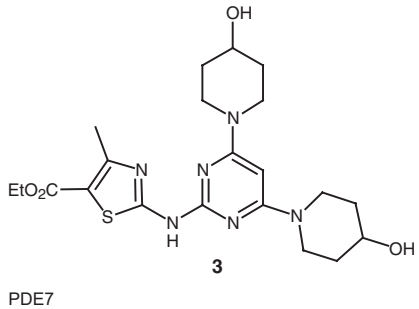


1

In another study it was demonstrated that the combined activity of dual PDE4/PDE7 inhibitors on leukocyte activation may be useful in treating a wide range of immune and inflammatory disorders [32,33]. Compared to lipopolysaccharide (LPS)-injected mice pretreated with vehicle, mice receiving **2** (7.5 mg/kg i.p.) or rolipram alone (5 mg/kg orally) had 52% and 54% reduction, respectively, in LPS-induced serum TNF-α. Mice treated with a combination of rolipram (5 mg/kg orally) plus **2** (7.5 mg/kg i.p.) showed 89% reduction in serum TNF-α. A similar experiment was conducted with **3** in the presence of cilomilast to show an additive effect on the reduction of TNF-α. This increase in activity could result in an increase in the therapeutic window with regard to nausea and emesis and represent an improvement over the administration of a PDE4 inhibitor as a single agent. This experiment did not however give any direct evidence of the efficacy of a specific PDE7 inhibitor on lung inflammation. However, murine and human PDE7 are similarly distributed in lungs and these studies are supportive evidence for a potential role of PDE7 inhibitors as therapeutic agents in airway inflammatory diseases.

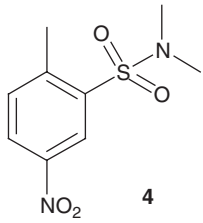


PDE7
IC_{50} M
0.030



PDE7
IC_{50} M
0.060

A new selective PDE7 inhibitor, BRL-50481 (**4**) was recently characterized as an *in vitro* tool in human pro-inflammatory cells [34]. Compound **4** showed only a modest inhibitory effect on human monocytes, lung macrophages and CD8 + T-lymphocytes. However, **4** acted additively with other cAMP-elevating drugs, especially when PDE7A1 was up-regulated. These findings suggest that PDE7 inhibitors could be beneficial in inflammatory/immune indications. Compound **4** has an IC_{50} value = 0.26 μM against PDE7A1 and is devoid of activity against PDE1B, PDE1C, PDE2, PDE3 and PDE5. Only modest activity against PDE4A4 was measured (IC_{50} = 62 μM).



PDE7 IC_{50} = 0.26 M

These studies support exploration of a potential role for specific PDE7 inhibitors in the management of various immunological and airway disorders.

2.5. PDE7 and other potential therapeutic uses

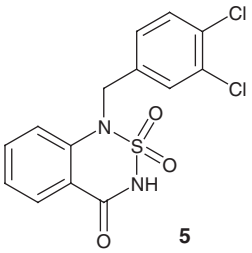
A potential therapeutic role has recently been suggested for PDE7 inhibitors in CNS disorders [8,17,23,35,36], including some controversial studies dealing with Alzheimer's disease [17,23,36]. Other potential therapeutic uses which target cardiovascular diseases [17,21,23], cancers [17,21,23,24], fertility [13,22], bone formation [37–39], as well as muscular dystrophy [40], have also been suggested.

3. SYNTHETIC PDE7 INHIBITORS

3.1. Recent medicinal chemistry developments toward selective PDE7 inhibitors

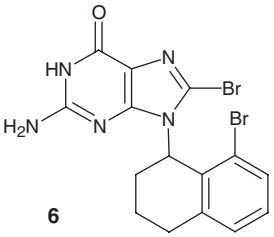
To date, no PDE7 inhibitor has advanced to clinical trials although progress in the design of selective PDE7 inhibitors has been evident.

The first generation of PDE7 inhibitors consisted of a series of benzo- and benzothienothiadiazines dioxides [41,42]; this group was followed by guanine derivatives [43]. The most relevant compounds, **5** and **6**, displayed weak inhibitory activity against PDE7 ($IC_{50} = 8$ and 1.31 μM, respectively).



5

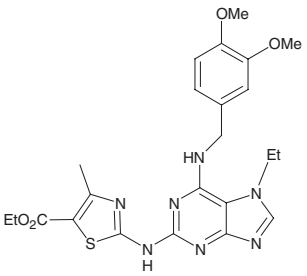
PDE7 IC_{50} = 8 M



6

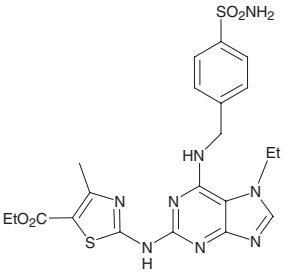
PDE7 IC_{50} = 1.31 M
< 14% inhibition for PDE4 and PDE3 at 10 M

High throughput screening (HTS) of compound libraries led to the discovery of new purine PDE7 inhibitors [44]. The initial lead had an IC_{50} value = 0.15 μM against PDE7 and a poor selectivity ratio (1.5 to 11) against the other PDEs tested (PDE1, 2, 3, 4 and 5). New derivatives were prepared by solid-phase synthesis in order to explore the SAR at the 2- and 6-position of the purine ring. As exemplified by **8** and **9**, the introduction of benzylsulfonamides at position 6 resulted in a dramatic increase in inhibitory activity.



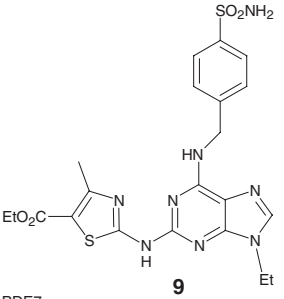
7

PDE7
IC_{50} M
0.15



8

PDE7
IC_{50} M
0.011



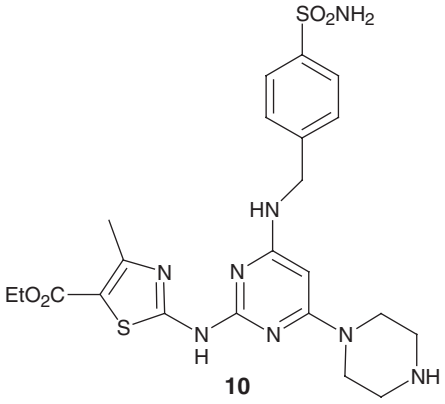
9

PDE7
IC_{50} M
0.010

The structural modifications leading to **8** and **9** resulted in approximately 50-fold and 16-fold increases, respectively, in selectivity PDE7 against PDE4. Compound **9**

displayed an excellent PDE7A selectivity profile with respect to PDE6, PDE8, PDE1 and PDE3 (100-fold, 1000-fold, 200-fold and 5000-fold, respectively). Interestingly, despite the low homology between the PDE5 and PDE7 isoenzymes, the lowest selectivity ratio (range of 0.38-15) for all the reported compounds was identified for PDE5 compared to other PDEs. The physicochemical properties of both compounds **8** and **9** were not appropriate for target validation *in vivo*.

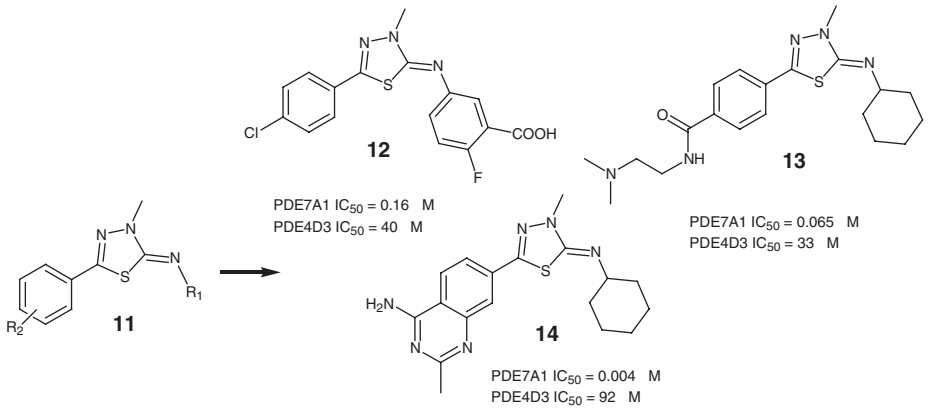
The purine scaffold was subsequently truncated to pyrimidine. The corresponding pyrimidine series was then optimized to yield **10** ($IC_{50} = 10\,nM$) [45] and another analog ($IC_{50} = 60\,nM$) (structure unavailable) which displayed acceptable pharmacokinetic parameters in mice and rats [46]. The latter compound was reported to inhibit T-cell proliferation *in vitro* ($IC_{50} = 200\,nM$) and to be selective *vs* a panel of receptors and ion channels. However, oral dosing (30 mg/kg, b.i.d.) of this pharmacological tool to a KLH-antibody mouse model for up to 10 days had no effect on antibody production, despite reaching high plasma concentrations (5 to 25 μM). Since no difference in T-cell proliferation was observed between incubation with T-cells and after administration of the PDE7 inhibitor to PDE7 knock-out mice or wild-type mice, it was suspected that this compound may inhibit T-cell growth by another mechanism [46].



10

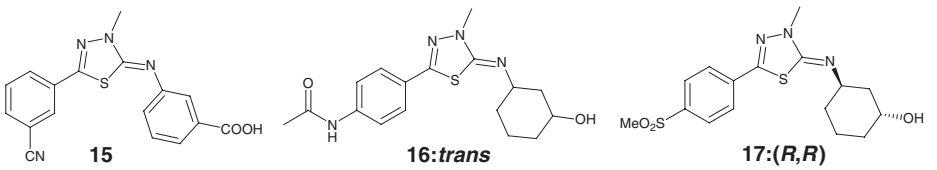
PDE7 IC_{50} = 10 nM

A potent and selective series of thiadiazole PDE7 inhibitors was developed *via* compound library screening and structure-activity relationship studies [47]. Starting from the generic template **11**, R1 and R2 were explored in detail leading to several highly selective compounds as illustrated by **12**, **13** and **14**. The cyclohexyl and the *meta*-benzoic group (R1) were preferred substituents to enhance potency and selectivity. The *meta*-benzoic acid derivative was found to be more than 150-fold selective *vs* PDE4D3 and at least 50-fold selective *vs* PDE1, PDE3A3, and PDE5.



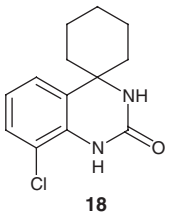
The relative position of the COOH group on the phenyl ring in **12** was critical for inhibitory activity. Moreover, bioisosteric replacements led to a dramatic decrease in selectivity. A broad SAR study encompassing R2 led to **13** and **14**. R2 groups with hydrogen bond donor (HBD) and hydrogen bond acceptor (HBA) properties at the *meta* and *para* positions were critical determinants for inhibitory activity associated with a significant level of selectivity over PDE4. Finally, 4-amino-quinazoline derivatives, exemplified by **14,** were prepared; the latter compound had an IC_{50} value = 3.9 nM *vs* PDE7 and was greater than 2000-fold selective against PDE4.

The optimization of the pharmacokinetic properties (especially rat bioavailability) of the thiadiazoles was also reported [48]. Metabolism-directed optimization studies guided the design of several potent inhibitors, which were used for *in vivo* target validation. The identification of the rat metabolites enabled the development of a successful strategy to minimize hepatic and extra-hepatic clearance. The combination of amide or amide bioisosteres (R2 groups) with (*R*)-hydroxy cyclohexyl or *meta*-benzoic acid fragments (R1 groups) afforded the potent PDE7 inhibitors exemplified by **15**, **16 (***trans*-isomer), and **17** (*R*,*R*-enantiomer) which displayed good rat pharmacokinetic properties.



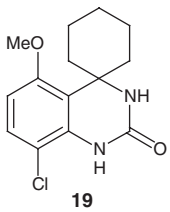
A series of spiroquinazolinone PDE7 inhibitors was identified by HTS [49,50]. Preliminary SAR studies around the relatively potent hit compound **18** (IC_{50} = 170 nM) revealed the importance of the 8-chloro substituent, as well as the preference for a spirocyclohexyl or cycloheptyl ring system. Further optimization led to two closely related series of 6-aryl substituted and 5-alkoxy

8-chloro-spiroquinazolinones. In the former, substitution of the 6-phenyl at the *para* or *meta* positions was tolerated. In order to improve solubility, ionizable side chains were introduced as exemplified by the potent and selective PDE7 inhibitor **20**.



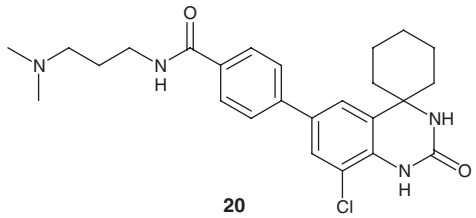
18

PDE7A1 IC_{50} = 0.17 M



19

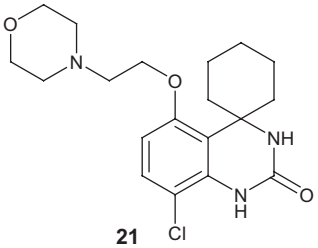
PDE7A1 IC_{50} = 0.014 M



20

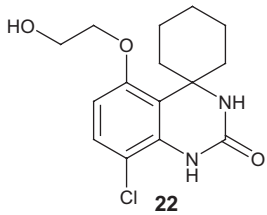
PDE7A1 IC_{50} = 0.024 M
PDE4D3 IC_{50} = 1.5 M

To reduce the overall hydrophobicity of the 5-methoxy 8-chloro-spiroquinazolinone lead **19**, polar, neutral, as well as acidic, and basic side chains were introduced on the methoxy group. Compounds **21**, **22** and **23** were reported to display an acceptable balance between inhibitory activity against PDE7, selectivity *vs* PDE1, 3, 4 and 5, as well as solubility. The latter three PDE7 inhibitors represent interesting, structurally related, pharmacological tools with differing physicochemical properties for *in vitro* assays. Analogs **22** and **23** have suitable *in vivo* pharmacokinetic profiles to be used in rat models for target validation [50].



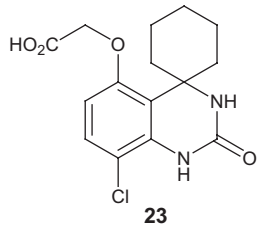
21

PDE7A1 IC_{50} = 0.038 M
PDE4D3 IC_{50} = 7.35 M



22

PDE7A1 IC_{50} = 0.055 M
PDE4D3 IC_{50} = 12 M



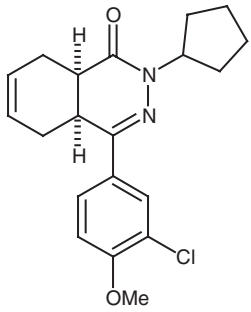
23

PDE7A1 IC_{50} = 0.046 M
PDE4D3 IC_{50} = 15 M

3.2. PDE7/4 dual inhibitors

Recently, IBFB-211913 (structure unavailable) was claimed as a new PDE4/7 inhibitor. It is reported under development for the treatment of asthma, autoimmune diseases and psoriasis [51]. Future data related to this compound will be helpful to assess and understand the intrinsic contribution of the PDE7 inhibition toward efficacy and side effects. In relation to this topic, several patents claimed the use of dual inhibitors (PDE7/PDE4) to synergize pharmacological effects and to increase the therapeutic index [32,33]. A series of phthalazinones have been disclosed as dual

PDE7/4 inhibitor [52]. The most potent example cited is compound **24,** which is 23-fold less active against PDE7 than PDE4.

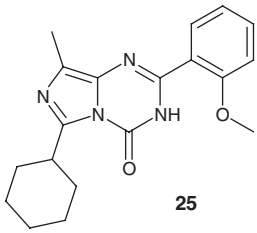


24

PDE7 IC_{50} = 18 nM
PDE4 IC_{50} = 0.78 nM

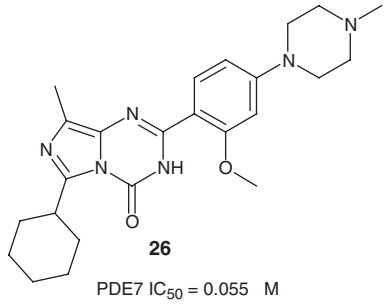
3.3. Other structurally diverse inhibitors

An additional number of structurally diverse PDE7 inhibitors, derived mainly from the recent patent literature (first generation of PDE7 inhibitors have been reviewed elsewhere [53]), provide useful structural information to assess the critical determinants for PDE7 inhibition. Among these is a novel series of imidazotriazinones exemplified by **25**, **26** and **27** [54]. The selectivities of these compounds are claimed to be more than 10-fold versus PDE4.



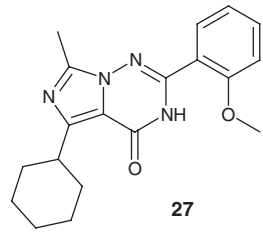
25

PDE7 IC_{50} = 0.34 M



26

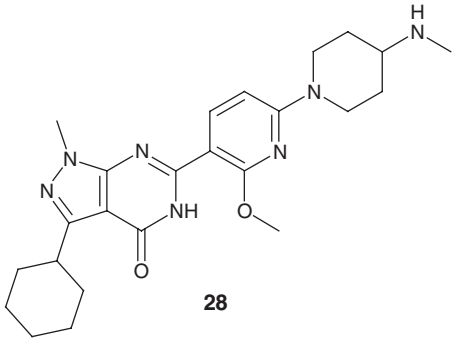
PDE7 IC_{50} = 0.055 M



27

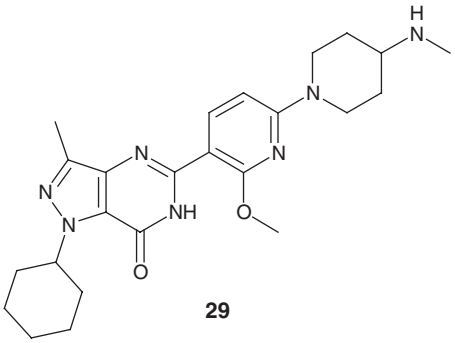
PDE7 IC_{50} = 0.49 M

Introduction of the piperazine moiety (**26**) resulted in a 6-fold increase in activity compared to **25**. Interestingly, both bioisosteres **25** and **27** displayed similar inhibitory activity. Pyrazolo analogues exemplified by **28** and **29** showed IC_{50} values in the 3 nM range and were found to be more than 370-fold selective versus PDE4 [55].



28

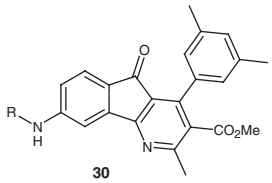
PDE7 IC_{50} = 0.0026 M
PDE4 IC_{50} = 1.2 M



29

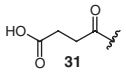
PDE7 IC_{50} = 0.0032 M
PDE4 IC_{50} = 0.98 M

A novel series of arylindenopyridines was recently described [56,57]. Compounds **30**, **35** and **36** exhibited high PDE7 inhibitory activity with sub-micromolar activity *vs* PDE4 and PDE5. Compounds within this chemical series are also A2a receptor antagonists. The level of inhibitory activity (<10 nM) was maintained in the same range when the R group (**30**) was substituted with a variety of side chains as illustrated by **31**, **32**, **33** and **34**. The selectivity of PDE7 *vs* PDE5 is dependent on the nature of the side chain (*cf* **33** and **34** with **31** and **32**).



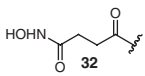
30

R



31

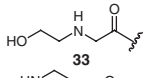
PDE7 IC_{50} = 0.0035 M
PDE4 IC_{50} = 0.954 M
PDE5 IC_{50} = 0.183 M



32

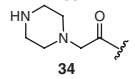
PDE7 IC_{50} = 0.0053 M
PDE4 IC_{50} = 0.875 M
PDE5 IC_{50} = 0.185 M

R



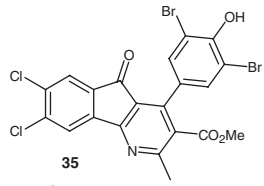
33

PDE7 IC_{50} = 0.0074 M
PDE4 IC_{50} = 0.684 M
PDE5 IC_{50} = 2.4 M



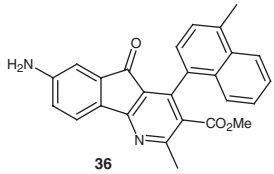
34

PDE7 IC_{50} = 0.008 M
PDE4 IC_{50} = 0.702 M
PDE5 IC_{50} = 3.7 M



35

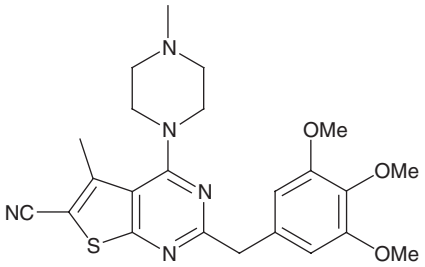
PDE7 IC_{50} = 0.0087 M
PDE5 IC_{50} = 0.557 M



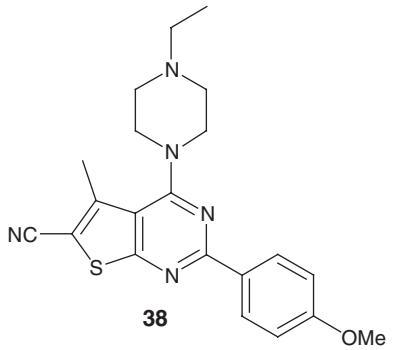
36

PDE7 IC_{50} = 0.005 M
PDE4 IC_{50} = 0.204 M
PDE5 IC_{50} = 2.33 M

Thienopyrimidine derivatives such as **37** and **38** were recently reported as sub-micromolar PDE7 inhibitors [58].

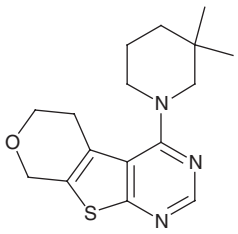


37

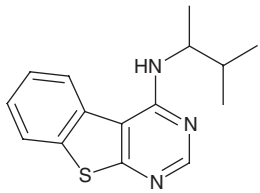


38

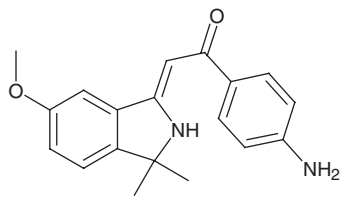
Although no activities were reported, tricyclic heteropyrimidine analogs including **39** and **40** were described [59,60]. Interestingly, their specific use as PDE7B inhibitors was claimed, particularly for osteoporosis, osteopenia and respiratory disorders such as asthma.



39



40

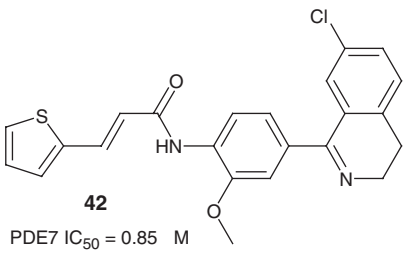


41

PDE7 IC_{50} = 23 nM

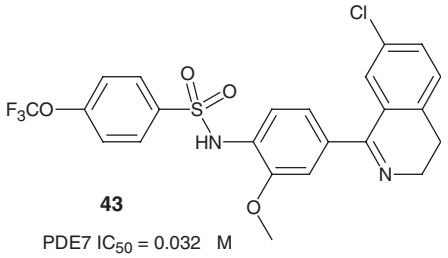
A large series of aza-heterobicyclic PDE7 inhibitors were reported in the patent literature [61]. As an example, compound **41** was claimed to have an IC_{50} value = 23 nM.

Phenyl dihydroisoquinolines, with a range of inhibitory activity against PDE7, were recently described [62,63]. Based on the activities disclosed, the sulfonamide derivatives such as **43** appear to be more potent than their amide counterparts, represented by **42**.



42

PDE7 IC_{50} = 0.85 M



43

PDE7 IC_{50} = 0.032 M

Several other patent applications with structurally diverse series have been published [53] but without associated biological data.

4. CONCLUSION

The first generation of phosphodiesterase inhibitors was inadequate as pharmacological tools to carry out *in vitro* and *in vivo* target validation studies on airway and immune diseases. However, significant additive effects with other cAMP-elevating drugs (especially PDE4 inhibitors) have been identified. The use of PDE7 inhibitors in combination with other cAMP elevating drugs represents an attractive approach to increase efficacy and perhaps to reduce any potential side effects of the latter. The development of new, potent, and selective PDE7 inhibitors has engaged many researchers over the past few years. These novel inhibitors should help to define the pathophysiological role(s) of this enzyme.

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Inhibitors of Anti-apoptotic Proteins for Cancer Therapy

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Contents

1. INTRODUCTION

Apoptosis, or programmed cell death, is the principal mechanism through which unwanted or damaged cells are safely eliminated. Just as diverse growth stimuli ultimately induce cellular proliferation through common pathways in the cell cycle, a set of evolutionarily conserved genes regulate the final aspects of the cell-death pathway. Balance between these proliferative and apoptotic processes is essential for normal tissue homeostasis. Although cancer has historically been considered a disease of uncontrolled cell division, abnormal resistance to apoptosis is now understood to contribute to tumor initiation, progression and resistance to chemotherapy [1].

A family of aspartate specific cysteine proteases called caspases drives apoptotic cell death. Members of this protein family normally exist as pro-enzymes that are activated by proteolytic cleavage and can be functionally subdivided into a hierarchy of 'initiator' and 'executioner' caspases. Initiator caspases (caspases 6, 8, 9, 10, 12) are activated during early apoptosis signaling and serve to propagate the death signal by cleaving and activating executioner caspases (caspases 2, 3, 7). The resulting proteolytic cascade leads to cleavage of numerous intracellular targets, and ultimately to cell death and the formation of apoptotic bodies that are rapidly engulfed and cleared by other cells [2,3].

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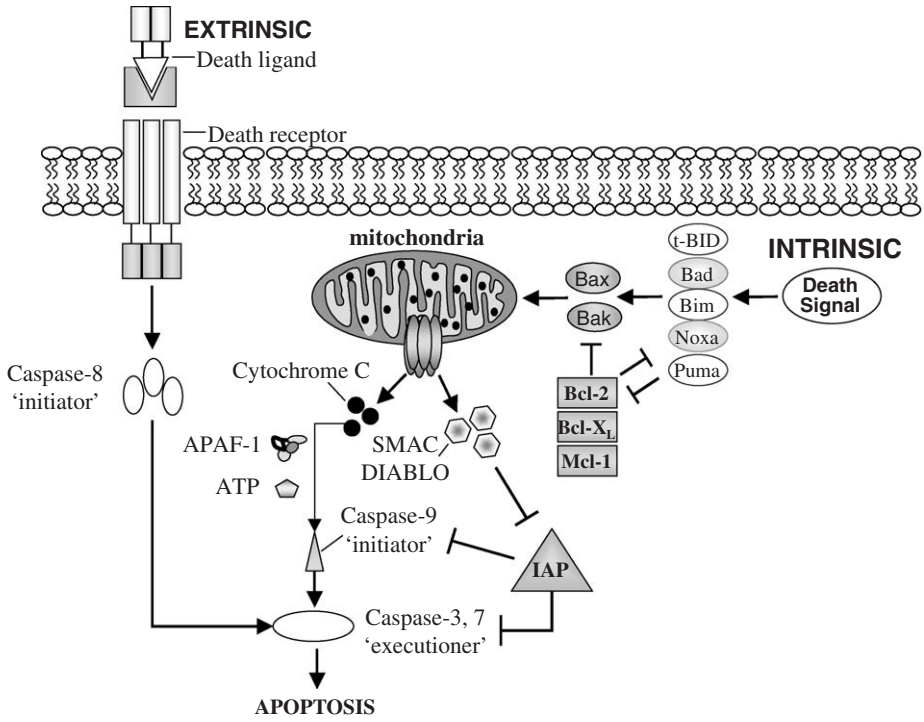


Fig. 1. Schematic representation of the intrinsic (mitochondrial) and extrinsic (death receptor) apoptotic signaling pathways

Two apoptosis signaling pathways exist that differ in the origin of their death signal, but converge upon a common pathway (Fig. 1). The extrinsic or death receptor pathway is initiated by an extracellular stimulus of a membrane bound receptor such as the action of Fas ligand on the Fas receptor. Upon surface activation, the cytoplasmic side of the receptor recruits and activates initiator caspases (e.g. caspase-8) that in turn activate executioner caspases (e.g. caspase-3). The intrinsic or mitochondrial apoptotic pathway responds to signals of stress or cell damage such as hypoxia, detachment, disregulated cell cycle, DNA damage or chemotherapy treatment. This results in the release of cytochrome *c* from the mitochondria into the cytosol where if forms a complex with Apaf-1 (apoptotic protease activating factor-1), dATP (or ATP) and the inactive initiator caspase procaspase-9. Within this complex, known as the apoptosome, caspase-9 is activated. Once activated, caspase-9 cleaves and activates executioner caspases (e.g. caspase-7) [4].

Programmed cell death is a highly conserved and tightly regulated process that is governed by the delicate checks and balances between families of pro-apoptotic and anti-apoptotic proteins. Upsetting this balance leads to deficient apoptotic signaling and is a common mechanism by which tumor cells can develop a survival benefit or resistance to chemotherapy. Two groups of proteins, members of the B-Cell

Lymphoma (Bcl-2) and inhibitors of apoptosis protein (IAP) families, are endogenous inhibitors of apoptosis that are overexpressed in many tumor cells. Members of the Bcl-2 and IAP families are non-enzymatic proteins that exert their inhibitory function through direct protein–protein interactions with their proapoptotic counterparts. Anti-apoptotic Bcl-2 family proteins act directly at the mitochondria and function to block cytochrome *c* release and can therefore inhibit only the intrinsic cell death pathway [5]. IAP proteins act further downstream by directly binding to and inhibiting both initiator and effector caspases and can therefore block both the extrinsic and the intrinsic pathways [6,7]. Small molecule antagonism of these endogenous inhibitors of apoptosis requires the disruption of high affinity protein–protein interactions. Affinities of small molecule inhibitors are derived from competition binding studies using peptides that mimic one of the protein binding partners. Fluorescent polarization assays (FPA) detect the displacement of fluorescently labeled peptide [8] while biosensor assays typically detect displacement of a protein from an immobilized peptide binding partner [9]. Differences in chemical shift perturbations observed in the NMR HSQC spectra of a target protein in the presence vs. absence of test compound cannot only approximate affinity, but also ensure binding in the expected region of the protein.

2. BCL-2 FAMILY PROTEIN INHIBITORS

The Bcl-2 family of genes encodes a family of closely related proteins that possess either pro- or anti-apoptotic activity and share up to four Bcl-2 Homology (BH) domains [10–13]. The anti-apoptotic family members (Bcl-X_L, Bcl-2, Bcl-w, A1/BFL-1, Mcl-1, Bcl-B) are characterized by three or four BH domains, designated BH1-4. The pro-apoptotic proteins can be further subdivided into those that incorporate three BH domains (Bax, Bak, Mtd/Bok) and the BH3-only proteins (Bad, Bik, Bid, Bim, Hrk, Blk, Bnip3, Noxa, Puma). The interplay between these three groups of proteins serves as the gateway to the intrinsic apoptosis pathway.

The multidomain pro-apoptotic proteins Bax and Bak are direct mediators of apoptosis and are absolutely required for the initiation of the mitochondrial apoptosis pathway [14]. Upon activation, these normally monomeric proteins oligomerize at the mitochondrial outer membrane, resulting in the release of cytochrome *c* and other apoptotic factors from the intermembrane space [15]. Anti-apoptotic Bcl-2 family proteins are primarily localized to mitochondria and inhibit cytochrome *c* release by blocking Bax/Bak activation [5]. They do so by the direct binding and sequestration of pro-apoptotic BH3-only proteins [16]. The BH3-only pro-apoptotic proteins act as molecular sentinels that are mobilized and activated in response to cellular damage. Some BH3-only proteins (e.g. Bim and Bid) can directly bind and activate Bax and Bak, while other (e.g. Bad) bind only to the anti-apoptotic proteins and act as trans-dominant inhibitors by displacing the BH3-only proteins that are capable of directly activating Bax and Bak [17].

The interactions between Bcl-2 family members are mediated through the binding of an amphipathic α-helix on a BH3-only protein to a hydrophobic surface groove

on its multidomain partner [18]. The three dimensional structures of Bcl-X_L, Bcl-2, Bcl-w and most recently Mcl-1 have been solved both alone and when bound by α-helical BH3 domain peptides derived from the pro-apoptotic proteins [19–22]. This work has not only enabled development of high throughput screening approaches for antagonists, but has also defined the critical binding interactions between these proteins, rendering them tractable drug targets.

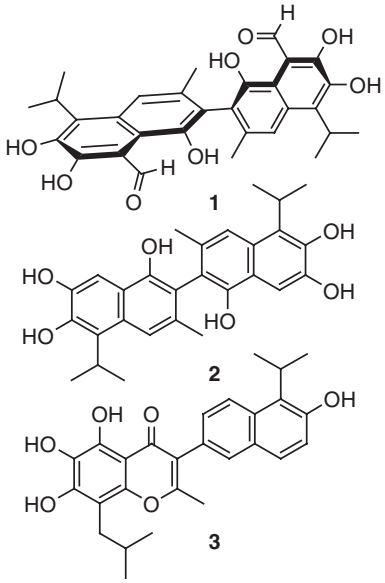
2.1. Peptide BH3 mimetics

The most direct approach to Bcl-2/Bcl-X_L inhibition is the use of peptides derived from proapoptotic BH3 domains. The activities of several synthetic BH3 domain peptides encompassing elements identified as critical for Bcl-X_L and Bcl-2 binding have been investigated as potential therapeutic agents. To overcome cellular permeability issues, these peptides have been conjugated to cell permeable moieties. CPM-1285 contains the mouse Bad BH3 domain (aa 140-165) conjugated at the N-terminus with decanoic acid [23]. This peptide conjugate exhibited high affinity for Bcl-2 ($IC_{50} = 130$ nM) and was shown to efficiently enter HL-60 human tumor cells by confocal microscopy and induce apoptosis *in vitro*. It also increased median survival time in a murine model of human myeloid leukemia. The internalization domain of the Antennapedia (Ant) protein has also been used as a cell membrane transporter of a Bak BH3 peptide. This cell permeable, fusion peptide containing a 16 amino acid Ant sequence and a 19 amino acid Bak sequence (aa 71-89) induced cytochrome *c* and caspase dependent apoptosis in HeLa cells and was able to reverse Bcl-X_L protection from Fas induced apoptosis [24].

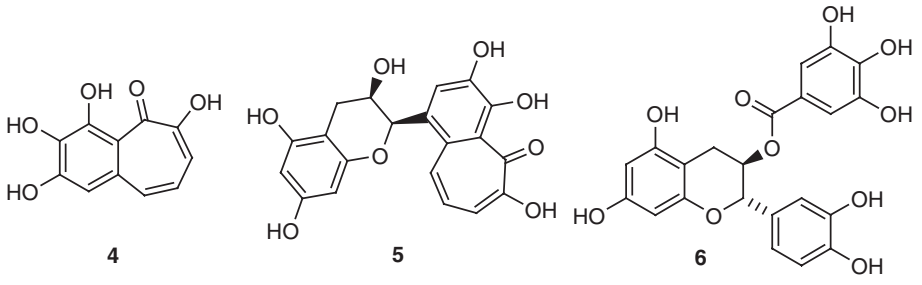
2.2. Natural product analogs

Gossypol is a natural product derived from cottonseed extracts that causes male infertility and has been studied extensively in humans as a male contraceptive. This compound is cytotoxic to a variety of cancer cell lines and was advanced to human clinical trial on this basis even though its mechanism of action was not clear. More recently, gossypol was discovered in a natural product library screens to bind Bcl-X_L [25]. Given its clinical history and this possible mechanistic rational, gossypol has been the focus of many recent investigations [25–30]. The (-)-enantiomer, **1**, is responsible for the majority of both the cytotoxic and spermicidal activities. It binds both Bcl-X_L ($K_i = 0.57\,\mu M$) and Bcl-2 ($K_i = 0.46\,\mu M$) and induces cytochrome *c* release, caspase activation and apoptotic death in numerous cell lines. (-)-Gossypol, **1**, reverses the protection afforded by both Bcl-2 and Bcl-X_L overexpression in Jurkat T leukemia cells with an IC_{50} of 18.1 μM and 22.9 μM, respectively, and dose dependently induces cytochrome *c* release from isolated mitochondria in these cell lines [31]. In the *in vivo* setting, **1** significantly enhanced the antitumor activity of X-ray irradiation leading to tumor regression in a PC-3 murine xenograft model of human prostate cancer [32] and potentiated the effect of CHOP therapy

(cyclophosphamide-adriamycin-vincristine-prednisone) in mouse xenograft models of diffuse large cell lymphoma [33].

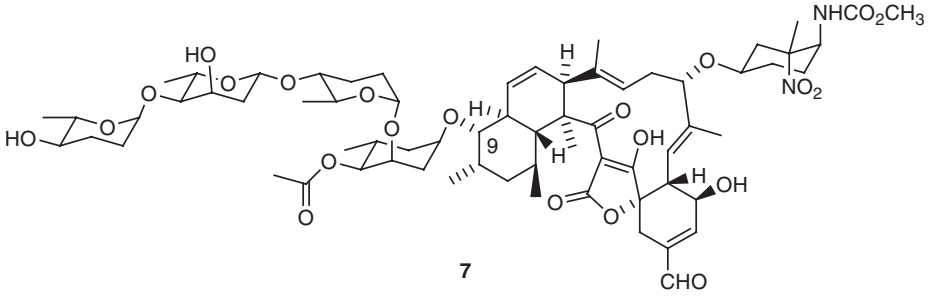


Guided by NMR structural analysis and molecular modeling, several analogs have been designed that lack the two aldehyde groups found in gossypol [34]. One of these, apogossypol **2**, retains moderate binding affinity for Bcl-X_L ($K_i = 2.3\,\mu M$). Time lapsed confocal microscopy experiments show the ability of **2** to displace a fluorescently labelled BH3-only protein (GFP-Bcl-G_s) from the mitochondria of cells containing wild-type Bcl-X_L but not those transfected with an inactive Bcl-X_L mutant (R139M). This compound has also been evaluated against 12 primary patient-derived samples of chronic lymphocytic leukemia to show a response in only half the samples with a composite LD_{50} of approximately $16\,\mu M$. Most recently, a synthetic analog **3**, designed based on the 3D structure of gossypol in complex with Bcl-X_L has been reported to bind both Bcl-2 ($K_i = 0.088\,\mu M$) and Bcl-X_L ($K_i = 1.49\,\mu M$) and to inhibit growth of human breast (MDA-MB-231, $IC_{50} = 1.54\,\mu M$) and prostate (PC-3, $IC_{50} = 1.82\,\mu M$) cancer cell lines [35].



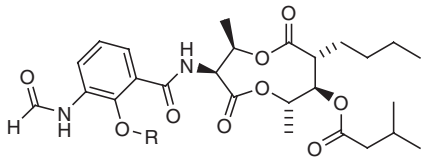
High throughput screens have identified three additional classes of polyphenols that bind Bcl-X_L [36]. NMR structural studies have confirmed binding of **4**, **5**, and **6** to the BH3 binding groove of Bcl-X_L. Purpurogallin, **4**, is an antioxidant found in edible oils and has moderate affinity for Bcl-X_L ($K_i = 2.2\,\mu M$). Theaflavanin, **5**, and (-)-catechin-3 gallate, **6**, are black and green tea extracts, respectively, with submicromolar affinities for both Bcl-X_L (0.25 μM, 0.12 μM, respectively) and Bcl-2 (0.28 μM, 0.40 μM, respectively). Although the gallate group of **6** is required for binding, a detailed structure activity relationship has not been reported. A mechanistic link between these effects and modulation of Bcl-2 family members has not been established.

Tetrocarcin A (TC-A), **7**, is a Gram-positive antibacterial agent isoloated from *Actinomycete* indentified in a cell-based high throughput screen of natural product libraries to reverse the protection afforded by Bcl-2 and Bcl-X_L overexpression against pro-apoptotic stimuli [37]. Modification of the C-9 sugar subunit produced compounds that maintain the ability to reverse Bcl-2 protection, but lack the parent antibacterial activity. Removal of the C-9 sugar moiety altogether or replacement with non-sugar substituents abolished both activities [38]. Although TC-A induces apoptosis in a concentration dependent fashion, this effect is independent of the expression level of Bcl-2 family member (Bcl-2, Bax or Bid). Direct binding of **7** to Bcl-2 family proteins has not been demonstrated. The mechanism of action of **7** remains controversial and has recently been postulated to involve activation of the ER-stress pathway [39,40].

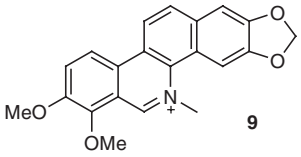


7

Antimcycin A, a *Streptomyces* derived antibiotic was identified from a screen of compounds with known effects on mitochondrial function for their ability to selectively kill cells with high versus low Bcl-X_L expression in isogenic cell lines [41,42]. High Bcl-X_L expression levels not only did not protect against antimycin A, but actually markedly enhanced antimycin A induced apoptotic cell death. The intrinsic fluorescence of antimycin A3, **8a**, increased proportionally in the presence of increasing concentrations of Bcl-2 protein with maximal effect at a 1:1 stoichiometry, suggesting a direct binding interaction. This effect was competitively reversed by addition of increasing concentrations of Bak BH3 peptide suggesting specific binding to the BH3 hydrophobic binding groove. Compound **8a** has an apparent K_d of 2.5 μM for Bcl-2. Although 2-methoxy antimycin A3, **8b**, has no effect on mitochondrial respiration, it retains binding affinity for Bcl-2 and the ability to selectively kill Bcl-X_L overexpressing cell lines. 2-Benzoyl antimycin A3, **8c**, has no detectable affinity for Bcl-2 and no effect on Bcl-X_L overexpressing cells.



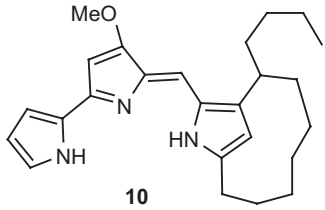
8a, R = H
8b, R = Me
8c, R = PhC(O)-



9

Chelerythrine, **9**, was identified in an FPA-based high throughput screen of a natural product library consisting of 107,423 extracts from a variety of sources [43]. It exhibits moderate Bcl-X_L binding affinity (FPA $IC_{50} = 1.5\,\mu M$) and disrupts Bak/Bcl-X_L interactions in an *in vitro* pulldown assay. Chelerythrine concentration dependently induces cytochrome *c* release from isolated mitochondria and shows specific killing at concentrations greater than $2\,\mu M$ in the Bcl-X_L overexpressing tumor cell line SH-SY5Y.

The GX15 series of Bcl-2 inhibitors is derived from the family of prodigiosin tripyrrole natural products that are produced by microorganisms such as *Streptomyces* and *Serratia* and contain the common 4-methoxy-2,2′-bipyrrole ring system found in **10**. The antibiotic, cytotoxic and more recently immunosuppressive activities of this family of natural products have been ascribed to a number of mechanisms of action and have recently been reviewed [44]. GX15 analogs have been shown by NMR studies and molecular modeling to competitively bind Bcl-2 [45] and **10** binds Bcl-w with three-fold higher affinity than Bik BH3 peptide [46]. An advanced lead in this series, GX15-070, reportedly binds Bcl-w ($K_d = 0.44\,\mu M$) and Mcl-1 ($K_d = 0.49\,\mu M$), disrupts Mcl-1/Bak interactions in SK-Mel melanoma cells and is cytotoxic ($EC_{50} = 1.7\,\mu M$) to primary patient derived B-cell chronic lymphocytic leukemia (CLL) cells. GX15-070 also exhibited *in vivo* antitumor efficacy in murine models of cervical and prostate carcinomas [47]. Phase I clinical trials for the treatment of CLL were initiated with GX15-070 in January 2005 [48].



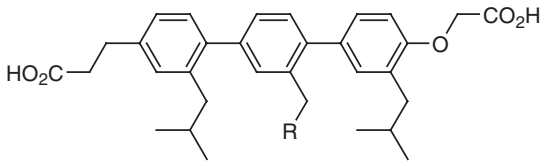
10

2.3. Small molecule inhibitors

2.3.1. Proteomimetics

Although there have been no reports of traditional peptidomimetic approaches to BH3 mimetics, several 'proteomimetics' have been reported that involve *de novo* design of molecular scaffolds to mimic the surface functionality projected along one face of an α-helix. These scaffolds mimic secondary protein structure and the proper

positioning of the critical *i*, $i+3$ or $i+4$ and $i+7$ hydrophobic side chains of the BH3 domain peptides. The terphenyl **11**, oligoamide foldamer **12**, and the terephthalamide **13** have all been shown to competitively displace a Bak derived peptide from Bcl-X_L with binding affinities of 0.114 μM, 1.60 μM and 0.78 μM, respectively [49–51].



R = 1-Napthalene

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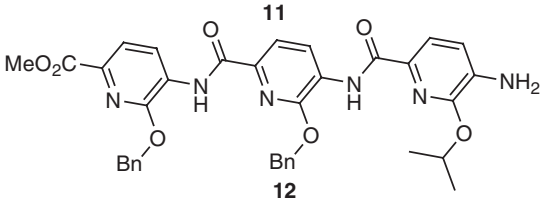

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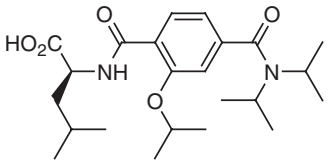
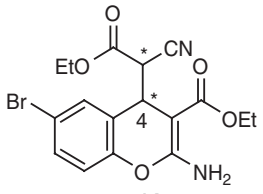


13

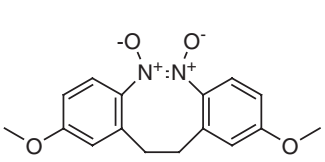
2.3.2. Leads discovered by virtual screening

A homology model of Bcl-2 based on the known X-ray and NMR structures of Bcl-X_L has been utilized by two different groups for virtual ligand screening. A screen of a 193,833 compound library from the MDL/ACD 3-D database culminated in the discovery HA14-1, **14**, which was evaluated as a mixture of diastereomers [52]. Compound **14** possesses modest affinity fro Bcl-2 ($IC_{50} = 9.0$ μM) and concentration dependently decreases viability of HL-60 tumor cells. Mechanism based activity is suggested by the ability of **14** to induce apoptotic cell death, activate caspase-3 and caspase-9 and decreased mitochodrial membrane potential and by the observation that this activity is dependent on the presence of Apaf-1.



14

A separate screening effort of the 3-D database of the NCI compound collection of 206,000 small molecules and natural products was conducted in a similar fashion. Binding confirmation of hits by FPA resulted in the identification of several compounds with low micromolar Bcl-2 binding affinities [53]. BL-11, **15**, exhibited modest affinity to Bcl-X_L ($IC_{50} = 9.0\,\mu M$) and Bcl-2 ($IC_{50} = 10.4\,\mu M$) and dose dependently induced apoptotic cell death ($IC_{50} = 10\,\mu M$) in HL-60 cells. This activity correlates with Bcl-2 protein levels in a panel of 4 human tumor cell lines [54]. Based on hits from this virtual screening campaign, medicinal chemistry efforts directed to improve binding affinity and cellular activity have led to the discovery of YC-137, **16**. Interestingly, YC-137 selectively binds Bcl-2 ($IC_{50} = 1.3\,\mu M$) over Bcl-X_L($IC_{50} > 100\,\mu M$). Consequently, **16** can reverse the protection afforded by Bcl-2 but not Bcl-X_L overexpression from IL-3 deprivation in FL5.12 and HCD-57 cells. The expression level of Bcl-2 also correlated with the apoptotic response to YC-137 in a panel of human breast cancer cell lines. Resistance to YC137 induced by prolonged exposure to sublethal concentrations was accompanied by decreased Bcl-2 protein levels but had no effect on Bcl-X_L protein levels. YC-137 activity was shown to be at least modestly tumor specific with no effect on normal epitheal cells, myoblasts or PMBCs at concentrations up to $0.5\,\mu M$, while peak apoptotic effect in tumor cell lines was seen at $0.3\,\mu M$ [55].



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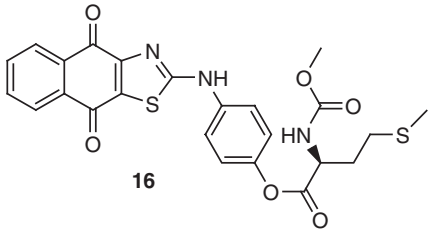
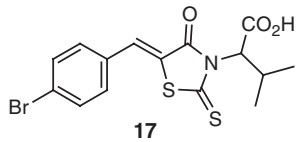


16

2.3.3. Leads discovered by chemical library screening

A high throughput screen of 16,320 commercially available chemicals employing a fluorescence polarization assay led to the identification of small molecule inhibitors BH3I-1, **17** and BH3I-2, **18** with Bcl-X_L K_i values of 2.4 and $4.1\,\mu M$, respectively [56]. Binding to the hydrophobic cleft of Bcl-X_L was confirmed by NMR structural studies that also suggested these compounds induce a protein conformational change similar to that seen upon Bak BH3 peptide binding. Both **17** and **18** concentration dependently disrupt the interaction of t-Bid with Bcl-X_L in an *in vitro* pulldown assay and disrupt Bcl-X_L/Bax and Bcl-X_L/Bad heterodimerization in a cellular context. The BH3I's induced cytochrome *c* release, caspase-3 and caspase-9 activation and apoptotic cell death in JK cells. More recently, a group of structurally related thiaolidenediones including Δ2-CG, **19**, has been reported to weakly bind both Bcl-X_L ($IC_{50} = 17\,\mu M$) and Bcl-2 ($IC_{50} = 22\,\mu M$) [57].



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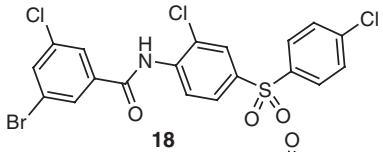

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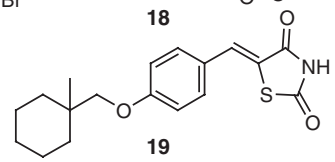
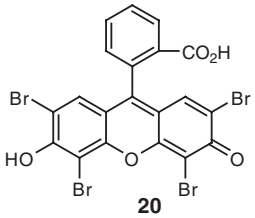


19

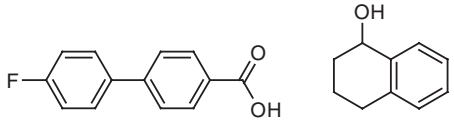
A screen of a 10,000 compound library based on a Biacore biosensor assay for inhibitors of the interaction between Bax and Bcl-X_L identified **20** as the only compound that induced > 50% inhibition. Several close structural analogs were less efficient inhibitors. The activity of **20** was confirmed by its ability to disrupt Bax/Bcl-X_L interactions in an *in vitro* pull-down assay. Compound **20** induces apoptosis at high concentrations in MCF7 human breast cancer cell line overexpressing Bcl-X_L and also increases the sensitivity of these cells to methylprednisolone [9].



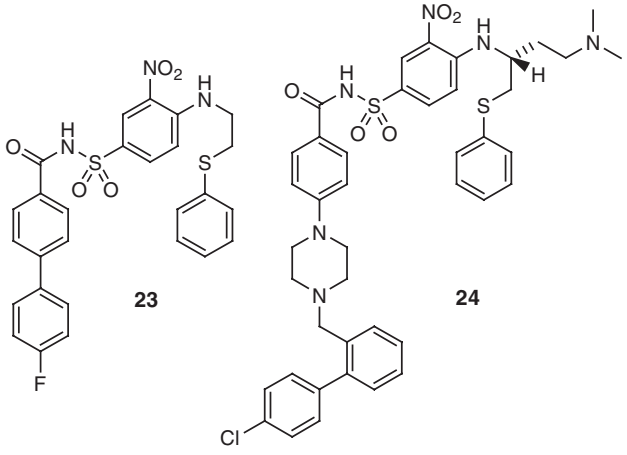
20

An NMR-based fragment screening approach identified 4-fluorobiphenyl-4 carboxylic acid, **21**, and 5,6,7,8-tetrahydronapthalen-1-ol, **22**, as weak ligands (0.30 mM and 4.3 mM, respectively) for two distinct but proximal binding sites within the hydrophobic binding groove of Bcl-X_L. Appropriate linkage of these two subunits through an acylsulfonamide tether followed by site-directed parallel synthesis led to the identification of **23** with an $IC_{50} = 36$ nM for Bcl-X_L. This activity was attenuated by a factor of > 280 in the presence of 1% human serum due to the tight binding of **23** to human serum albumin domain III (HSAIII). Structure-based design utilizing NMR solution structures of analogs bound to HSAIII, Bcl-X_L and Bcl-2 led to the discovery ABT-737, **24**, that binds with high affinity ($K_i \leqslant 1$ nM) to Bcl-X_L, Bcl-2 and Bcl-w, but not to Mcl-1 and A1. This affinity is maintained in the presence of 10% human serum (Bcl-X_L, $IC_{50} = 35$ nM). ABT-737 does not directly induce cytochrome *c* release from isolated mitochondria, but reverses the protection afforded by both Bcl-2 and Bcl-X_L from activating BH3 only stimuli such as t-Bid. Importantly, this activity is completely Bax/Bak dependent. This compound also efficiently disrupts the interaction of Bcl-X_L and BH3 only proteins in a cellular context based on

mammalian two hybrid and colocalization of fluorescently labeled proteins. ABT-737 potentiates the effects of chemotherapy and radiation in tumor cell lines *in vitro*, but exhibits potent single agent activity against a subset of tumor types including small cell lung cancer, leukemias and lymphomas. The activity against leukemia and lymphomas has been extended to primary patient derived samples where it induces potent cell killing ($IC_{50} < 100\,nM$) in 17 of 19 follicular lymphoma and chronic lymphocytic leukemia samples tested. The less active antipode of **24** was employed as a loss of function control in all assays and showed little to no effect. ABT-737 significantly improved survival in a murine tumor model of disseminated disease using DoHH-2 lymphoma cell line and induced complete regression of established tumors in xenograft models of SCLC (H146, H1963). Upon removal of treatment, the tumors did not grow back in a high percentage of animals [58].



21 **22**



23 **24**

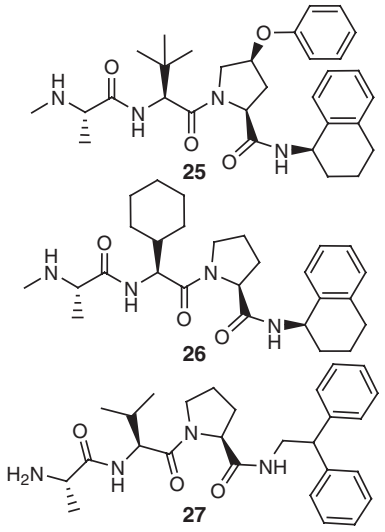
3. INHIBITORS OF XIAP

Human X-linked IAP (XIAP), the best-characterized among the IAP family members, is believed to directly inhibit caspases via its baculovirus IAP repeat (BIR) domains. The BIR3 domain of XIAP binds directly to the small subunit of caspase-9 [6,59]. As evident from the X-ray structure of the complex, XIAP sequesters caspase-9 in an inactive monomeric state, thus preventing formation of catalytically active caspase-9 [60]. A region containing the BIR2 domain of XIAP directly binds to and inhibits the executioner caspases 3 and 7 which prevents the proteolytic cascade that results in apoptosis [61–63]. Recently, a mammalian protein, SMAC (also known as DIABLO), was found to trigger apoptosis by acting as an endogenous antagonist of

XIAP [64,65]. SMAC binds to the BIR3 domain of XIAP in the same binding groove that bind caspases thus preventing these interactions. The structural basis for the binding of SMAC to XIAP has been elucidated by NMR and X-ray structural analysis [66–68]. The SMAC N-terminus binds the BIR3 domain ($K_d \approx 500$ nM) in an extended conformation with only the first four amino acid residues (AVPI) contacting the protein. In various proof-of-concept studies, SMAC-derived peptides as well as antisense oligonucleotides sensitized malignant cell lines to chemotherapy [14–22] [69–77]. Thus, the SMAC N-terminus has been utilized as a starting point for the design of peptidomimetic XIAP inhibitors.

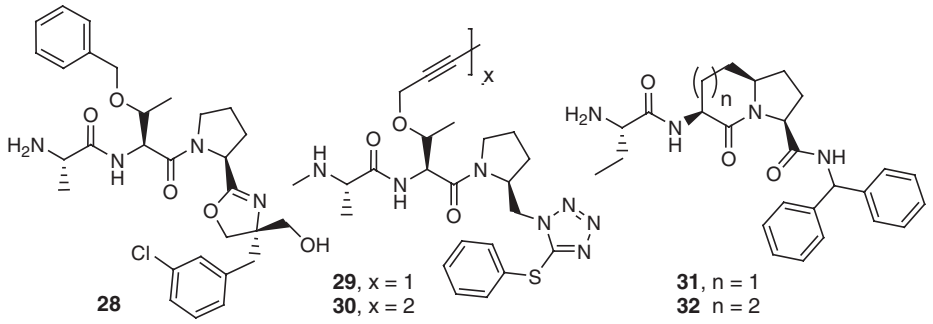
3.1. Peptidomimetic analogs based on SMAC N-terminal peptide

Based on the NMR structure of the SMAC N-terminal peptide in complex with the BIR3 domain of XIAP, a series of capped tripeptides comprised of unnatural amino acids was developed that binds with high affinity to the BIR3 domain of XIAP [78]. Structure-based design was utilized to improve the affinity of these BIR3 binders to the single-digit nanomolar range, as exemplified by compound **25** ($K_d = 5$ nM). Compound **26** ($K_d = 16$ nM) promotes cell death in several human tumor cell lines, with the highest activity observed in the human breast cancer cell lines BT549 ($EC_{50} = 7$ nM) and MDA-MB-231 ($EC_{50} = 13$ nM). This activity was extended to the *in vivo* setting in a murine xenograft tumor model of breast cancer (MDA-MB-231) where **26** induced significant tumor growth delay. Structurally-related capped tripeptides, such as **27**, have also been reported to inhibit XIAP [79] as well as ML-IAP ($K_d = 30$ nM), a potent anti-apoptotic protein that is strongly up-regulated in melanoma [80].

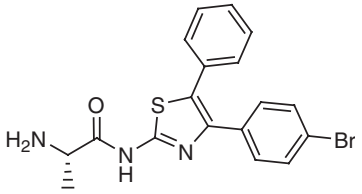


Computer-simulated conformational analysis of the SMAC-derived tetrapeptide AVPF was utilized for the design of non-peptidyl replacements of the PF dipeptide portion [81]. Oxazoline **28** was identified from a library of 180 peptidomimetics to

bind XIAP BIR3 with roughly the same affinity as the parent AVPF peptide ($K_i = 0.39\,\mu M$). Interestingly, an alkynyl substitution of a related tetrazole PF mimetic was found to have similar XIAP binding affinities when in the monomeric (**29**, $K_d = 0.51\,\mu M$), or dimeric (**30**, $K_d = 0.12\,\mu M$) forms. However, in a caspase-3 de-repression assay, based on overcoming XIAP-mediated suppression of caspase-3 in HeLa cell extracts, the dimer, **30,** is much more active. This discrepancy has been directly attributed to the bivalent nature of **30**. Similar to SMAC, which is a native homodimer, compound **30** may simultaneously interact with adjacent BIR domains of XIAP. Compound **30** also forms strong complexes with several different IAP family members including XIAP, cellular IAP-1, and cellular IAP-2. Compound **30** synergizes with both TNFα and TRAIL to induce caspase activation and cell death in HeLa and T98G human glioblastoma cells, respectively, with no effect on normal cells. The design of the conformationally constrained XIAP antagonists **31** and **32** ($K_i = 0.35\,\mu M$ and $0.025\,\mu M$, respectively) was also based on the 3D structure of the SMAC N-terminal peptide in complex with XIAP BIR3 domain [82,83]. While neither of these compounds exhibited single agent cytotoxicity, high micromolar concentrations of **31** potentiated cisplatin-induced apoptosis in PC-3 human prostate cancer cells and **32** ($20\,\mu M$) reversed the protection of high XIAP levels against chemotherapy treatment in Jurkat leukemia T cells.



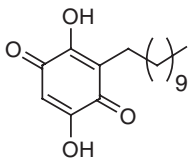
The importance of the N-terminal alanine of SMAC for binding is evident from both the SAR of peptide libraries and the 3D structure of SMAC peptide bound to the BIR3 domain. Keeping only this terminal alanine in place, a series of Ala-capped 5-membered heterocyclic amines that mimic AVPF binding was prepared [84]. Thiazole **33** binds to XIAP BIR3 with similar affinity as the SMAC N-terminal peptide ($K_d = 0.74\,\mu M$). NMR analysis of **33** bound to XIAP BIR3 indicates a binding mode similar to that of the SMAC peptide.



33

3.2. Leads discovered by virtual screening

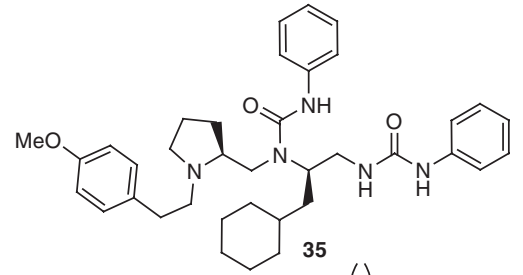
Computational screening of a 3D structure database of natural products was the starting point for the discovery of embelin, **34**, isolated from the Japanese Ardisia herb [85]. Embelin binds the BIR3 domain of XIAP with an $IC_{50} = 4.1\,\mu M$ and NMR analysis confirms several key interactions with residues crucial for binding to SMAC and caspase-9. Embelin (25–50 μM) induces cell death and caspase-9 activation in PC-3 prostate cancer cells with high XIAP expression levels. In XIAP-transfected Jurkat cells, embelin (50 μM) reverses the protection afforded by over-expression of XIAP against etoposide-induced apoptosis.



34

3.3. Leads discovered by chemical library screening

Screening of several combinatorial libraries using an enzyme de-repression assay that measures relief of XIAP-mediated suppression of caspase-3 led to the identification of a series of polyphenylureas exemplified by **35** [86,87]. Unlike the peptides derived from the SMAC N-terminus, the active polyphenylureas were shown to inhibit XIAP by binding to the BIR2 domain of XIAP, consequently leading to the activation of downstream executioner caspases-3 and 7. These compounds, but not inactive controls, induced rapid apoptosis in several tumor cell lines. Urea **35** induced significant tumor growth delay in PPC1 prostate cancer and HCT116 colon cancer murine xenograft models, but displayed little toxicity to normal tissues at high concentrations.



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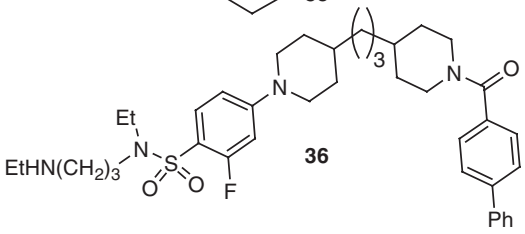

36

Using a similar high-throughput screening approach to that described above, a series of compounds, exemplified by phenylsulfonamide **36**, was identified from a combinatorial library [88]. Compound **36** disrupts the XIAP/caspase-3 interaction *in vitro* and synergizes with death receptor stimulation to bypass the apoptotic block resulting from the loss of the pro-apoptotic protein Bax in the colon carcinoma cell line HCT116.

4. CONCLUSIONS

Targeting tumor growth by inducing or restoring normal apoptotic signaling pathways has only recently emerged as a potential approach to cancer chemotherapy. Although many cytotoxic agents ultimately induce apoptosis, there are no marketed drugs that specifically affect the regulation of apoptosis. Because many agents initiate the apoptotic signalling pathway by a variety of mechanisms, it is often difficult to differentiate direct cytoxicity from antagonism of anti-apoptotic proteins. As the understanding of these signaling pathways evolves, so too will the ability to characterize the functional effects of lead compounds. Development of small molecule antagonists of the endogenous antiapoptotic proteins such as those outlined here is a tremendous challenge due in large part to the need to inhibit hydrophobic, large surface area protein–protein interactions and the difficulty in conclusively establishing mechanism of action. Nonetheless, a variety of promising and structurally diverse leads have been discovered and are in varied stages of development. Even though this field is in its infancy, the evolving understanding of the structural basis for these interactions, coupled with new screening techniques such as 3-D computational and fragment-based screening approaches, offers great promise in developing inhibitors of antiapoptotic proteins for the treatment of cancer.

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AKT Kinase and Hsp90 Inhibitors as Novel Anti-cancer Therapeutics

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Contents

1. INTRODUCTION

One of the greatest challenges of oncology drug development has been to identify agents that target tumors while maintaining acceptable levels of toxicity in normal tissues. While selection of an appropriate target enzyme is an important first step, developed drugs typically also inhibit unintended targets: some predictable based on target homology and some unpredictable. For targets whose disease biology makes them particularly attractive for drug development, multiple approaches to their inhibition result in compounds with a wide range of possible specificity profiles. An example of such an attractive target is the protein kinase AKT. The widespread loss of PTEN (phosphatase and tensin homolog) activity in tumors as well as reports of activating mutations or amplifications in PI3-kinase and the AKT isoforms makes AKT an obvious target for oncology drug development. Consequently, both ATP-competitive and allosteric inhibitors are being explored as AKT inhibitors. Likewise, compounds in development such as Hsp90 (heat-shock protein 90) inhibitors, while not intended only as AKT inhibitors, do result in loss of AKT protein as a consequence of Hsp90 inhibition. In this review, we will provide an update on compounds in development as AKT and Hsp90 inhibitors and comment particularly on the wide-ranging specificity patterns that accompany the various molecules that share inhibition of AKT as part of their mode of action.

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2. AKT

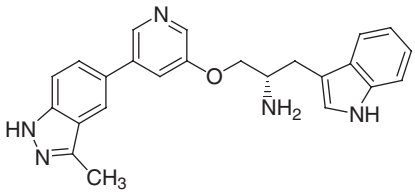
AKT is an AGC group serine/threonine kinase in the PI3-kinase signaling pathway. The kinase domain bears considerable homology to a number of other kinases of the AGC group including protein kinases A (PKA) and C (PKC), making generation of selective, ATP-competitive inhibitors problematic. PKA is of particular concern because of its well-defined role in signaling from many G-protein coupled receptors, and because it is suspected to "cross-talk" with other signaling pathways [1,2].

Another consideration for an AKT inhibitor is its pattern of inhibition among the three isoforms of AKT (AKT1, AKT2 and AKT3). While the kinase domain is highly conserved among the AKT isoforms, the sequence of the N-terminal pleckstrin homology (PH) domain is less highly conserved [3]. The PH domain controls the membrane localization of AKT and helps to maintain the kinase domain in an inactive state in the absence of cellular stimuli, so it provides a target for allosteric AKT inhibitors that may result in isoform selectivity.

The pattern of inhibition among the three AKT isoforms will undoubtedly influence therapeutic index, yet the optimal inhibitor selectivity profile is difficult to predict with our current understanding of AKT biology. For example, AKT2 has been most closely linked in function to insulin signaling, causing concern that its inhibition may lead to insulin insensitivity, yet AKT2 has also been found to be amplified more frequently in human tumors than AKT1 or AKT3 [4–6]. Data from generation of isoform selective knockout mice suggests that the three AKT isoforms do not have redundant functions, and may help determine the optimal selectivity profile for an inhibitor [7]. In this report we wish to summarize information on AKT inhibitors available since the most recent comprehensive review [8], and wherever possible, report the selectivity profile of the inhibitors.

2.1. ATP competitive inhibitors

A series of 3,5-disubstituted pyridines have been claimed as AKT inhibitors [9,10]. Among them is A-443654 (**1**) containing indazole and indole heterocycles linked by the pyridine ring [10]. The basic amino chiral center has specific S configuration. Compound **1** was reported as an ATP competitive pan-AKT inhibitor ($K_i = 0.16$ nM for AKT1) with at least 40-fold selectivity against other tested kinases. This compound displayed significant dose-dependent anti-tumor activity in multiple tumor models, either as monotherapy or in combination with other anti-tumor agents. AKT was inhibited within tumors at concentrations achieved during dosing. While increases in insulin secretion were observed with this compound, it did not result in increased blood glucose levels, and the dose limiting toxicity was significant weight loss.



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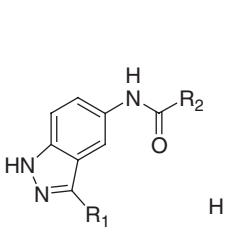

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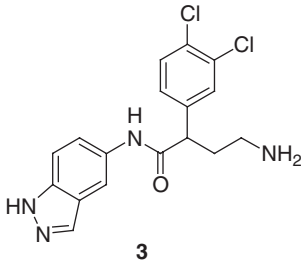
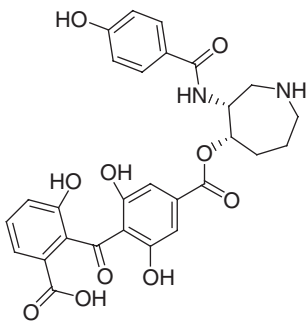


3

Another series of 5-amidoindazoles with general structure **2** was claimed as AKT3 inhibitors [11]. More than 300 analogs with qualitative IC_{50} data for AKT3, PKA, PDK1 and ROCK have been disclosed. Some of these compounds such as **3** appear to have selectivity versus PKA (IC_{50} $<1\,\mu M$ for AKT3, $>5\,\mu M$ for PKA).

Natural product (-)-balanol (**4**) was first isolated as a fungal metabolite [12,13] and found to be a potent ATP competitive inhibitor of PKA ($K_i = 3.9\,nM$) [14]. Later, a series of balanol derived novel azepane compounds were reported to be potent AKT inhibitors by Breitenlechner and co-workers [15]. In an attempt to improve plasma stability of the initial AKT hit **5** ($IC_{50} = 5\,nM$ for AKT1, 5 nM for PKA), analogs were designed and synthesized to replace the ester moiety which could be hydrolyzed by esterase *in vivo*. As a result, a double amide **6** has much improved plasma stability ($T_{1/2} = 69\,h$, in mouse plasma at 37 °C) while maintaining *in vitro* potency ($IC_{50} = 4\,nM$ for AKT1, 2 nM for PKA).



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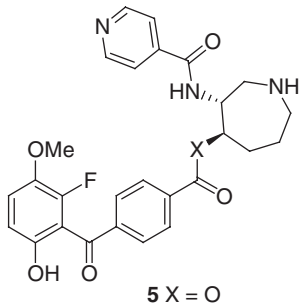
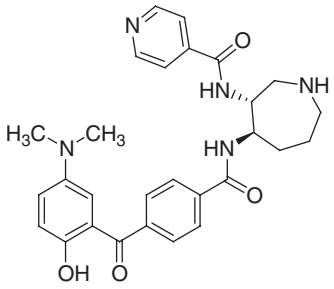


5 X = O
6 X = NH

More recently, the same group has reported their results to achieve AKT selectivity of these azepane analogs [16]. These inhibitors mimic ATP but extend further into a site not occupied by ATP. In this new site, defined by the glycine-rich loop and the activation loop, selectivity over PKA can be achieved by the introduction of bulkier substituents. As shown by the SAR trend of analogs **7** ($IC_{50} = 23$ nM for AKT, 30 nM for PKA), **8** ($IC_{50} = 20$ nM for AKT, 400 nM for PKA), and **9** ($IC_{50} = 20$ nM for AKT, 1900 nM for PKA), compound **7** is non-selective. However compounds **8** and **9**, with a bulky piperidine ring on acetophenone, are 20-fold and 95-fold selective of AKT versus PKA, respectively. Analysis of co-crystal structures with PKA showed that because of steric hindrance from the PKA-specific phenylalanine F187, the bulkier piperidine moiety of inhibitor **9** adopts an energetically unfavored envelope conformation to avoid steric clash of the two methyl groups with F187. This explains the low affinity of the inhibitor for PKA. This work demonstrates that it is possible to achieve selectivity for AKT versus PKA with inhibitors that bind to the kinase domain. There is no AKT isoform selectivity data reported for these compounds.



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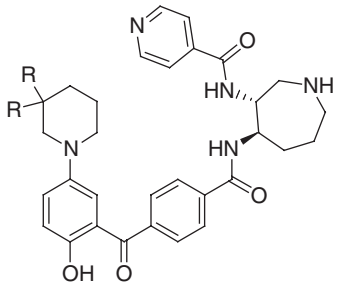
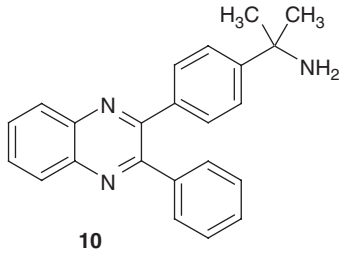


8 R =H
9 R =CH_3

2.2. Allosteric inhibitors

PH domain-dependent AKT inhibitors have been reported since early 2000 [17–23]. Recently Lindsley and co-workers have disclosed more details on two series of non-ATP competitive, PH domain-dependent, selective allosteric inhibitors of AKT [24]. The two series of inhibitors were developed from initial hit 2,3-diphenylquinoxaline **10** ($IC_{50} = 3.4$ μM for AKT1, 23 μM for AKT2, >50 μM for AKT3, PKA, PKC and SGK). To improve potency and physical properties, an iterative focused library approach was employed to modify the gem-dimethyl region as well as the quinoxaline core. This effort led to two pyrazinones **11** ($IC_{50\_} = 0.76$ μM for AKT1, 24 μM for AKT2, >50 μM for AKT3) and **12** ($IC_{50} = 21.2$ μM for AKT1, 0.325 μM for AKT2, >50 μM for AKT3) as potent AKT1 specific and AKT2 specific inhibitors respectively. Neither compound inhibited PKA, PKC and SGK (IC_{50s} >50 μM). The two compounds were evaluated individually and in combination. In a caspase-3 assay using A2780 cells with doxorubicin, a 1:1 mixture of **11**:**12** resulted in a 10-fold increase in caspase-3 activity in contrast to a 3-fold increase when **11** and **12**

were dosed alone, suggesting that a maximal apoptotic response requires inhibition of both AKT1 and AKT2. This notion was further supported by a dual AKT1/AKT2 inhibitor chosen among a series of 6,7-substituted quinoxalines. The tricyclic analog **13** ($IC_{50} = 58$ nM for AKT1, 210 nM for AKT2, 2119 nM for AKT3) was selective for AKT1 and AKT2 both *in vitro* and in cells (cell-based IPKA assay, $IC_{50} = 305$ nM for AKT1, 2086 nM for AKT2, $> 25{,}000$ nM for AKT3).



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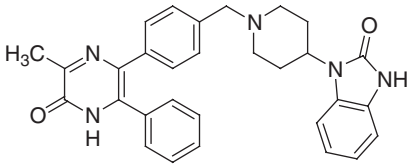
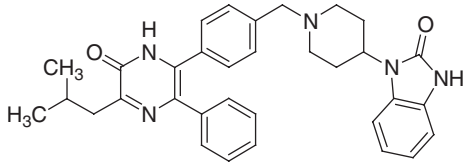
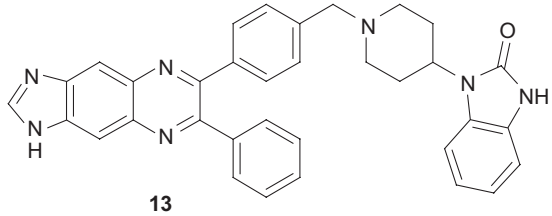


11



12

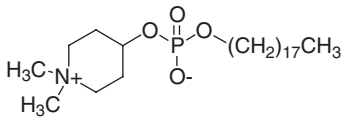
When tested in caspase-3 assays, compound **13** displayed a similar profile as when using a 1:1 mixture of **11**:**12**. This compound sensitized LnCaP cells to induction of apoptosis by TRAIL, leading to a 6–10-fold activation of caspase-3 compared to control or TRAIL alone. Dosing in mice led to plasma concentrations of 1.5–2 μM, a concentration sufficient to inhibit IGF stimulated phosphorylation of AKT1 and AKT2 immunoprecipitated from the mouse lung.



13

Perifosine **14** is a synthetic, substituted heterocyclic alkylphosphocholine that has been shown to disrupt AKT membrane localization and activation—possibly by interfering with the interaction of natural D-3 PtdIns (Phosphatidylinositol) phosphates with the PH domain of AKT [25]. Perifosine is one of several lipid analogs that may utilize this mechanism of action, though perifosine combines the best characterization of mechanism of action and progress into the clinic [25–27]. Treatment of a number of cell lines with perifosine results in growth inhibition with GI_{50} values of 1–10 μM. Perifosine does not inhibit other kinases that have been

tested or other enzymes in the PI3 kinase pathway suggesting that it may be selective for AKT. The effect of 14 on other proteins that interact with PtdIns-P3 via a pleckstrin homology domain has not been reported.



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Phase I trials with orally administered loading dose/maintenance dose schedule have demonstrated stable drug levels and long compound half-life [28]. Toxicities were primarily gastrointestinal, but were lessened by prophylactic anti-emetic treatment. Perifosine has advanced into a number of Phase II clinical trials for solid tumor indications.

3. HEAT-SHOCK PROTEIN 90 (HSP90)

The serendipitous discovery in 1994 [29] that the anti-tumor antibiotics geldanamycin (GA) and herbimycin A (HA) are ATP-competitive inhibitors of Hsp90, instead of the tyrosine kinases as originally believed, has sparked intense interests in the role of this family of chaperon proteins in tumor development and progression. Since then, a long list of intracellular signaling molecules with oncogenic potential have been found to require association with Hsp90 to achieve their active conformation, correct cellular location and stability [30]. Hsp90 has now emerged as one of the most attractive anti-cancer therapeutic targets in that inhibition of this single target uniquely blocks multiple cancer-causing signaling pathways simultaneously. This is accomplished by inhibitor-induced destabilization and eventual proteosome-mediated degradation of a magnitude of oncogenic proteins such as AKT, Raf-1 and Her-2. Since Hsp90 was reviewed in *ARMC* in 2003 [31], tremendous progress has been made in understanding the structure and function of Hsp90 as well as developing novel Hsp90 inhibitors. These advances will be the focus of the current review.

3.1. Hsp90 structure

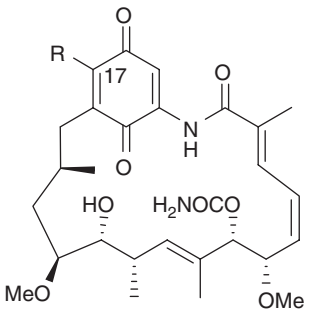
The Hsp90 chaperone is comprised of three domains: a 24-28 kDa N-terminal domain, a 38-44 kDa middle region, and an 11-15 kDa C-terminal domain [32]. The N-terminal domain contains an ATP-binding site and has weak ATPase activity [33]. Recent data suggest that occupancy of the N-terminal ATP pocket opens a second putative ATP-binding site in the C-terminal domain. Together, the conformational changes that occur upon binding and hydrolysis of ATP, regulate the molecular machinery necessary for stabilization and maturation of client proteins [33]. The ATP competitive inhibitors of both binding sites have been identified and are the subject of this review.

3.2. Hsp90 N-terminal domain inhibitors

The crystal structures of the N-terminal domain bound to ATP, ADP, and several inhibitors of Hsp90 have been solved [34–36]. All binding molecules share several common features in this binding site. They all make a direct interaction with Asp93, which itself is involved in an interaction with a tightly bound water molecule. This water molecule donates a hydrogen bond back to the binding molecules. The unusual bent (C-shaped) conformation of bound ATP is mimicked by several of the known inhibitors. The unique shape of the ATP-binding pocket implies that a high degree of selectivity among other ATP-binding proteins should be possible. Indeed, we and others have observed that various structurally distinct Hsp90 inhibitors exhibit literally no activities toward a panel of protein kinases evaluated. It is important to note that all structural information is based on the N-terminal domain of uncomplexed Hsp90. The structure of full length Hsp90 remains to be determined and the active form of Hsp90 is comprised of a complex multi-chaperone system [37] which may impart structural changes to the ATP-binding site. Nevertheless, several inhibitors of this ATP-binding site have been identified and are at various stages of preclinical and clinical development.

3.2.1. Geldanamycin and derivatives

The natural product geldanamycin (GA), **15**, is a member of the benzoquinone ansamycin antibiotics that was isolated in the early 1970's [38]. It has been known for decades that GA possesses potent anti-tumor activities both *in vitro* and *in vivo*. However, it was not until recent years that the actual cellular target of GA was determined to be Hsp90. Despite the activity in tumor xenograft models, progression of GA was stopped due to unacceptable levels of hepatotoxicity, which is believed to be caused by the benzoquinone moiety of GA instead of Hsp90 inhibition per se, as the structurally unrelated radicicol derivatives (see below) have similar biological activity but are not hepatotoxic [39].

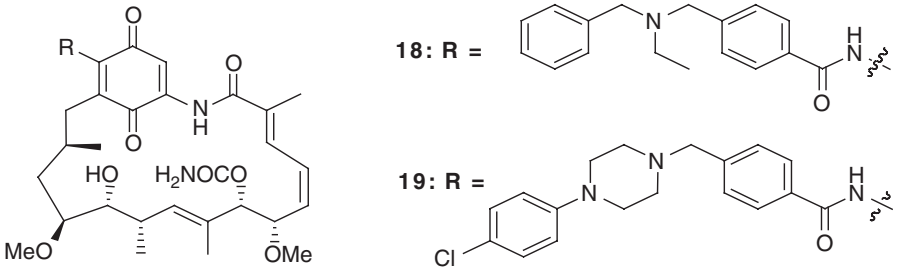


15 R = OMe
16 R = $NHCH_2CH{=}CH_2$
17 R = $NHCH_2CH_2N(CH_3)_2$

Replacing the potentially reactive C-17 methoxyl with allyl amine resulted in 17-AAG, **16**, which showed similar cellular effects but lower hepatotoxicity than the parent. In Phase I, the drug was shown to have schedule dependent toxicity, with hepatotoxicity as a DLT (dose limiting toxicity) [30]. Poor solubility requires the use of DMSO as a solvent and remains an issue with the current formulation. KOS-953, an improved formulation of 17-AAG, has recently begun Phase I investigations. In addition, the allyl group is extensively metabolized *via* oxidation and cleavage. Despite the limitations of 17-AAG, the drug has advanced to Phase II clinical trials in multiple indications.

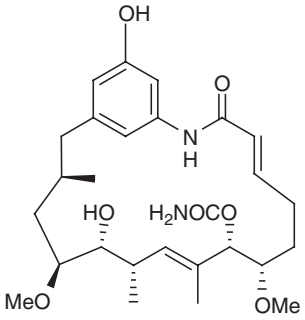
A follow-on compound to 17-AAG, the more soluble C-17 dimethylaminoethylamine derivative 17-DMAG, **17**, has similar activity *in vitro* and shows oral efficacy in tumor xenograft models [40,41]. The dimethylaminoethyl group also does not appear to undergo extensive metabolism in pre-clinical models [42]. The drug has advanced to Phase I clinical trials.

Several additional C-17 derivatives are in preclinical evaluation. Tian and coworkers recently reported the synthesis and evaluation of a large number of 17-aminogeldanamycin derivatives [43]. They found that the binding affinity to Hsp90 was not significantly affected by changing the C-17 substituent, and used this position to improve the physical properties. They reported several potent analogs (IC_{50} <100 nM, SKBr3 cell growth inhibition) with at least 5-fold increase in solubility compared to 17-AAG. Le Brazidec *et al.* also recently reported the synthesis and evaluation of C-17 derivatives of geldanamycin [44]. Several amides, carbamates and ureas were evaluated for potency and improved pharmaceutical properties. Two compounds, **18** and **19**, had comparable activity to 17-AAG and showed activity in several animal models when administered i.p. Despite the potential improvements that these derivatives may show over 17-AAG, they all contain the benzoquinone moiety and thus hepatoxicity is likely to continue to be a limiting factor.



McDaniel and coworkers recently reported several derivatives of geldanamycin which resulted from genetic engineering of the geldanamycin polyketide synthase (GdmPKS) gene cluster in *S. hydgroscpocus* [45]. In the course of this work, one analog, KOSN1559, **20**, was identified with 8-fold greater binding affinity ($K_i = 16$ nM) than 17-AAG. The compound also showed sub-micromolar activity in an SKBr3 cell proliferation assay (0.86 μM). Of particular interest with these

derivatives is the lack of benzoquinone moiety and thus these compounds may offer significantly improved toxicity profiles over 17-AAG.



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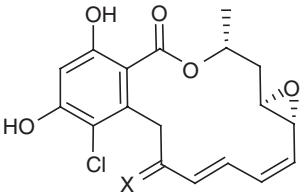
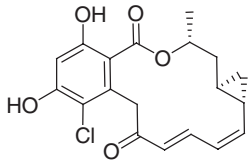
3.2.2. Radicicol and other recorcinol inhibitors

The antifungal antibiotic natural product radicicol, **21**, isolated from the fungus *Monosporium bonorden* in the mid 1950's [46], was also believed in the early 1990's to inhibit v-src tyrosine kinase [47]. It was subsequently shown to exert its effect by inhibition of Hsp90 and x-ray crystallographic studies of the N-terminal domain confirmed the binding of radicicol to the ATP-binding site [48]. Despite having promising *in vitro* activity, radicicol was inactive *in vivo* due to instability in serum. Efforts to address this instability issue have led to several derivatives.



21 X = O
22 X = NOH
23 X = $NOCH_2CON(CH_3)_2$

Ikuina and coworkers prepared derivatives, **22** and **23**, which replaced the biologically unstable carbonyl with an oxime. These compounds were shown to have more potent antiproliferative activities than radicicol and significant *in vivo* activities in several human tumor xenograft models [49]. The clinical development of these compounds, however, was not pursued. Danishefsky and co-workers, in their attempt to replace the potentially problematic epoxide and carbonyl, prepared several compounds **24, 25** and **26,** which showed activity as measured by depletion of Her-2 levels in MCF-7 breast cancer cell [50].



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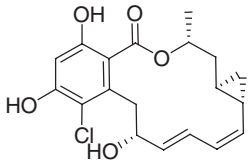

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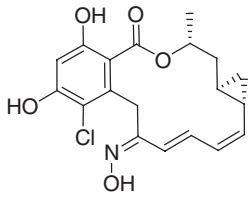

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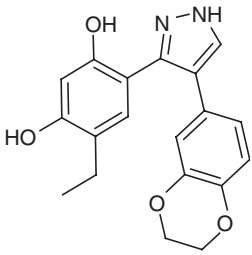
3.2.3. Dihydroxyphenylpyrazoles

In an attempt to limit the liabilities posed by the natural product inhibitors of Hsp90, several groups have pursued novel, small molecule scaffolds. Rowlands reported the novel pyrazole CCT018159, **27** [51], which maintains the critical binding resorcinol moiety of radicicol [52]. The compound inhibits the ATPase activity of Hsp90 with a low micromolar IC_{50}; it also inhibits the proliferation of several tumor cell lines at similar concentrations (~8 μM). The typical signature of Hsp90 inhibition has been observed for this class of compound (up-regulation of Hsp70, depletion of Raf and Her-2), but the *in vivo* activity and toxicity has not been reported. These compounds have been reported to suffer from poor pharmacokinetic properties, presumably related to metabolism of the phenols. Recently, a series of analogs with low nanomolar affinity for Hsp90 have been reported [53].



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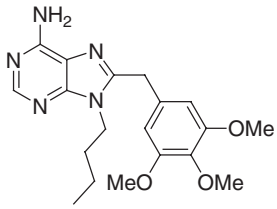
3.2.4. Purine based inhibitors

An additional series based on a purine scaffold was reported by Chiosis and colleagues [54]. The first in class compound, **28**, was shown to inhibit Hsp90 with low affinity. The generation of a small library around this scaffold resulted in **29**, a compound with similar potency (3–6 μM) against a wide range of tumor cell lines. This is not in agreement with the notion that certain tumors (e.g. BT474 breast tumor over-expressing Her-2) are more sensitive to Hsp90 inhibition as demonstrated with 17AAG [55]. PU24FCl inhibited tumor growth in an MCF-7 breast cancer xenograft model with concomitant depletion of Hsp90 client proteins Akt, Raf-1, Her-2 and Her-3 [54].



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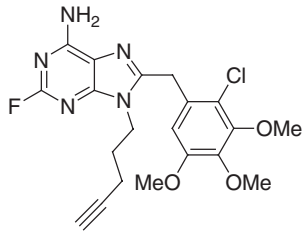

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The crystal structures of these compounds in complex with the N-terminal domain of Hsp90 [56] show that a conformational change in the ATP-binding pocket occurs upon binding of these compounds. These compounds adopt the characteristic bent shape of ligands specific to this binding site.

3.3. Hsp90 C-terminal domain inhibitors

While the majority of attention has been given to discovering inhibitors of the N-terminal domain, the C-terminal domain ATP binding pocket also offers an opportunity to develop inhibitors which may impair Hsp90 function. The crystal structure of this domain has yet to be determined; however, there are several compounds that are believed to bind to this region.

Novobiocin, an antibiotic known as an inhibitor of DNA gyrase B, also interacts with Hsp90. The binding site has been mapped to a region in the C-terminus [57]. Cells exposed to micromolar concentrations of novobiocin show decreases in the client proteins Her-2, Raf-1, mutant p53 and v-src [58]. Cisplatin has also been shown to bind to the C-terminal domain of Hsp90 [57] and induces a conformational change in the chaperone structure [59]. It has been proposed recently that there is an interesting yet very complex interaction between N-terminal and C-terminal ATP binding sites [60]. The C-terminal binding site becomes optimally accessible only after occupancy of the N-terminal ATP pocket by either ATP or Hsp90 inhibitors; conversely, occupancy of the C-terminal site(s) inhibits the binding of ATP to the N-terminal site. In addition, the two ATP pockets display a distinct nucleotide binding specificity. Whereas the N-terminal site is relatively restricted to adenine nucleotide with intact adenine ring, the C-terminal site can accommodate both purine and pyrimidine nucleotides. Both GTP and UTP have been found to be specific binders of C-terminal site [60]. This knowledge will undoubtedly help future development of more potent and specific C-terminal Hsp90 inhibitors.

4. CONCLUSION

AKT and Hsp90 are each cancer therapeutic targets that influence cellular growth, translation, proliferation, metabolism and survival. The compounds reviewed in

this report represent many approaches to inhibit them, though it is still unknown which will represent avenues to cancer therapy with an acceptable pattern of selectivity, PK, PD, efficacy, and toxicity.

The prominent position of the PI3-kinase pathway in cancer has resulted in numerous programs targeting AKT for cancer therapy, yet development of such a compound has proven challenging. This may be due, in part, to the close similarity between AKT and other kinases of the AGC group kinases. No small molecule AKT inhibitor has yet entered clinical trials, so we are still awaiting definition of the necessary selectivity profile and PK/PD parameters to allow for efficacy without undue toxicity. The AKT inhibitors reviewed in this report suggest that progress in this field is being made in targeting this critical pathway.

The concept of Hsp90 as a cancer target was initially met with skepticism. As interference of Hsp90 function will affect a wide array of client proteins, it was suspected that the pleiotropic Hsp90 inhibitors would cause undue toxicity in human patients. Quite surprisingly, data from Phase I trials suggest that 17AAG is well tolerated at concentrations that clearly cause target modulation. Further bolstering this finding is the recent report that histone deacetylase (HDAC) inhibitors, which exhibit remarkable anti-tumor activities with manageable toxicities in clinical trials, also block the chaperone function of Hsp90 (via hyperacetylation) and induce concurrent degradation of multiple client proteins in both cultured tumor cells and human patients, in a manner similar to that of ATP competitive Hsp90 inhibitors [61,62]. These observations support the notion that stressed tumor cells are more sensitive to Hsp90 inhibition than normal cells. It is conceivable that our increased understanding of the molecular basis of tumor selectivity of Hsp90 inhibition will ultimately drive the discovery and development of inhibitors with superior antitumor efficacy and improved safety.

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Novel Strategies in HIV Prevention-Development of Topical Microbicides

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Contents

1. INTRODUCTION

1.1. Microbicides as a novel approach to prevent HIV infection

Sexual transmission of HIV continues to fuel the HIV/AIDS pandemic with greater than 90 percent of adolescent and adult HIV infections resulting from heterosexual intercourse [1]. Worldwide, approximately half of the 42 million people living with HIV/AIDS are women [1,2]. A variety of biological and socioeconomic factors contribute to the vulnerability of women to infection such as anatomic and histological differences between female and male genital tissues [3] as well as inconsistent condom use due to partner consent or cultural acceptance [4]. As a result, women are at risk for acquiring HIV from exposure to infectious seminal fluid during intercourse with high-risk sex partners [5,6]. In the absence of an effective prophylactic vaccine or therapy, scientific efforts over the past ten years have focused on the development of safe, effective formulations of anti-HIV agents to reduce sexual and perinatal transmission of HIV [2,7].

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1.2. HIV infection in the genital tract

The current lack of a preventive vaccine or effective microbicide is reflective of the limited understanding of those factors in the genital mucosa that influence HIV transmission or susceptibility to infection. Male-to-female heterosexual transmission is two to eight times more efficient compared to female-to-male, with a male-to-female per contact infectivity estimated to be 0.0009 [8,9]. Receptive anal intercourse results in an estimated per contact infectivity of 0.0082 [10]. The reason for the increased rate of HIV infection from penile-anal sex compared to penile-vaginal sex may be due to differences in the architecture of vagina/cervix compared to the rectum/colon [3].

1.2.1. Vaginal intercourse

Women are at risk for acquisition of HIV during vaginal intercourse due to trauma to the genital epithelium, extended exposure to infectious seminal fluid, and the greater availability of HIV targets in the genital mucosa. In theory, an effective topical microbicide could be applied vaginally prior to sexual intercourse where it could spread across the surfaces of the vagina and cervix and protect against subsequent HIV infection. The vagina and ectocervix are covered with multiple layers of stratified squamous epithelial cells that form an effective, but not absolute, barrier to HIV/SIV infection. This is demonstrated by the inability of ectocervical epithelial sheets to transcytose HIV [11] and the need to use 10,000 times more SIV in Rhesus macaques to establish infection through nontraumatic vaginal challenge compared to intravenous challenge [12,13]. On the other hand, the endocervix is a more fragile barrier since it is composed of a single layer of mucus-secreting, simple columnar epithelial cells. While cervical epithelial cell lines have been shown to be infected *in vitro* [14], recent work indicates that primary cervical epithelial cells and cell lines are refractory to cell-free and cell-associated HIV infection [15]. Although it is not clear whether HIV crosses the epithelial barrier by nontraumatic mechanisms (i.e. direct infection or transcytosis) or through micro-tears in the vaginal/cervical epithelium, the virus gains access to the underlying immune cell targets in the lamina propria of the mucosa. Once virus has crossed the epithelium, these underlying immune cells become infected or carry the virus to the local lymph nodes. Although a variety of studies suggest the role of multiple cell types during initial infection, collectively these studies suggest that dendritic cells, CD4+ T cells, and macrophages are important liaisons between virus entry and dissemination throughout the body.

1.2.2. Anal intercourse

Though anatomically and structurally different, the anus/rectum shares striking histological similarities with the cervix/vagina. Like the transition zone from vagina/ectocervix to endocervix, the epithelium changes from a stratified layer of cells in the anus to a single layer of simple columnar cells in the rectum/colon. As in

vaginal intercourse, trauma induced during anal intercourse likely results in tears in the epithelium, allowing access of HIV to underlying immune target cells. Unlike cervical cells, *in vitro* data indicate that infectious HIV can transcytose through intestinal cell lines in micro-vesicles [16]. Other studies have shown that intestinal epithelial cell lines can be productively infected *in vitro* [17,18], although there is no conclusive evidence that HIV infection of intestinal epithelial cells occurs *in vivo*. Virus that breaches the epithelial layer, as observed in the female genital tract, gains access to an enriched population of HIV target cells (dendritic cells, CD4+ T cells, and macrophages) within the lamina propria of the rectum/colon.

While the precise mechanisms by which HIV infects the vaginal or rectal mucosa are yet to be defined, it is increasingly clear that the innate protection provided by the mucosal epithelium should be maintained. Results from a well-known clinical trial with COL-1492, a vaginal spermicide with known *in vitro* anti-HIV activity, demonstrated the toxic effect of topically applied nonoxynol-9 (N-9) (Fig. 1) and emphasized the need for the development of safe, nontoxic candidate microbicides as well as improved pre-clinical testing of these compounds [19]. More recent studies with N-9 indicate sloughing of entire epithelial sheets from the vagina and rectum within minutes of application, thus leaving a denuded mucosa vulnerable to infection with HIV and other mucosal pathogens [20,21]. Ideally, an effective microbicide should be nontoxic to the mucosal epithelium while maintaining efficacy against HIV infection – even if the epithelial barrier is breached by trauma or other means.

2. REQUIRED AND ADVANTAGEOUS CHARACTERISTICS FOR A MICROBICIDE

2.1. Broad-spectrum versus HIV-specific

While the major focus of microbicide development has been HIV since HIV/AIDS is a lethal and incurable disease, other sexually transmitted infections (STIs) are also significant causes of morbidity and mortality [22]. In fact, the spread of these diseases are also facilitated by the same factors driving the worldwide spread of HIV – such as lack of woman-controlled prevention methods and a lack of widespread use of condoms. Non-HIV STIs include curable infections such as trichomoniasis, Chlamydia, gonorrhea, syphilis, and chancroid, as well as noncurable infections caused by Herpes virus and human papillomavirus [22]. Since STIs are important co-factors for HIV transmission, candidate microbicides have been developed with overlapping mechanisms of action and potential activity against other sexually transmitted pathogens. Overall, women at risk for HIV

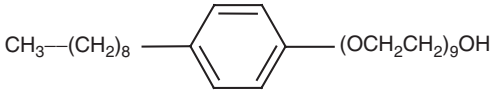

Fig. 1. Nonoxynol-9 (N-9).

infection are generally concerned about acquiring other STIs and have a high interest in broad-spectrum microbicides [23].

2.2. Spermicidal versus non-spermicidal

Despite the toxicity observed after use of the spermicide N-9, candidate microbicides in development are tested for spermicidal activity [24]. Women prefer the option to use a product that may or may not have contraceptive activity while maintaining efficacy against HIV and other STIs [23,25].

3. CONSIDERATIONS FOR ACCEPTABILITY OF A TOPICAL MICROBICIDE

In addition to a minimum set of safety and efficacy requirements, a topical microbicide should have additional characteristics that will increase its acceptability to end users [25,26]. Although protection against HIV infection and enhancement of sexual pleasure are key components of acceptability, a variety of additional desired qualities can differ not only between women and men, but also between different countries and cultures [25]. Additional characteristics include level of lubrication (i.e. lubricated versus "dry" sex), color, odor, and taste, as well as effect on vaginal or rectal health.

4. TARGETS FOR TOPICAL MICROBICIDES

4.1. Genital environment modulators

4.1.1. Enhanced innate factors

Cervicovaginal secretions have intrinsic antimicrobial activity that may be important in protecting women against sexually transmitted infections. Defining these innate factors and identifying how they protect against infection is essential for microbicide development because candidate topical microbicides must not interfere with the innate protective activity, and these factors could be exploited to facilitate the development of novel microbicides. Candidate components for this anti-viral activity include defensins, (secretory leukocyte protease inhibitor) SLPI, lactoferrin or mucins [27-30].

4.1.2. Engineered lactobacilli

In healthy women of child-bearing age, the protective mucosa in the vagina is populated with microflora typically dominated by lactobacilli, and their dominance over pathogenic anaerobes is positively associated with vaginal health [31].

Depletion or disturbances of vaginal lactobacillus flora has been associated with establishment of opportunistic infections like bacterial vaginosis and an increased risk of acquiring HIV type 1 (HIV-1) [32]. The principal lactobacillus species isolated from the vaginal mucosa of healthy women are *Lactobacillus crispatus*, *Lactobacillus jensenii*, and *Lactobacillus gasseri* [33,34]. These three species are efficient colonizers of the vaginal mucosa and likely exist in a natural ''biofilm'' composed of bacteria and extracellular matrix materials [35].

The vaginal microflora of women is part of a dynamic ecosystem. Through genetic engineering, the vaginal microflora may be further enhanced to form an efficient protective shield against the transmission of sexually transmitted diseases like HIV. One approach involves the genetic modification of natural human isolates of lactobacilli to express high-affinity HIV-binding proteins. These proteins can be associated either with the bacterial surface or secreted into the mucosal biofilm matrix, enabling the mucosal layer to bind viruses and impede their access to underlying epithelial cells. Importantly, this may lead to prolonged exposure of viruses to inactivating substances naturally secreted by lactobacilli, such as lactic acid and hydrogen peroxide, thereby significantly reducing the numbers of infectious viral particles.

The expression of heterologous proteins has been achieved in Gram-positive bacteria, including lactobacilli, lactococci, and streptococci [36,37]. The engineering of a natural colonizing vaginal isolate of *L. jensenii* to express a secreted form of the prototypical HIV-binding protein, CD4, was described by Chang *et al*. [38]. The authors demonstrated that 2D CD4 produced by these bacteria exhibit full biological activity *in vitro*, defined by the ability to bind gp120 and to inhibit HIV-1 viral entry. This work provides an important first step toward the development of engineered lactobacilli, applied onto the vaginal mucosa, to block the sexual transmission of HIV in women.

4.1.3. Plant derived antibodies

Recombinant antibodies can be used to diagnose, treat and prevent disease by exploiting their specific antigen-binding activities. A large number of drugs currently in development are recombinant antibodies, and most of these are produced in cultured rodent cells. Although such cells produce authentic functional products, they are expensive, difficult to scale-up and may contain human pathogens. Plants represent a cost-effective, convenient and safe alternative production system and are slowly gaining acceptance. Five plant-derived therapeutic recombinant antibodies (plantibodies) are undergoing clinical evaluation, three of which can be used as prophylactics [39,40].

4.1.4. Surfactants

Surfactants are designed to destroy the membrane of a pathogen, rendering it noninfectious. The disadvantage of surfactants, however, is that they also disrupt the macro- or microenvironment of the vagina and cervix after repeated exposure. C31G is a broad-spectrum antibacterial agent that shows contraceptive properties

in vitro [41]. It is the most advanced of the surfactants, has been tested in several clinical safety studies [42], including male tolerance studies, and is now being testing in a phase III clinical trial. C31G in its formulated form, Savvy, has a high rate of spread and dissolution in the mucous of the vagina and cervix. Because C31G also has anti-spermicidal activity, its acceptance as a topical microbicide in populations where planned contraception is not culturally accepted could be reduced. The other leading surfactants (Z-14, SLS and SDS) are not as advanced as C31G. Nonoxynol-9 (N-9), a widely used spermicide, has been shown to be ineffective for HIV prevention since a clinical trial in African women demonstrated that women who used N-9 before intercourse had a 50% greater rate of new HIV infection than women with placebo [19].

4.1.5. Polyanionic polymers

Polyanionic polymers exhibit their mechanism of action by inhibiting the initial virus-cell interaction. This class of compounds, in most circumstances, mediates inhibition of HIV replication by interaction with the positively charged V3 loop of gp120 [43,44]. It has been shown that the gp120 from X4 viruses are more highly positively charged than gp120 with R5 tropism and therefore, polyanionic compounds are generally more effective against X4-tropic viruses.

PRO2000 is the most advanced compound in the polyanion polymer class based on its preclinical and clinical status. The compound is active against herpesvirus 1 and 2, HIV, and SIV. It is currently in two Phase I trials for safety and tolerability as a vaginal gel (HIVNET 020) and in men in combination with BufferGel (HTPN 032). Phase II/III trials are being planned. The second most advanced compound is Carraguard, a formulated carrageenan, which has successfully completed safety and early efficacy trials. A phase III clinical trial is also planned [2].

There are three other very notable lead compounds in the polyanionic polymer class, cellulose acetate phthalate (CAP), β-cyclodextrin (BCD), and SPL-7013. CAP is unique because it can be a component of pharmaceutical formulations. CAP binds to a site on HIVgp120 independent of the CD4 binding site [45]. The interaction of CAP and gp120 results in the formation of a six-helix hybrid, a stable non-functional conformational virus entry intermediate in the entry pathway [46]. Thus, CAP prevents the binding of gp120 to CD4. However, it is not known whether CAP will be effective against HIV that enters the cell independent of CD4.

The second unique compound group is β-cyclodextrin. BCD depletes cholesterol from cell and virus membranes, thus changing their fluidity and inhibiting the function of membrane rafts. The replication of HIV is intimately tied to the use of rafts in the cell membrane [47,48]. Rafts are aggregations of lipids in the cell membrane which help support and position membrane proteins and receptors for their various functions [49]. CD4 and the HIV coreceptors are present in cholesterol containing rafts, and depletion of cholesterol inhibits virus entry [50]. Changes in virus infectivity associated with Nef function [51] are also dependent upon functional rafts [52]. Additionally, HIV release and assembly is raft-dependent,

suggesting that BCD may also be able to prevent the release of virus from infected cells [53,54].

The final member of this class of compounds is the dendrimer SPL-7013, also known as VivaGel. This compound is active against herpesviruses and HIV by blocking attachment and entry [55,56].

4.1.6. Proteins

This class of compounds includes a wide variety of lead topical microbicides, such as negatively charged albumins, monoclonal antibodies (PRO140 and PRO549), small peptides (T-20 and T-1249), bovine β-lactoglobulin, modified chemokines (APO RANTES and PSC RANTES), the genital tract defense molecules (protegrins and β-defensins), and cyanovirin-N [56].

Cyanovirin-N is a highly structured 11,000 kDa protein derived from blue-green algae. It is a mannose binding protein that specifically binds to HIV gp120 and prevents virus entry [57]. Cyanovirin-N is extremely stable, as a result of multiple internal disulfide bonds, and will retain antiviral activity following boiling. It has been tested in non-human primate models for its efficacy but not in clinical trials [58].

4.1.7. Reverse transcriptase inhibitors

RT inhibitors of both the nucleoside reverse transcriptase inhibitors (NRTI) and non-nucleoside reverse transcriptase inhibitors (NNRTI) classes have been proposed as potential topical microbicide candidates. NNRTIs are small molecules which do not need to be phosphorylated for antiviral activity and mediate inhibition of the HIV reverse transcriptase enzyme by binding to a defined enzyme domain (NNRTI pocket) [59]. NNRTI candidates for topical microbicides are the thiocarboxanalide derivative UC-781 and SJ-3366, a HEPT-like homocyclic pyrimidinedione (Samjin Pharmaceutical Inc.). UC-781, although highly active against HIV, was not advanced to clinical trials due to resistance and solubility issues [60]. UC-781 became a topical microbicide candidate following demonstration of virucidal activity, long-term protection in pre-treated cells, and reduction in the infectivity of virus released from chronically infected cells [61-63]. UC-781 mediates these activities in part due to high affinity binding coupled with a low off-rate for the HIV-1 reverse transcriptase enzyme [63].

SJ-3366 has a dual mechanism of action; it inhibits virus entry and reverse transcription. It has a TI > 2,000,000 for HIV-1 and inhibits HIV-2 replication.

DABO (3,4-dihydro-2-alkoxy-6-benzyl-4-oxopyrimidine), MC 1047 and MC1220 have been proposed as candidate topical microbicides based upon their ability to prevent virus breakthrough in a long-term tissue culture assay.

A final class of NNRTIs are thioureas. They inhibit reverse transcription of HIV and are additionally able to immobilize sperm [64,65].

In contrast to NNRTIs, NRTIs are chain terminators of HIV reverse transcription and function to prevent conversion of HIV genomic RNA into DNA [66].

PMPA or its prodrug have been used to treat HIV/AIDS disease, and *in vitro* PMPA demonstrates a broad range of activity against clinical isolates and multi-drug NNRTI resistant isolates [67]. It is also active against vaginal challenge in the SIV and HIV-2 macaque model [68]. A phase I safety and acceptability trial in both uninfected and HIV infected women using PMPA in a vaginal gel formulation is currently under way (HPTN 050).

4.1.8. NCp7 nucleocapsid zinc finger inhibitors

The two zinc-fingers of the HIV p7 nucleocapsid (p7NC) protein are essential for virus replication, and mutations in the zinc fingers lead to replication incompetent viruses [69-71]. The zinc fingers participate in multiple aspects of HIV replication, including reverse transcription [72,73], integration [74], and virus maturation and release [75,76]. Compounds that interact with the zinc fingers have virucidal properties either directly through interaction with the zinc-coordination amino acids of the finger or indirectly through inhibition of virus polyprotein maturation by restricting HIV protease enzyme processing of disulfide bond modified and cross-linked polyprotein precursors [77]. An example of a zinc finger inhibitor is aldrithiol-2 (AT-2), that covalently modifies the essential zinc fingers in the nucleocapsid (NC) protein of human immunodeficiency virus type 1 (HIV-1) or simian immunodeficiency virus (SIV) virions, thereby inactivating infectivity. [78]. Therefore, the virucidal agent AT-2 has the potential to be developed as a microbicide.

4.1.9. Coreceptor inhibitors

The molecules that have potential as topical microbicides include CCR5 (R5) and CXCR4 (X4) inhibitors. Since most of the mucosal infections are transmitted by R5 specific viruses, the development of R5 inhibitors is predominant over X4 inhibitors. The R5 inhibitors currently under investigation for systemic use are SCH-C and SCH-D (Schering Plough), TAK-779, TAK-220, and TAK-652 (Takeda Pharmaceuticals), UK 427,857 (Pfizer), and ONO-4128 (ONO/GSK). CXCR4 inhibitors currently being evaluated are AMD070 and AMD3100 (AnorMED, Canada).

5. TOPICAL MICROBICIDES UNDER DEVELOPMENT

5.1. Preclinical

Microbicides currently under pre-clinical development are Alkyl sulfates, tobacco-derived antibodies and fusion proteins (HIV, HSV, HPV), anti ICAM-1 antibodies, betacyclodextrin, the entry/fusion inhibitors C85FL, K5-N, and OS(H), cyanovirin-N, lime juice, mandelic acid condensation polymer (SAMMA), MC1220 (lead compound in the dihydroxy alkyl benzyl oxopyrmimidine series), the vaginal defense enhancer MucoCept HIV, Novaflux proprietary product, and porphyrins (Table 1).

Table 1. Microbicides in Pre-clinical Trials (modified from the Alliance for Microbicide Development's Microbicide Research and Development Database (MRDD))

Product	Developer	Infectious agents *in vitro* and/or animal model data
Porphyrins	Emory University	HIV-l, *Herpes simplex*, *Neisseria gonorrheae*
Alkyl sulfates	Pennsylvania State University	HIV-l, *Herpes simplex*, human papillomavirus, *Chlamydia trachomatis*, *Neisseria gonorrheae*
Antibodies and fusion proteins	Mapp Biopharmaceutical, Inc.	HIV-l, *Herpes simplex*
Anti ICAM-1 antibody	Johns Hopkins University	HIV-l
Betacyclodextrin	Johns Hopkins University	HIV-l
C85FL	Cornell University, Weill Medical College	HIV-l
Cyanovirin-N	Biosyn, Inc.	HIV-l
K5-N, OS(H)	San Raffaele Scientific Institute	HIV-l
Lime juice	University of Melbourne	HIV-l
Mandelic acid condensation polymer (SAMMA)	Mount Sinai Medical School	HIV-l, *Herpes simplex*, human papillomavirus, *Chlamydia trachomatis*, *Neisseria gonorrheae*, *Candida albicans*
MC1220 (Lead compound of Dihydroxy Alkyl Benzyl Oxopyrimidine series)	Idenix Pharmaceuticals, Inc.	HIV-l, Hepatitis B, *Herpes simplex*, *Haemophilus ducreyi*, *Neisseria gonorrheae*, *Trichomonas vaginalis*, *Candida albicans*, *Bacillus subtilis*, *Staphylococcus aureus*, *Candida tropicalis*, *Streptococcus*
MucoCept HIV	Osel, Inc.	*Candida albicans*, HIV-l
Novaflux proprietary product	Pennsylvania State University	HIV-l

Table 2. Microbicides in Clinical Trials (modified from Alliance for Microbicide Development's Microbicide Research and Development Database (MRDD) and [2])

Product	Developer	Infectious agents *in vitro* and/or animal model data	Clinical trial phase
Pro2000/5 Gel	Indevus Pharmaceuticals, Inc.	HIV-1, *Herpes simplex*, *Chlamydia trachomatis*, *Neisseria gonorrheae*	Phase 1
Protected lactobacilli in combination with BZK	Biofem, Inc.	HIV-1, *Chlamydia trachomatis*, *Neisseria gonorrheae*, *Candida albicans*, *Streptococcus agalactiae*, *Escherichia coli*, *Pseudomonas aeruginosa*	Phase 1
Savvy (C31G)	Biosyn, Inc.	HIV-1	Phase 3
Tenofovir/PMPA gel	Gilead Sciences, Inc.	HIV-1	Phase 2
TMC120	International Partnership for Microbicides	*Neisseria gonorrheae*, *Haemophilus ducreyi*, HIV-1, *Herpes simplex*, human papillomavirus, *Candida albicans*	Phase 1
UC-781	Biosyn, Inc.	HIV-1	Phase 1
ACIDFORM gel	Global Microbicide Project	(Vaginal Defense Enhancer)	Phase 1
BufferGel™	Reprotect, LLC	HIV-1, *Treponema pallidum*, *Herpes simplex*, *Chlamydia trachomatis*, *Haemophilus ducreyi*, *Neisseria gonorrheae*, *Trichomonas vaginalis*, BV-associated bacteria, *Staphylococcus aureus*, human papillomavirus	Phase 1
Carraguard®	Population Council	HIV-1	Phase 1
Cellulose acetate 1,2-benzenedicarboxylate (cellacetate/CAP)	New York Blood Center (NY, USA)	HIV-1, *Chlamydia trachomatis*, *Herpes simplex*, *Haemophilus ducreyi*, *Neisseria gonorrheae*, *Trichomonas vaginalis*, *Candida albicans*, *Garnerella vaginalis*	Phase 1
Cellulose sulfate gel	Global Microbicide Project	HIV-1	Phase 1
Human monoclonal antibodies C2F5, C2G12, C4E10	Polymun Scientific	HIV-1	Phase 1
Invisible Condom	Laval University	HIV-l, *Herpes simplex*, human papillomavirus, *Chlamydia trachomatis*, HIV-1, *Neisseria gonorrheae*	Phase 1/2
Lactin-Vaginal (Lactin Vaginal Capsule)	University of Pittsburgh	HIV-1, *Chlamydia trachomatis*, *Neisseria gonorrheae*, *Trichomonas vaginalis*, *Candida albicans*	Phase 1
Polystyrene sulfonate gel (PSS gel)	Global Microbicide Project/TOPCAD/CONRAD	HIV-1	Phase 1

Table 2. Continued

Product	Developer	Infectious agents *in vitro* and/or animal model data	Clinical trial phase
VivaGel (SPL7013 gel)	Starpharma, Ltd.	HIV-1 *Herpes simplex*, *Chlamydia trachomatis*	Phase 1
Dextran/Dextrin sulfate/ Emmelle™	Institute of Tropical Medicine, Imperial College School of Medicine, Medical Research Council, England	HIV-1, *Herpes simplex*, *Chlamydia trachomatis*	Phase 1/2
Praneem tablet	M/S reproductive Technologies (New Delhi, India)	HIV-1	Phase 1/2

For more information on candidate microbicides and trials, consult the Alliance for Microbicide Development's Microbicide Research and Development Database (MRDD) at www.microbicide.org.

5.2. Clinical

There are a variety of compounds currently under Phase I clinical trials. Compounds in Phase I clinical trials include ACIDFORM gel, BufferGel, Carraguard, Cellulose acetate 1,2-benzenedicarboxylate (cellacetate/CAP), Cellulose sulfate gel, human monoclonal antibodies C2F, C2G12, C4E10, Lactin Vaginal Capsule, polystyrene sulfonate gel (PSS gel), PRO2000/5 gel, protected Lactobacilli in combination with BZK, TMC120, UC-781, and VivaGel (SPL7013). ''Invisible Condom'' is in Phase 1/2 clinical trials, Tenofovir/PMPA gel in Phase 2, and Savvy (C31G) in Phase 3 clinical trials (Table 2).

6. CONCLUSIONS: THE FUTURE OF TOPICAL MICROBICIDE DEVELOPMENT

In the light of the absence of a licensed HIV vaccine, the development of an effective, topical microbicide has become more important. Topical microbicides have the advantage over vaccines in that they could be available over the counter, they do not need to be administered by a health care professional, and they can be manufactured cheaply and in large quantities. However, their effectiveness will greatly depend on the responsibility of the individual user. Therefore, education, individual and community customs, religious cultures, and other socio-economic influences play a major role in the potential success of microbicides to prevent HIV infections.

Many chemical and natural substances are currently under investigation for development as topical microbicides. Some drugs that are highly effective against HIV *in vitro*, but are prohibited for systemic use (for example dextran sulfate), are being considered as topical microbicides. Natural substances, such as lime juice, may also be highly effective in preventing sexually transmitted infectious diseases. Generally,

microbicides are preferred that are broadly active against a variety of sexually transmitted microorganisms.

So far, several microbicides have advanced to Phase III clinical trials, but no microbicide is currently approved by the Food and Drug Administration or any other licensing agency. As soon as the first microbicide is approved for prescription or over-the-counter use, the impact of topical microbicides on the prevention of HIV and other infectious diseases can be monitored.

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New Developments in HIV Therapeutics

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Contents

1. INTRODUCTION

Although there are currently 21 individual drugs and several combinations thereof that have been approved by the Food and Drug Administration (FDA) for marketing in the US for the treatment of HIV/AIDS, there remains a pressing need for new therapeutics in this area. Until recently, all AIDS drugs inhibited either of the viral enzymes reverse transcriptase (RT) or protease. RT inhibitors are further classified as either nucleoside (NRTIs) or non-nucleoside (NNRTIs). The majority of treatment regimens use a variety of combinations of at least two, and as many as five, of these drugs. Nonetheless, current therapy is plagued by the emergence of viral populations that are often resistant to other drugs in the same class. This often severely limits treatment options for patients who have experienced treatment failure. It is now common for newly infected individuals to be infected with HIV that contains mutations that compromise the efficacy of more than one of the approved drugs [1]. A second major limitation of current therapy is related to convenience and tolerability. Many drugs have sub-optimal dosing requirements including high pill burden, food and drug interactions, and the requirement for dosing up to three times daily. Poor tolerability is also problematic for many patients, and can include variations in lipid and glucose levels, liver enzyme elevations, gastrointestinal disturbances, neurological effects, and effects on renal function [2]. These complications

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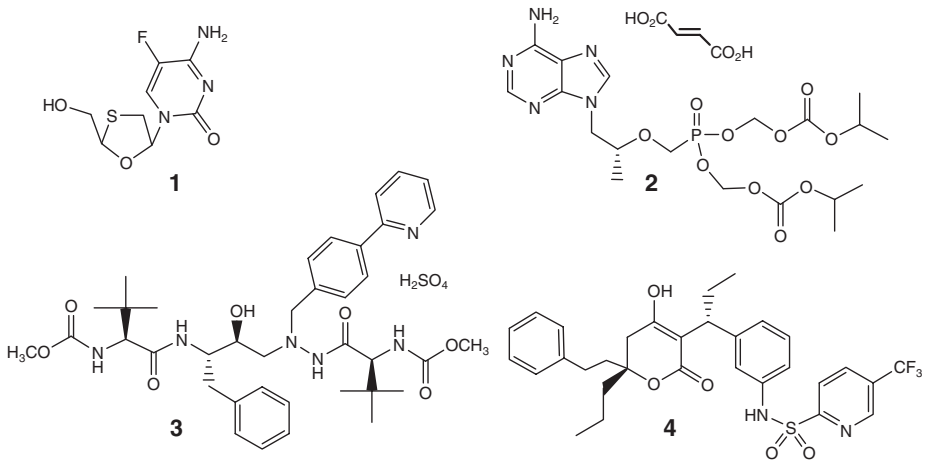
can have a negative impact on patient compliance – a key to the successful management of HIV. Poor compliance is directly related to the development of resistance and therapeutic failure. All of the issues mentioned above highlight the need for the introduction of new drugs for the treatment of HIV/AIDS. Optimally, these new drugs should be directed at new viral or cellular targets that have not yet been compromised by clinical resistance. The ideal drug should also have a low pill burden, be taken no more than once daily, and should be free of significant drug interactions, food effects, and side effects. Additionally, the optimal drug should have a high barrier to the development of resistance.

2. FDA-APPROVED DRUGS SINCE 2003

Of the new drugs approved during the past two years, only the 36-amino-acid polypeptide enfuvirtide (T-20) works by a new mechanism of action. Enfuvirtide is thought to prevent viral fusion by competitive binding to a region of the viral envelope glycoprotein gp41. It is indicated for use in treatment-experienced patients with uncontrolled viral replication, and is given by subcutaneous injection twice daily. Mutations in the gp41 region that cause resistance have been documented in patients treated with enfuvirtide in combination with other antiretroviral agents [3].

Two new NRTIs with once-daily oral dosing have recently been approved. Emtricitabine **1** (FTC) is the 5-fluoro derivative of lamivudine and presents a similar resistance profile. Tenofovir disoproxil fumarate **2** (TDF) is a guanosine analog incorporating an acyclic phosphonomethyloxy group as a sugar mimic. Unlike the other NRTIs, **2** requires only the intracellular addition of two phosphates to become the active form of the drug. A formulation containing a fixed combination of **1** and **2** has been approved for marketing as Truvada®, and is indicated for dosing as a single tablet once daily. A NRTI combination product that combines abacavir with lamivudine (Epzicom®) has been approved. It is also indicated for dosing as a single tablet once daily.

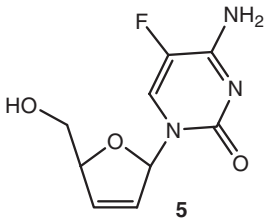


An important addition to the protease inhibitor (PI) class is atazanavir sulfate **3** (ATV), which is the first drug from this category to be approved for once-daily dosing. Like most other PIs, the pharmacokinetic profile of **3** is significantly improved when administered with ritonavir. Another advantage is that **3** has only minor effects on cholesterol and triglyceride levels. The second new entry from the protease class is fosamprenavir calcium (Lexiva®), which is a phosphate prodrug of amprenavir. This new tablet formulation has considerably decreased the pill burden of the parent drug, and may be taken with meals without compromising oral absorption. Tipranavir **4** (TPV) is a structurally distinct PI that has been submitted for FDA approval.

3. NEW COMPOUNDS DIRECTED AT CLINICALLY VALIDATED TARGETS

3.1. NRTIs

In a 10-day study in antiretroviral naïve patients, daily doses of 50–200 mg of the 5-fluorocytidine analog **5** (RVT, D-d4FC) reduced plasma viral RNA from 1.67 to 1.7 $\log_{10}$ copies/mL [4]. When administered as add-on therapy to treatment-experienced patients at 200 mg/day, 4 of 8 patients achieved an undetectable viral load (<400 copies/mL) [5]. The oxathiolane derivative of cytidine **6** (SPD-754) has also been shown to decrease viral loads in naïve patients in a 10-day monotherapy study, with no evidence of drug-related resistance [6].



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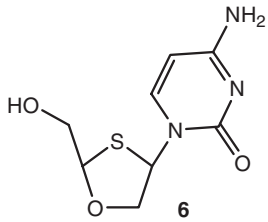

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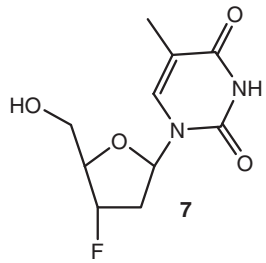

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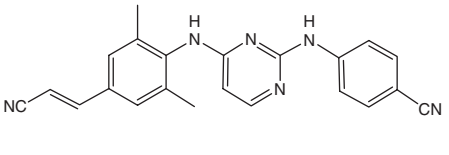
The dideoxythymidine compound **7** (alovudine, MIV-310) was recently reported to have significant antiviral activity in a cohort of 15 patients with at least two thymidine-associated mutations who were failing current therapy [7]. When added to their current therapy at the remarkably low oral dose of 7.5 mg/day over a four-week period, the median decrease in viral load was 1.13 $\log_{10}$. The four patients taking stavudine as part of their regimen experienced a 0.57 $\log_{10}$ drop in viral load, versus a 1.88 $\log_{10}$ drop in the 11 other patients.

3.2. NNRTIs

Because NNRTIs have become a cornerstone of HIV treatment, and because cross resistance is common between the three marketed drugs in this class, the need for

new NNRTIs that have a robust resistance profile is pressing. Significant progress has been made in the discovery of such compounds. The diarylpyrimidine **8** (rilpivirine, R278474, TMC-278) has been reported to be active against a panel of viruses including L100I, K103N, Y181C, Y188L, G190S and K103N/Y181C, many of which are insensitive to the marketed NNRTIs. It is significantly more potent than efavirenz and its predecessors TMC-120 and TMC-125, has good oral bioavailability, and has been evaluated in one-month oral toxicity studies in rats and dogs [8]. In a recent clinical study in patients not currently receiving antiviral treatment, oral doses of between 25–150 mg of **8** in PEG-400 solution were administered once daily for 7 days. All doses produced a 1.0–1.3 $\log_{10}$ drop in viral load by day 8, with trough plasma levels well above the protein-adjusted IC_{90}. The compound was generally well tolerated, and no evidence of resistance was observed [9].



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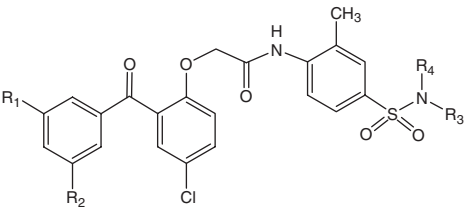
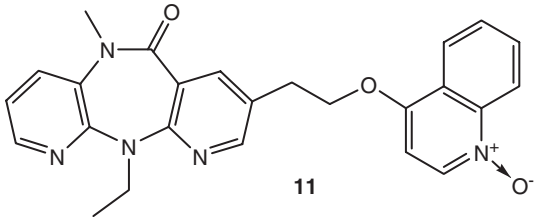


9 $R_1 = CF_3$, $R_2 = F$, $R_3 = R_4 = H$

10 $R_1 = CN$, $R_2 = Cl$, $R_3 = COCH_2CH_3$, $R_4 = Na$

A second class of NNRTI with a broad spectrum of activity against mutant viruses is exemplified by the benzophenone **9** (GW4511). Twelve mutant viruses containing single RT mutations known to cause resistance to various NNRTIs were vulnerable to **9** at concentrations less than 10-fold higher than the IC_{50} for wild type [10]. A number of other mutant viruses containing double mutations such as K103N/Y181C and K103N/L100I were also sensitive to **9**. Results from a Phase I clinical trial of the prodrug **10** suggest that twice-daily dosing will be required in subsequent clinical studies [11].

The third family of broadly active NNRTIs is from the dipyridodiazepinone class. Phase I studies of compound **11** (BILR 355) have shown modest human pharmacokinetics; however, co-administration with the P-450 inhibitor ritonavir may allow the option of once-daily dosing. Compound **11** has an EC_{50} of 1.3 nM against wild-type HIV-1, and has an EC_{50} of 3.5–235 nM versus a panel of clinically relevant single and double mutant viruses [12].



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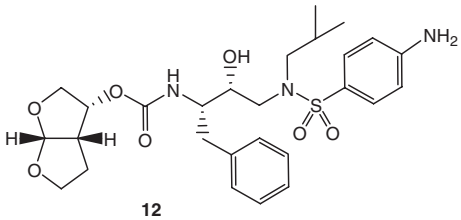
3.3. PIs

New PIs are being designed to address the problem of PI cross-resistance. Among compounds reported to be in clinical development, compound **12** (TMC114, darinavir) displays good antiviral potency against a range of clinical HIV isolates, including many that are resistant to marketed PIs. In a Phase 2 clinical study of treatment-experienced patients failing a regimen that included a PI, switching the PI to ritonavir-boosted TMC114 resulted in a median viral load reduction of 1.2 to 1.5 $\log_{10}$ after 14 days of treatment [13]. Compound **13** (VX385, GW0385) reportedly has excellent potency against wild-type virus and is active against the majority of PI-resistant isolates in preclinical antiviral assays. However, clinical pharmacokinetic studies suggest that twice-daily dosing and boosting with ritonavir may be necessary for efficacy against PI-resistant HIV isolates [14].



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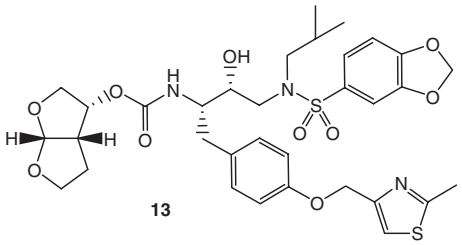

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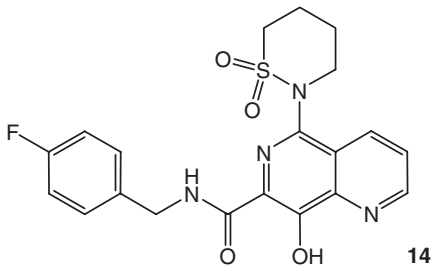
3.4. Integrase inhibitors

HIV integrase, a virally encoded enzyme that covalently inserts the viral DNA into a host cell chromosome, is required for viral replication, and therefore, an attractive target for antiretroviral drugs. Despite many years of research, clinical proof-of-concept for integrase inhibitors has only recently been reported [15]. In a placebo-controlled clinical trial of 30 treatment-naïve and treatment-experienced HIV-infected subjects, those who received either 200 mg or 400 mg twice-daily of the naphthyridine carboxamide integrase inhibitor **14** (L-000870810) displayed an average reduction in viral load of >1.7 $\log_{10}$ after 10 days of treatment. Although clinical development of this compound was discontinued owing to findings from long-term preclinical toxicology studies, another investigational HIV-1 integrase inhibitor has been advanced into clinical development. The finding that an integrase inhibitor can be a potent antiretroviral drug provides strong impetus to develop other compounds in this mechanistic class.



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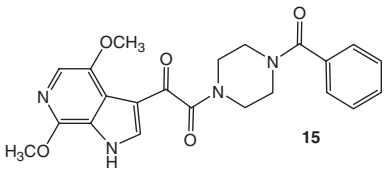
3.5. Entry inhibitors

Blocking the entry of HIV virions into target cells represents a fundamentally new way of inhibiting HIV replication, as exemplified by enfuvirtide. Compounds with two different mechanisms of action distinct from that of enfuvirtide have recently displayed clinical efficacy. HIV enters host cells by sequentially binding its envelope glycoproteins (gp120:gp41) to the host protein CD4 and to one of two co-receptors CCR5 and CXCR4. Some HIV isolates use only CCR5, some can use either CCR5 or CXCR4, and a few use only CXCR4. Compound **15** (BMS488043) blocks HIV entry by binding to the viral protein gp120, thereby preventing gp120 from binding productively to CD4. In a clinical trial, 8 of 12 subjects receiving 1800 mg of **15** twice daily displayed viral load reductions of >1 $\log_{10}$ [16]. This variability in efficacy is consistent with the preclinical observation that unselected primary HIV isolates vary in sensitivity to the related compound BMS-806 by more than three orders of magnitude.

A second entry inhibitor mechanism targets one of the co-receptors, CCR5, rather than a viral protein. Whereas molecules binding CD4 could theoretically inhibit entry of any HIV isolate, CCR5 antagonists can only block entry of those isolates that use CCR5 exclusively. Three CCR5 antagonists currently in clinical development are compounds **16** (UK427857, maraviroc), **17** (GW-873140, ONO 4128) and **18** (Sch-D, Sch-417690) which are reportedly in or approaching Phase 3 clinical trials. In Phase 2 studies of HIV-infected subjects whose viruses used only CCR5, 100 mg of **16** twice daily produced a median viral load reduction of 1.42 $\log_{10}$ after 10 days of treatment. Interestingly, at least two patients who showed poor antiviral responses were subsequently found to have circulating virus that could use the co-receptor CXCR4. The poor virologic response in these patients was likely explained by outgrowth of the CXCR4-using variant during CCR5 antagonist therapy [17]. Compound **17** showed similar efficacy in Phase 2 monotherapy studies, with a 100 mg twice-daily regimen producing a median viral load drop of 1.5 $\log_{10}$ [18]. Average viral load decreases of at least 1.0 $\log_{10}$ were observed for HIV infected patients receiving either 10, 25 or 50 mg of compound **18** twice daily over a 14 day period [19].



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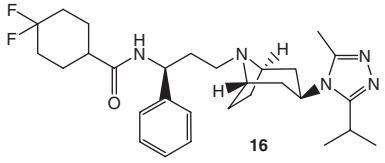

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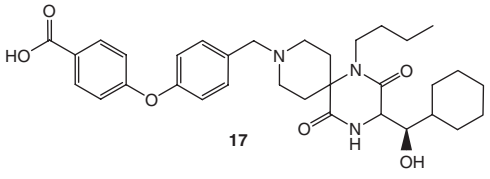

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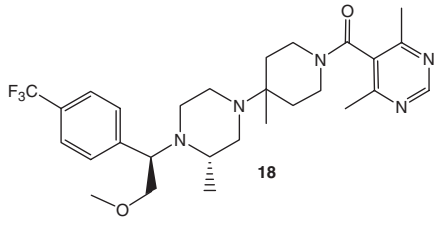
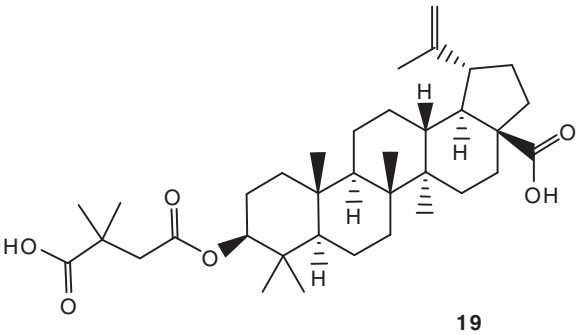


18

3.6. Maturation inhibitors

The triterpene derivative **19** (PA-457) is an antiviral agent whose antiviral mechanism of action was only recently elucidated. This compound blocks the ability of HIV protease to cleave a specific site in the gag protein, the CA-SP1 cleavage site, which in turn prevents full maturation of newly made virions into an infectious form. Compound **19** does not appear to act directly on HIV protease, but the precise biochemical mechanism of inhibition is still unknown. In recently disclosed clinical data, administration of single oral doses of **19** (75, 150, or 250 mg) elicited a viral load reduction of up to 0.7 $\log_{10}$ in HIV-infected subjects [20]. The compound has been granted fast-track approval status by the FDA.

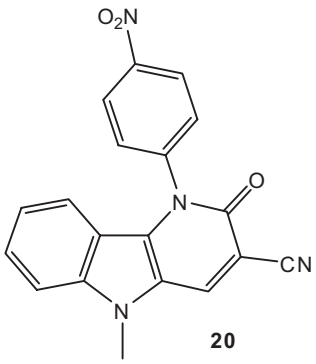


19

4. LOOKING FORWARD: ANTIVIRAL AGENTS WITH NEW MECHANISMS OF ACTION

4.1. Nucleotide-competitive RTIs

Recently a family of compounds represented by **20** has been reported to inhibit HIV RT by a novel mechanism. Enzyme kinetics have shown that **20** is a competitive inhibitor of the incoming nucleotide, does not act as a chain terminator, and inhibits RT with an IC_{50} of 290 nM and an EC_{50} of 30 nM in an antiviral assay [21].



20

4.2. Additional targets

As new therapeutic agents such as those described in Section 3 above enter the armamentarium of antiretroviral drugs, HIV variants displaying resistance to those agents will almost certainly emerge. Worse yet, if history is a guide, many of these resistant viruses will also display intraclass drug resistance. One approach to circumventing intraclass resistance is to develop agents in different target classes. Many possible antiviral targets have been identified, and more are being discovered all the time. Among viral proteins, one particularly attractive target is the RNase H [22,23], an enzyme that resides in a discrete domain of RT. This conserved enzyme, which is absolutely required for viral replication, has an active site architecture similar to that of integrase, and indeed similar classes of compounds have been found to inhibit both enzymes [24]. HIV also has a number of accessory genes (vif, vpr, vpu, rev, tat, and nef) that play important roles in replication and/or pathogenesis. Each of these represents a potential antiviral target, and though in some cases the biology and biochemistry are poorly understood, some inhibitors of these pathways have been reported. One example can be found in the recent description of small molecules reported to block binding of tat to the host co-activator protein PCAF [25].

Beyond viral proteins, clear opportunities exist to target cellular proteins with antiviral drugs, as exemplified by CCR5 antagonists. Host proteins have been implicated as important players in several stages of the HIV replication cycle. As one example, the cellular protein tsg101 is essential for the process of releasing new viruses from an infected host cell [26]. There is little doubt that many other essential host proteins will be identified by the application of siRNA screening to HIV infection assays, and those host proteins may well represent the future of antiretroviral drugs.

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Antibacterials for the Treatment of Gram Positive Infections

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Contents

1. INTRODUCTION

More than any other advances in medicine in the last century, those made in the 1940s in antibiotics were responsible for the increase in lifespan enjoyed by the inhabitants of developed countries. By the mid 1950s there were several antibiotics available for the treatment of bacterial infections, particularly those caused by Gram positive organisms and researchers in the 1960s and 1970s focused on Gram negative bacteria. In recent decades the increased occurrence of life-threatening antibiotic-resistant Gram positive infections has put the spotlight, in both the scientific [1] and the popular press [2,3], on the incidence of morbidity and mortality ascribed to them.

Gram positive bacteria are responsible for a wide range of clinical infections, from community-acquired otitis media, the common ear infections of young children, to nosocomial bacteremias and soft tissue infections prevalent in patients with indwelling catheters and implanted prostheses. The Gram positive anaerobe, *Clostridium difficile* is the most common cause of colitis in hospitals and old folks homes.

Gram positive bacteria, by virtue of their prokaryotic nature and reasonably permeable cell wall present the medicinal chemist with a wide variety of targets and hence several different structural types of anti-Gram positive agents are known.

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Although the discovery of novel pharmacophores has slowed, it has not yet stalled and, moreover, new modifications of older types have yielded therapeutic advantages in many cases.

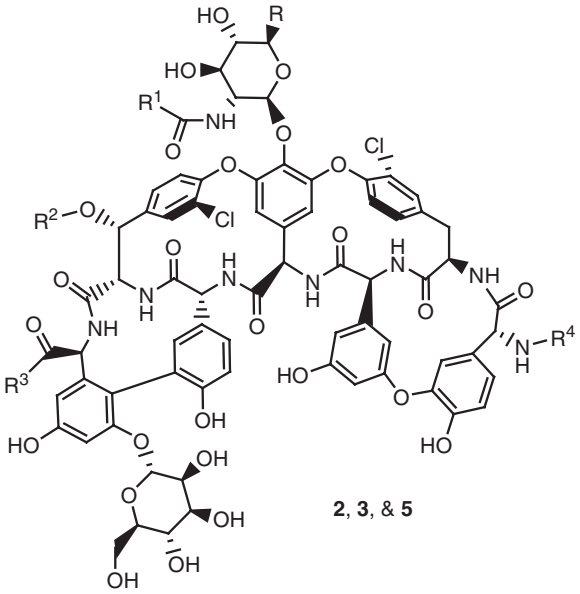
2. GLYCOPEPTIDES

For many years the two glycopeptides in clinical use, vancomycin **1** and teicoplanin **2**, were considered the bastion of defense against systemic infections of resistant *staphylococci* and *enterococci,* but as this bulwark has collapsed [4,5] there has been a concerted effort at second generation glycopeptides. These have been approached by classical and enzymatic semi-synthesis and via genetic manipulation of the producing organism and mutasynthesis. Many of these transformations have been enabled by the sequencing of several biosynthetic gene clusters [6,7], and these results have led to analogs of vancomycin with modified sugars [8], variants in the lipoacyl group [9] of teicoplanin **2** and A-40926 **3** and a difluoro analog [10] of balhimycin **4**. The glucosyl transferases have been heterologously expressed and shown to accept UDP and TDP derivatives of a wide variety of modified sugars in transfer reactions to the 4-OH of the trihydroxyphenyl glycine moiety of both vancomycin **1** and teicoplanin **2**. Moreover similarly obtained vancosaminyl transferase was able to attach both vancosamine and 4-*epi*-vancosamine to the attached modified sugar [8]. Some of the semisynthetic derivatives are more advanced in development and show, not only increased potency and efficacy against resistant strains, but also very different pharmacokinetics.

Drug	Terminal Plasma Half-life	Typical suggested regimen
Vancomycin **1**	4 to 6 hours	1 g every 12 hours for 7 to 10 days
Teicoplanin **2**	3 to 7 days [11]	12 mg/kg day 1, followed by 6 mg/kg/day [12]
Dalbavancin **5**	9 to 12 days	1 g followed by single 1/2 g after 7 days [13]
Oritavancin **6**	$>$ 10 days	1.5–3 mg/kg daily for 3 to 7 days [14]
Telavancin **7**	7 to 9 hours [15]	7.5 to 15 mg/kg daily for 7 days [16,17]

Dalbavancin **5** had MIC_{50}s of 0.06, 0.03, 0.008, 0.016 mg/L against *Staph. aureus,* coagulase-negative *staphylococci,* β-hemolytic and viridans group *streptococci*, respectively, and inhibited all vancomycin-susceptible *enterococci* at 0.25 mg/L in a study of 1129 Gram positive clinical isolates [18]. It was the most potent of several

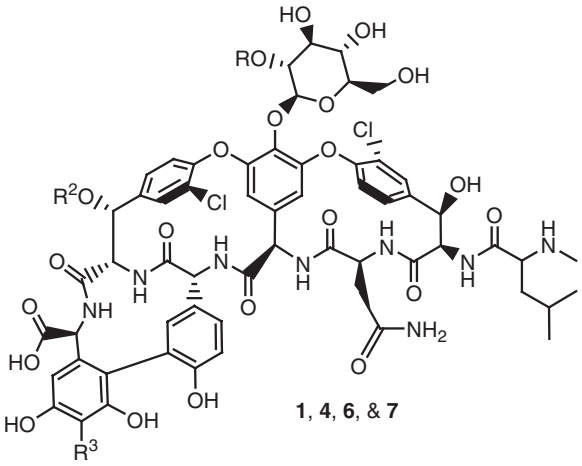
agents tested against 146 strains of *staphylococci* [19], and has shown, statistically significant, superior efficacy and safety on the regimen above to vancomycin **1** in the treatment of catheter-related septicemia from Gram-positive pathogens [20].



2, **3**, & **5**

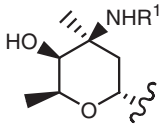
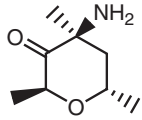
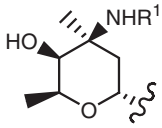
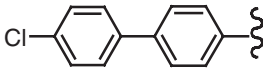
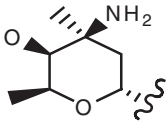
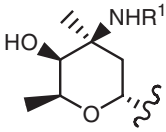
Antibiotic	R	R^1	R^2	R^3	R^4
Teicoplanins **2**	$-CH_2OH$	Variant~C_9H_{17}-	β-2-Acetylamido-glucopyranosyl-	-OH	-H
A-40926 **3**	-COOH	$CH_3(CH_2)_8$-	-H	-OH	$-CH_3$
Dalbavancin **5**	-COOH	$(CH_3)_2CH(CH_2)_8$-	-H	$(CH_3)_2N(CH_2)_3NH$-	$-CH_3$

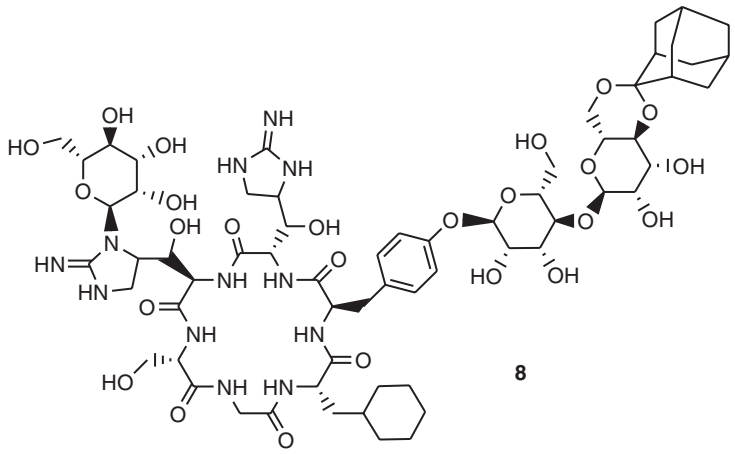
Several derivatives of *N*-decylaminoethylvancomycin were prepared with modifications at the carboxyl group and via aminomethylation of the 3,5-dihydroxyphenyl ring. [21] On the basis of tissue distribution in rats, following a single i.v. dose of 50 mg/kg, and 7 days at 25 mg/kg/day, as well as antibacterial potency, telavancin **7** was selected for clinical studies. In a comparative study against 401 clinical isolates of Gram-positive aerobes, telavancin **7** had MICs of $\leqslant$ 1 mg/L for almost 90% of the strains and with the exception of a group of 29 strains of Vancomycin-susceptible *Enterococcus faecalis*, where teicoplanin **2** was more potent, it was more potent than vancomycin **1** or teicoplanin **2** against all groups [22]. The side-chain of oritavancin (LY333328) **6** has been implicated in membrane bound dimerization of the antibiotic enabling it to bind both the terminal dipeptides and depsipeptides of vancomycin-susceptible and -resistant strains [23]. Oritavancin **6** has been shown to be efficacious and synergistic with ceftriaxone in a rabbit model of pneumococcal meningitis [24].



1, **4**, **6**, & **7**

The mannopeptomycins are a family of glycopeptides, structurally very different from the classical glycopeptides discussed above, and they have been shown to bind to lipid II, but perhaps not surprisingly at a different target than vancomycin **1** or mersacidin. [25]. A semisynthetic mannopepto-mycin, AC 98-6446 **8**, has been shown to have potent *in vitro* activity against a large battery of resistant and sensitive *staphylococci, enterococci* and *streptococci* [26].

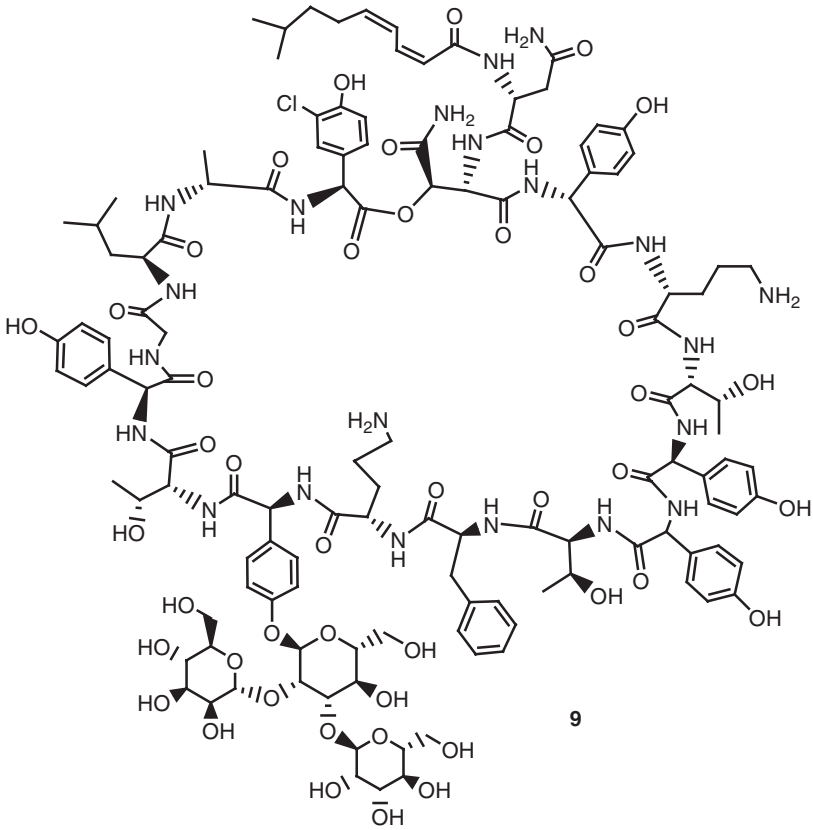
Antibiotic	R	R^1	R^2	R^3
Vancomycin **1**		-H	-H	-H
Balhimycin **4**	-H	N/A		-H
Oritavancin **6**				-H
Telavancin **7**		$-(CH_2)_2NH(CH_2)_9CH_3$	-H	$-CH_2NHCH_2PO_3$



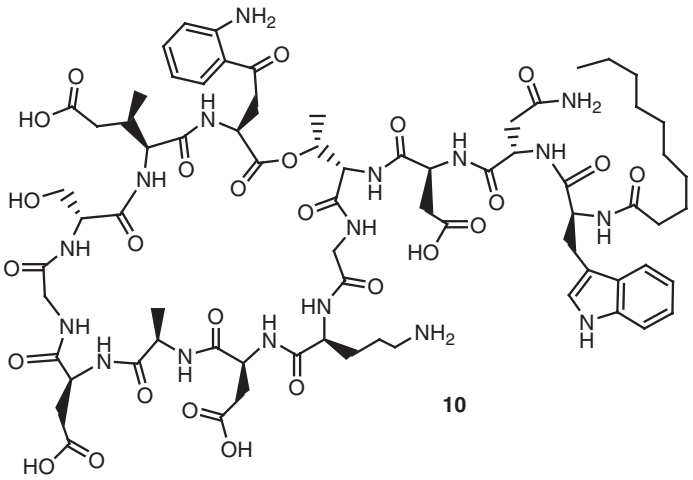
8

3. LIPOPEPTIDES AND LIPOGLYCOPEPTIDES

The only lipoglycopeptide under serious study has been the ramoplanin **9** and it has been advanced both as a prophylactic for patients at high risk of VRE infections [27] and for the treatment of clostridial colitis. It is probably the most potent anti-Gram positive antibiotic tested in man with virtually no resistance seen or developed *in vitro*, but it has little or no activity against Gram negatives [28]. The mode of action has been implicated as binding to lipid II in a 2:1 complex and inhibiting bacterial transglycosylases [29]. The major component A2 of the antibiotic complex and two minor components A1 and A3 have been the subject of total synthesis studies [30,31]. These synthetic studies have been extended to analogs, which provide crucial SAR data. The β-hydroxyasparagine moiety can be replaced with a diaminopropionic acid to give, rather than a depsipeptide, a cyclic peptide of improved *in vitro* potency and base stability. Substituting α,γ-diaminobutyric acid for HOAsn, and hence expanding the size of the peptide ring, led to loss of activity. Replacing the acyl chain with an acetyl group reduced activity significantly [32]. The lack of activity of the ring expanded analog has been attributed to its much greater tendency to self aggregate [33].



9



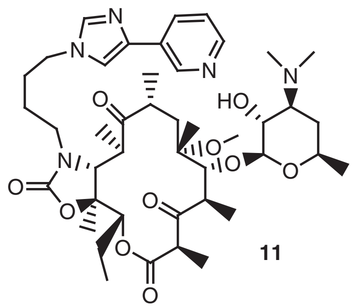
10

The lipopeptide, daptomycin, (Cubicin^{TM}) **10** has been approved by the USFDA for the treatment of skin and soft tissue infections and is the first of the class. The

cyclase from the analogous calcium-dependent antibiotic from *Streptomyces coelicolor* was used to cyclise several lipopeptide analogs of daptomycin **10** and define several aspects of the SAR. Activity was invariably calcium dependent and two of the four aspartic acid residues were essential for activity [34].

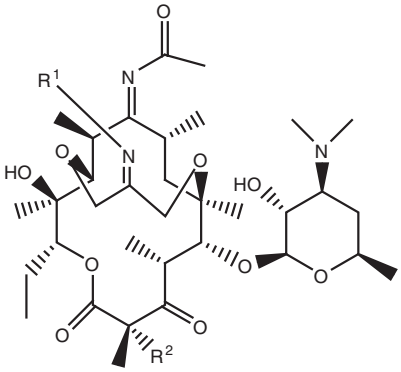
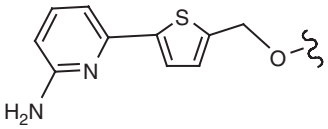
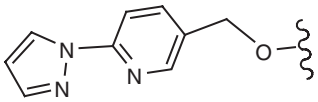
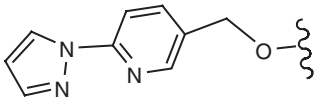
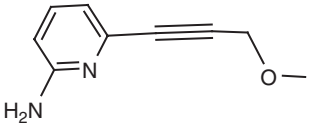
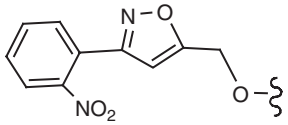
4. MACROLIDES

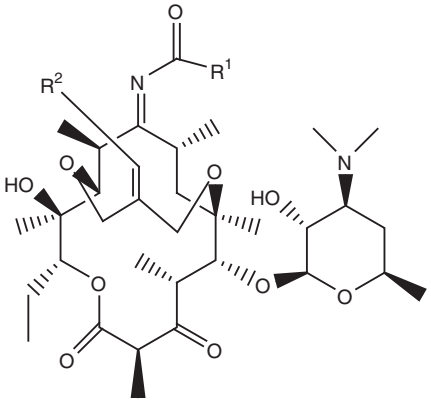
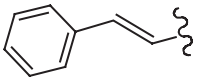
The good clinical efficacy in upper respiratory tract infections of telithromycin **11**, and the low incidence of resistance, particular in the Far East where *S. pneumoniae* have a very high incidence of resistance to macrolides [35] is explained by further binding of the aryl side chain to sites on the 23S rRNA. However site-directed mutagenesis of likely ribosomal binding nucleotides G745, G748, A752 (domain II) and A2058 (domainV) did not identify this, although they did distinguish telithromycin **11** binding from that of tylosin [36].



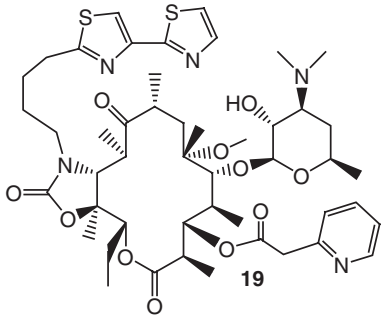
11

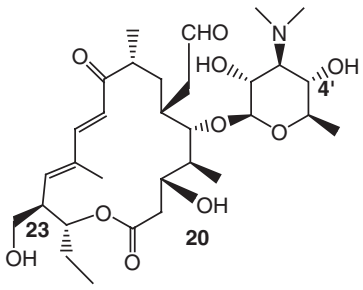
Several novel 6,11-**O**-bridged ketolides have been synthesized and evaluated [37–44]. EP-13159 **12** had very potent activity against both macrolide-sensitive and -resistant *S. pneumoniae*, and a $t_{1/2}$ of 12.4 hours in the dog. EP-13417 **13** was shown to accumulate significantly in the lung tissue in the mouse, as also did EP-13420 **14**. Both EP-13420 **14** and EP-13543 **15** were of equal efficacy to telithromycin **11** in mouse systemic *S. aureus* and *S. pneumoniae* models and superior in rodent lung infection models of *H. influenzae. In vitro* potency was highly dependent on the geometric isomer of the oxime and isoxazoles such as EP-13428 **16** were prepared in high abundance of the desired *E* isomer. Although both EP-1304 **17** and EP-12578 **18** were susceptible to *erm*-mediated resistance both were potent against inducible resistant strains. Ribosomal binding studies indicate that these bridged macrolides bind at the same site on the *E. coli* ribosome as erythromycin, protecting A2058 and A2059 (domain V) from methylation. Those with an extended side chain also protected A752 (domain II) [45].

	Antibiotic	R^1	R^2
	EP-13159 **12**		-F
	EP-13417 **13**		-F
	EP-13420 **14**		-H
	EP-13543 **15**		-H
	EP-13428 **16**		-H

	Antibiotic	R^1	R^2
	EP-1304 **17**	CH_3-	-H
	EP-12578 **18**	-OCH_3	

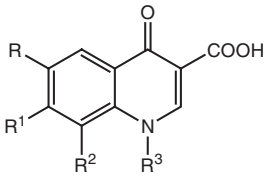
A series of descladinosyl-6-**O**-methylerythromycin A-11,12-carbamate-3-**O**-esters showed good *in vivo* activity in a mouse lung *S. pneumoniae* infection. FMA 1082 **19** was comparable to telithromycin **11** in this model and gave high levels in lung tissue following an oral dose [46]. Derivatives of tylosin have been prepared in which each of the neutral sugars have been replaced. From the monoglycoside, OMT **20**, replacement of the original 4'-sugar with arylalkyl groups overcame *erm* and *mef* resistance in *S. pneumoniae* and somewhat less efficiently in *S. pyogenes* [47]. Acylation (carbamates) or alkylation at the 23 position was less effective at overcoming resistance but did maintain good activity against *H. influenzae* [48]. An extensive effort to extend many of the beneficial SARs from ketolides to azalides has not been met with major success [49].

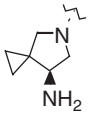
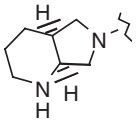
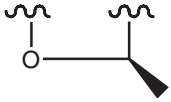
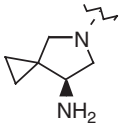
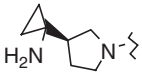
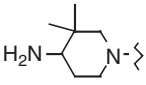




5. QUINOLONES

Quinolones have long since emerged from their Gram negative urinary tract infections origin to encompass a broad spectrum of bacteria infections. Recently the mantra that a 6-fluoro substituent was essential for high potency has collapsed. Quinolones are a major focus of antibacterial research, with several new agents in various stages of development. However the potency and efficacy of advanced agents is such that many of the newer agents have needed to focus on specific applications. DX-619 **25**, which was the subject of 20 presentations at the last two ICAACs, and which has potent broad spectrum activity, [50,51] appeared to be focused heavily on *staphylococci* [52–58] and somewhat less so on other gram positives [59–64]. The compound showed superior efficacy to linezolid **31** and vancomycin **1** in animal models of *S. aureus* and VRE endocarditis, [65] an LD_{50} i.v. in mice, rats and monkeys of >100 mg/kg, an NOAEL of 5 mg/kg/day for 4 weeks in rats and monkeys and no chondrotoxicity in dogs at 40 mg/kg/day for 8 days [66].



Quinolone	R	R^1	R^2	R^3
Sitafloxacin **21**	-F		-Cl	
Pradofloxacin **22**	-F		-CN	
AM-1939 **23**	-F			
DK-507k **24**	-F		$-OCH_3$	
DX-619 **25**	-H		$-OCH_3$	
WCK 1152A **26**	-F		$-OCH_3$	

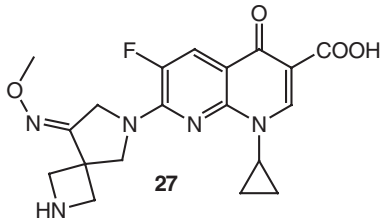
Pradofloxacin **22**, which appears to be focused on the domestic pet market, is highly bactericidal and this is ascribed to its potency in inducing the SOS system [67].

AM-1939 **23** shows potent Gram positive activity, superior to that of marketed quinolones both *in vitro* and in efficacy models in mice against *S. aureus* and *S. pneumoniae* [68].

DK-507k **25** and sitafloxacin **21** are probably the most potent quinolones against a broad range of Gram positive organisms, and the latter is slightly more potent against most of the Gram negatives, however DK-507k was still more efficacious than ciprofloxacin in a rat urinary tract model of *Pseudomonas aeruginosa* colonization of a foreign implant [69,70]. DK-507k **24** and sitafloxacin **21** were the most potent of 10 agents tested against 261 pneumococcal strains including 26 quinolone resistant strains [71]. DK-507k **24** was as efficacious as clinafloxacin in a mouse thigh model with *S. aureus* 2017 (ciprofloxacin-resistant MRSA) and more efficacious against the 1417 MRSA strain [72].

WCK 1152 **26** is bactericidal against *S. pneumoniae* [73] and more active than moxifloxacin in a mouse pneumonia model with fluoroquinolone-sensitive and -resistant strains [74] and against *Legionella pneumophila* in a guinea pig model [75].

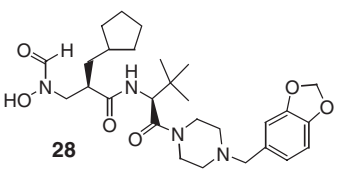
Described as a quinolone, although actually a naphthyridine, DW-224a **27** is clearly positioned for respiratory tract infections. It has potent activity, comparable to gemifloxacin , against a battery of 353 quinilone sensitive *pneumonococci* [76] and against 29 quinolone resistant strains for which the resistance mutation had been characterized [77]. It has potent activity against a battery of predominantly clinical isolates of *Mycoplasma pneumoniae, M. hominis, Legionella pneumophila* and *Chlamydophila pneumoniae* [78].

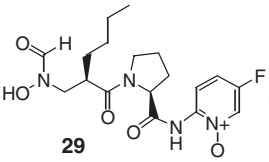


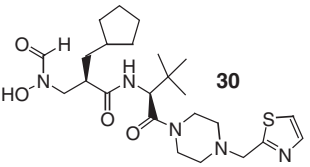
27

6. DEFORMYLASE INHIBITORS

BB-83698 **28** is the first peptide deformylase inhibitor to reach the clinic where it showed pharmacokinetic parameters congruent with those seen in animal studies and exhibited no adverse effects at levels projected to be therapeutic [79]. Several new peptide deformylase inhibitors with potent activity against *staphylococci* and *streptococci* have been described. LBM415 **29** has an *in vitro* spectrum with activity against both antibiotic-sensitive and -resistant respiratory tract pathogens [80,81], and has good tissue distribution following i.v. or oral administration [82] and efficacy in rodents models [83]. GBB-200061 **30** is reported to have similar properties [84].

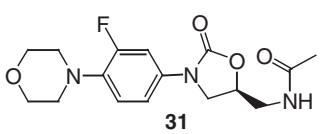


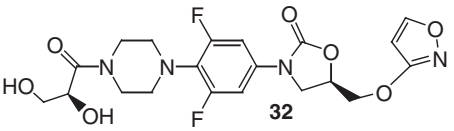




7. OXAZOLIDINONES

Several new oxazolinones have been prepared, extending the results on replacement of the acetamido group of linezolid **31**, which previously led to AZD2563 **32** [85], to substituted or unsubstituted triazoles or tetrazoles. It has been shown that triazoles with a 4- substituent bind more weakly monoamine oxidase Type A than do other oxazolidinones [86].



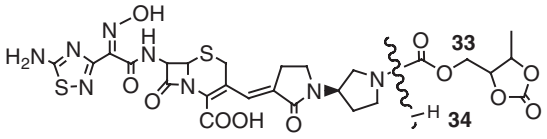


8. B-LACTAMS

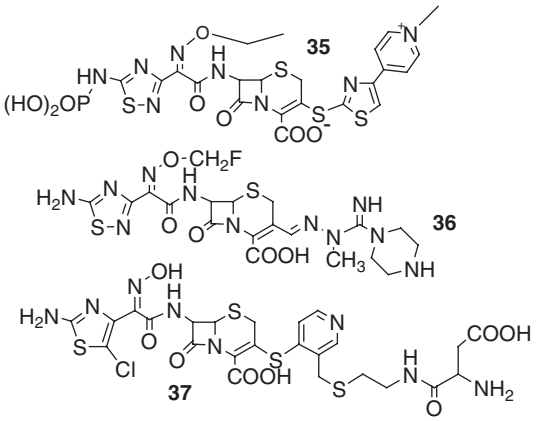
β-Lactams continue to provide the pharmacophore, which has spawned the greatest number of new antibacterials warranting clinical studies. During the last 5 years, 15 new β-lactams with impressive anti-Gram positive activity have been reported at ICAACs.

8.1. Cephalosporins

In that time, out of 9 new parenteral cephalosporins reported, 6 have entered human clinical trials in the US, Europe or Japan. No reports have appeared on RWJ-333441 and S-3578, in the last two years and it is assumed that clinical trials have been halted. Ceftobiprole medocaril (BAL5788) **33**, is a prodrug of Ro 63-9141 **34** showing favorable activity against Pen-resistant *S. pneumoniae* (PRSP) ($MIC_{90} = 0.125\,\mu g/mL$) [87-89] and is currently in USFDA Fast Track Phase II trials for treatment of resistant infections.

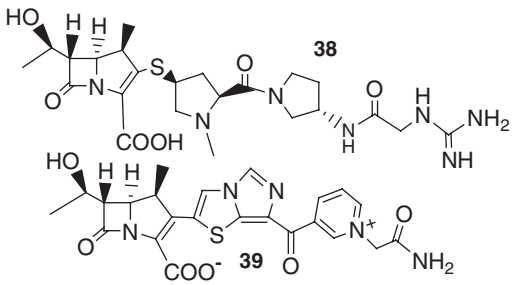


The common feature of the other 3 cephalosporins with this indication, in clinical studies, PPI-0903 **35** [90,91], CB-181963 **36** [92–95] and RWJ-442831 **37** [96], is a considerable activity against MRSA ($MIC_{90} = 2–4\,\mu g/mL$) and PRSP ($MIC_{90} = 0.25–1\,\mu g/mL$).



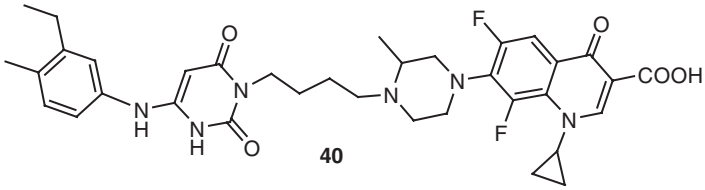
8.2. Carbapenems

Of the 6 reported parenteral carbapenems two are currently in clinical studies. Ro4908436 (US), identical to CS 023 **38** (Japan), is in Phase I studies in both countries. As well as strong activity against MRSA and PRSP, [97–99] CS 023 **38** is also active against *Pseudomonas aeruginosa,* which often causes concomitant infections with MRSA. ME1036 **39** is in Phase I in Japan and has very strong activity against PRSP ($MIC_{90} = 0.03\,\mu g/mL$) along with its considerable activity against MRSA and, in addition, it shows good activity against BLNAR-type resistant *Haemophilus influenzae*. [100,101]. ME1036 **39** is projected as a useful agent for the treatment of resistant respiratory or ear & nose infections both in community-acquired and nosocomial settings.



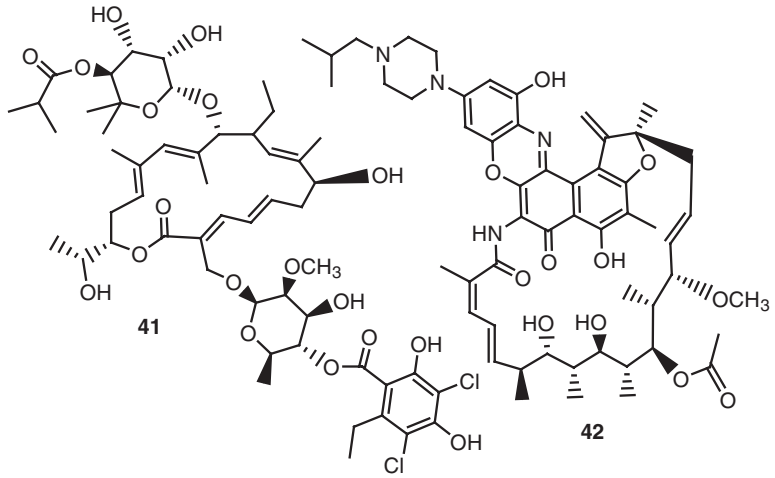
9. OTHER STRUCTURAL TYPES

Several instances exist where only one or two examples of a structural class have been the subject of recent noteworthy studies. The attachment of quinolones or naphthyridines to other antibacterial pharmacophores has been extended to a new type. MBX-251D **40** comprises a quinolone attached to a DNA polymerase IIIc inhibitor. It was potent against all Gram positives tested except for strains having both gyrase and Pol IIIc mutations, and it was efficacious by the i.v. route in mouse i.p. infection models of *S. aureus* Smith, MRSA and VRE [102]. Similarly, a series of oxazolidinone-quinolone hybrids has been made with several members highly potent against gram positives including strains resistant to linezolid **31** and/or ciprofloxacin [103,104].

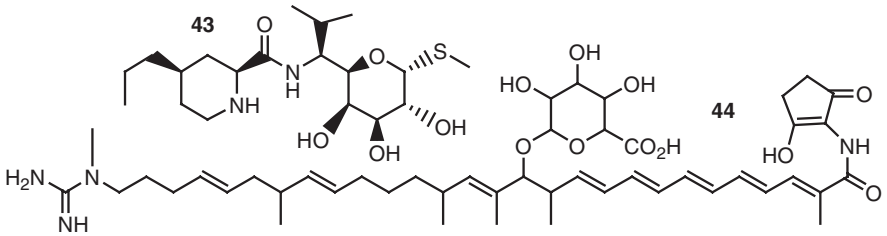


40

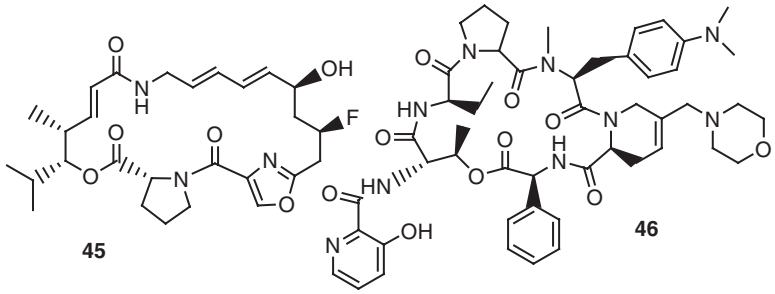
Increased attention has been directed at *Clostridium difficile*-associated diarrhea and colitis as outbreaks of resistant strains with increased mortality have emerged. These conditions were typically treated with oral vancomycin **1** or metronidazole, and although these were highly effective, 20% relapse after the end of treatment is common. Other antibiotics, bacitracin, fusidic acid, and rifampicin were not therapeutically superior, however teicoplanin **2** showed a somewhat lower relapse rate [105]. Also the economic cost of this infection has come under scrutiny and is considerable [106,107]. OPT-80 (tiacumicin B) **41** is an atypical macrolide, which shows very potent activity towards *C. difficile.* In a comparison with vancomycin **1**, metronidazole, moxifloxacin, fusidic acid and linezolid **31**, against 207 clinical isolates OPT-80 **41** was by far the most potent drug, with MICs against all strains of $\leqslant 0.0625$ mg/L. [108] It, **41**, is very potent against most other Gram positive anaerobes, with the exception of *C. ramosum*, and also potent against *staphylococci*, but is virtually inactive against Gram negatives [109,110]. In the hamster model OPT-80 **41** was curative and not subject to relapse as seen for vancomycin **1** [111]. Rifalazil **42** and the rifamycin derivative, ABI-0966N, are also highly potent against *C. difficile* and curative in the hamster model [112] as is ramoplanin **9** [113]. OPT-80 **41** is very well tolerated orally in both monkeys and rats with an NOEL following 28 days p.o. administration was >90 mg/kg/day [114].



A novel 7-*des*-chloro-7-methyl analog of clindamycin, (VIC-105555) **43**, and has been prepared, and shows markedly better potency [115,116], pharmacokinetics in animals [117,118] and efficacy [119,120] than clindamycin. The compound is susceptible to *erm*-mediated resistance.



A new pharmacophore has been discovered by genome scanning for biosynthetic capability leading to NCEs [121]. ECO-00501 **44** is a straight-chain Type I polyketide, potent against sensitive and resistant *staphylococci* and *streptococci* [122]. It, **44**, has a mode of action resembling that of large cationic peptides and shows efficacy in murine models [123].



The new semisynthetic streptogramin, XRP 2868, consisting of a 70/30 mixture of RPR132552 **45** and RPR202868 **46** has been shown to have potent activity against Gram positive *cocci* and other respiratory tract pathogens irrespective of their resistance to other antibiotics [124]. It is also very potent and compared well against established agents against Gram positive anaerobes, *lactobacilli* and pathogenic actinomycetes [125].

10. CONCLUSIONS

Progress over the last few years in the search for antibacterials has countered any claims that the discovery of new agents for this indication is past. As well as major improvements in spectra, resistance susceptibility, and ADME characteristics in new members of well-established classes, there have been agents discovered, and/or developed, with new mechanisms of action. In the former category we have several glycopeptides, macrolides, quinolones, β-lactams, the new lincosamide, VIC-1055555, and the new streptogramin, XRP 2868, as well as the recently US approved novel tetracycline, tigecycline, which was not described here. In the latter category, the approval of daptomycin introduces a new chemical class into therapy, and the discovery of ECO-00501, and the development of ramoplanin, provide new classes of antibiotics while the synthetic approaches to the novel targets of Pol III and peptide deformylase open areas for other new antibacterials. History indicates that bacteria will develop resistance mechanisms against all of these agents in time and our ability to combat these pathogens will depend on our commitment of resources and the quality of our science.

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Progress on New Therapeuties for Fungal Nail Infections

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Contents

1. INTRODUCTION

Onychomycosis is a fungal infection of the toe and finger nails, with the majority of cases involving infection of the toe nails [1]. The disease is mostly caused by a class of fungi known as the dermatophytes, which are also responsible for skin fungal infections. Dermatophytes flourish on dead keratinized tissue and normally infect the stratum corneum layer of skin, scalp hair and nails [2]. Non-dermatophyte species including yeasts and molds can also be involved. The dermatophytes account for around 90% of all cases of onychomycosis [1,3] and include *Trichophyton*, *Microsporum* and *Epidermophyton* species. However, *Trichophyton rubrum* and *Trichophyton mentagrophytes* are by far the major causative agents accounting for 60–70% of the cases [1,3,4]. The fungi can infect the nail plate, nail bed and surrounding skin folds (proximal fold at the cuticle and lateral folds on either side of the nail plate). Onychomycosis damages the nail plate causing thickening and discoloration. In more advanced cases, the nail plate lifts away from the nail bed, termed onycholysis, which causes discomfort and sometimes can be painful.

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Clinical presentations of onychomycosis have been divided into four categories: distal subungual (infection occurs at or near the tip of the nail plate and involves the underlying nail bed), proximal subungual (infection is at or near the cuticle and involves the underlying nail bed), superficial (infection is in the nail plate only with no nail bed involvement) and total dystrophic onychomycosis (whole nail involvement and considered a combination of the other types) [5]. Between 6.5 to 13.8% of the population in North America is reported to be infected with this disease and the prevalence increases with age [1,4,6,7]. One study reported 48% of 70 year olds are infected with onychomycosis [6].

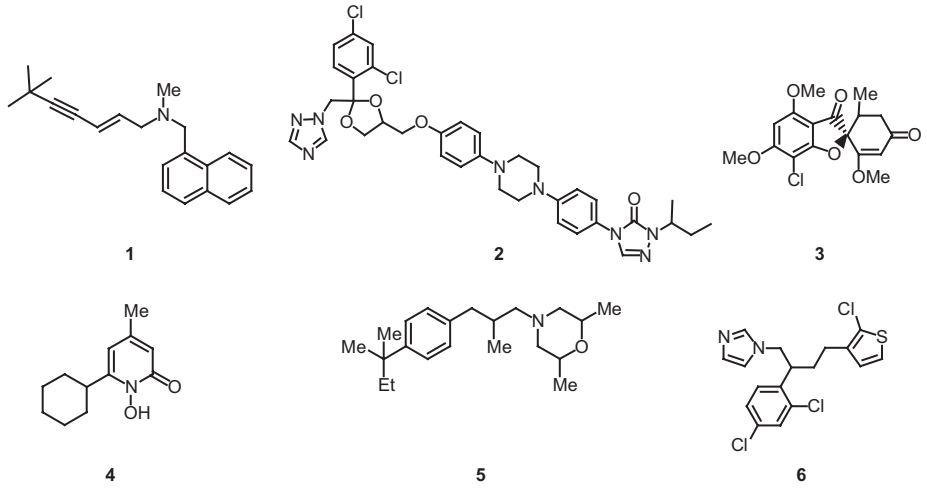
2. DRUG THERAPY

Onychomycosis is difficult to permanently cure. Treatment failures and relapses are common, which exacerbate the problem [8–10].

In order for an antifungal drug to be effective, it must presumably disseminate throughout the nail plate, nail bed and other locations occupied by the fungi, and reach concentrations that will eliminate the pathogen. This can be especially difficult when the nail plate has lifted from the bed (onycholysis). Unlike damaged skin that can repair itself, the nail plate cannot, therefore results of therapeutic treatment are not evident until new nail growth occurs and is clear of infection. Toe nails typically take about 1 year to fully grow out.

Because of the length of time required to observe new nail growth, clinical trials typically take around 9–12 months (either 3 months systemic treatment with 6–9 months follow up or 6–9 months topical treatment with 3–6 months follow up). During this time, the infected nails can be monitored for growth of new clear nail and for presence of viable dermatophytes. Efficacy is usually recorded in one of three ways: mycological cure, clinical cure or complete cure. Standard definitions of these cures are not completely uniform; each report usually provides the criteria that were used in the study. A mycological cure is defined by the extent of eradication of the fungi. It is assessed by removing a section of nail and screening for the presence of dermatophytes by microscopy and by culturing the nail for growth of dermatophytes *in vitro*. A clinical cure is defined by the extent of new nail growth at the proximal fold which is visibly clear of infection. A complete cure is defined when a patient has a mycological cure and clinical cure. Obviously, a complete cure is most desirable but hindering this is the fact that in many cases more than one digit is infected and not every digit may be cleared of infection.

Onychomycosis is treated both systemically and topically. Current systemic treatments include terbinafine **(1)**, itraconazole **(2)** and griseofulvin **(3)**. Current topical treatments include ciclopirox **(4)**, amorolfine **(5)** and tioconazole **(6)**.



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2.1. Systemic treatments

Currently, systemic treatment is the most effective method of curing onychomycosis. Even so, between 20–25% of patients fail to respond [11]. Terbinafine **(1)** and itraconazole **(2)** are the two systemic treatments of choice with terbinafine showing greater efficacy than itraconazole and lower rates of recurrence [11–15].

Terbinafine **(1)**, a representative of the allylamine class of antifungal agents, inhibits squalene epoxidase [16,17] and thereby prevents the biosynthesis of ergosterol, a key ingredient in the fungal cell wall. Terbinafine is active against dermatophytes, *M. furfur*, *Aspergillus* species and some *Candida* species including *C. parapsilosis*; however, it is fungistatic against *C. albicans* [2]. A single oral dose of 250 mg terbinafine given to humans produces peak plasma concentrations of 1 μg/mL within two hours [14]. It is >99% protein bound and has a half-life of about 36 hours. It is administered at a dose of 250 mg once daily for 6 weeks for finger nails or 12 weeks for toe nails [14]. One study showed that terbinafine localizes in the stratum corneum *via* sebum [18]. Terbinafine has a cLogP of 6.5 and a molecular weigh of 292 Da.

Itraconazole **(2)**, which is from the azole class of antifungal agents, inhibits lanosterol 14 α-demethylase and thus stops the biosynthesis of ergosterol. It has broad spectrum activity against species including dermatophytes, *Candida* species, *Aspergillus* species and *M. furfur* [2]. Blood levels of itraconazole after a single 200 mg dose given to humans reached a peak level of 0.2–0.3 μg/mL after 4–5 hours [15]. It is 99.8% protein bound and has a half-life of 21 hours. It is administered either 200 mg once daily for 12 weeks or 200 mg twice daily for 7 days followed by 3 weeks with no treatment and repeated for three months. Like terbinafine,

itraconazole also localizes in the stratum corneum via sebum but at much lower levels [18,19]. Itraconazole has a cLogP of 3.3 and molecular weight of 706 Da.

Griseofulvin **(3)**, isolated from *Penicillium griseofulvin* in 1939 [20], has a limited spectrum of activity. It is fungistatic against dermatophytes only and works by binding to microtubular proteins thus inhibiting cell mitosis. It has a cLogP of 2.2 and a molecular weight of 353 Da.

The commonly used antifungal agent, fluconazole, has also been prescribed, off-label, for the treatment of onychomycosis.

2.2. Topical treatments

Treatment of onychomycosis by topical methods has been met with limited success and reasons for this will be explored in more detail in Section 3. As with treating skin fungal infections such as tinea pedis (athletes foot), topical application for onychomycosis would seem the obvious choice. However, unlike the stratum corneum, the nail plate is a more difficult barrier to penetrate, requiring the drug to have much different physicochemical properties than are required for skin penetration. The two main topical treatments used today are ciclopirox and amorolfine, both of which are formulated in lacquers that are painted onto the infected nails. The lacquer dries to leave a water-insoluble film on top of the infected nail, which then acts like a drug depot releasing the drug into the nail plate [21,22]. Tioconazole has also been used but has been largely replaced by ciclopirox and amorolfine.

Ciclopirox **(4)** is a hydroxypyridone antifungal agent and is believed to work by inhibiting metal dependant enzymes that degrade intracellular toxic peroxides. It does this by chelating the polyvalent cations (Fe^{3+} or Al^{3+}) required by these enzymes [23–25]. Ciclopirox has antifungal, antibacterial and anti-inflammatory activities [25]. It is administered to the infected nails daily and due to the slow growth of nails, this treatment continues for at least 6 months. Ciclopirox has a cLogP of 2.5 and a molecular weight of 207.

Amorolfine **(5)** is a morpholine antifungal agent and works by inhibiting ergosterol biosynthesis. Amorolfine is administered once or twice weekly to the infected nails for 6 to 12 months. Amorolfine has a cLogP of 5.8 and molecular weight of 317.

The relative lack of clinical efficacy seen by topical antifungal treatments has led to a substantial research effort to understand the reasons for this failure. The most common belief is that treatment failure following topical therapy for onychomycosis results from the inability of the drug to penetrate and disseminate throughout the nail. This topic will be explored in more depth in Sections 3 and 4. Other factors that have been implicated include lack of microbiological activity in the presence of keratin [26,27], lack of microbiological activity against the dormant dermatophytes in the nail keratin [28] and poor penetration of drug into the dermatophytoma, a thick mass of fungi and nail debris, that builds up between the nail plate and nail bed [29].

2.3. Combination and booster treatments

Since there are no current antifungal treatments available that will provide a complete cure, practitioners are attempting combination therapy and/or booster therapy in an attempt to improve efficacy rates [30–32]. Combination therapy includes the use of oral plus oral therapy e.g. oral teribinafine plus oral itraconazole either in parallel or sequentially; oral plus topical therapy, e.g. oral terbinafine plus topical ciclopirox lacquer; or other dual, triple or quadruple combinations. However, these studies show only marginal improvement at best and further studies are warranted. Booster therapy involves giving a second course of systemic treatment, terbinafine or itraconazole, 6–9 months after systemic treatment began [30].

3. DRUG PENETRATION THROUGH THE NAIL

3.1. Composition of the nail plate

The human nail anatomy consists of nail plate, nail bed and nail matrix. The nail plate consists of three layers: the dorsal and intermediate layers derived from the matrix, and the ventral layer derived from the nail bed [33,34]. The upper (dorsal) layer is a few cell layers thick and consists of hard keratin. It constitutes the main barrier to drug diffusion into and through the nail plate. The intermediate layer constitutes three quarters of the whole nail thickness, and consists of soft keratin. Below the intermediate layer is the ventral layer of soft keratin, a few cells thick, that connects to the underlying nail bed, in which many pathological changes can occur. Thus, in the treatment of nail diseases, achieving an effective drug concentration in the ventral nail plate is of great importance. The nail bed consists of non-cornified soft tissue under the nail plate, and is highly vascularized. Beneath the nail bed at the proximal fold is the nail matrix, which is a heavily vascularized thick layer of highly proliferative epithelial tissue that forms the nail plate.

The human nail is approximately 100 times thicker than the stratum corneum of the skin, and both are rich in keratin. However, they exhibit some physical and chemical differences [35,36]. The nail possesses high sulphur content (cystine) in its hard keratin domain, whereas the stratum corneum does not. The total lipid content of the nail ranges from 0.1% to 1%, as opposed to approximately 10% for the stratum corneum.

Under average conditions, the nail contains 7% to 12% water, in comparison to 25% in the stratum corneum. At 100% relative humidity, the maximum water content in the nail is approximately 25%, in sharp contrast to that in the stratum corneum, which can increase to 200–300%.

The nail's unique properties, particularly its thickness and relatively compact construction, make it a formidable barrier to the entry of topically applied agents [37]. In one study, the concentration of an applied drug across the nail dropped about 1000-fold from the outer surface to the inner surface [38]. As a result, the drug concentration presumably had not reached a therapeutically effective level in the inner ventral layer.

The existing clinical evidence suggests that a key to successful treatment of onychomycosis by a topical antifungal product lies in effectively overcoming the nail barrier.

3.2. Nail penetration models

To achieve an effective drug concentration into and through the human nail plate, development of an appropriate *in vitro* method to explore the physicochemical characteristics and permeability of the nail is of importance.

Walters and his colleagues pioneered the study of the permeability characteristics of the human nail plate in the early 1980's. They designed an *in vitro* method utilizing a stainless steel diffusion cell that permitted the exposure of nail plate to a bathing medium that was stirred by small motors mounted above the cell. Their most important research finding is that, as a permeable membrane, the hydrated human nail plate behaves more like a hydrophilic gel membrane in its barrier properties than as a lipophilic membrane such as stratum corneum [37]. This finding also explains the behavior of some solvents that promote diffusion through the skin horny layer but have little promise as accelerants of nail plate permeability [39].

Mertin and Lippold [35,36,40] modified Franz diffusion cells to measure nail permeability characteristics and drug uptake into nails. For onychomycosis treatment, they indicated, not only the flux of an antifungal drug through the nail plate is of importance, but also the antifungal potency, which is expressed as minimum inhibitory concentration (MIC). An efficacy coefficient, E, was therefore introduced, which should be maximized for high therapeutic effectiveness. Thus, for maximum efficacy, a high flux of drug through the nail and a low MIC are desired characteristics.

$$E = \text{Flux}/\text{MIC}$$

Kobayashi and his coworkers [34] investigated the permeation characteristics of drugs with different lipophilicity through three layers of the human nail plate (the dorsal, intermediate, and ventral nail layers), using a modified side-by-side diffusion cell. The data suggested that the upper (dorsal) layer functions as the main nail barrier to drug permeation, exhibiting low drug diffusivity.

However, most published *in vitro* nail study methods required the human nail sample to be in contact with an aqueous solution on either or both sides during incubation. Consequently, the human nail plate is artificially hydrated beyond normal levels. Methods of nail sample preparation have also used scalpel or sand paper to remove nail samples, which is not only time consuming, but also may not be accurate [34,41]. Recently Hui *et al.* [42–44] developed a novel experimental system that simulates the *in vivo* conditions of therapeutic, non-occluded application of drug to a human nail. In this device, the human nail (top center) surface was open to air, while the inner (ventral) surface made contact with a small saline-wetted cotton ball, which acted as both a nail supporting bed and a moisture supplier. The average of hydration of the wetted cotton balls, 118 ± 9.4 AU, resembles the average hydration of a human nail bed, 99.9 ± 8.9 AU, as measured in fresh human cadavers. After completion of the dosing and incubation phase, the

nail plate was transferred to a micrometer-controlled nail sampling instrument that enables accurate and reproducible sampling of the inside of the nail with high mass balance efficiency (Fig. 1).

Data shows that the average depth of nail sampling from the inner center surface was well controlled at 0.26 ± 0.05 mm (corresponding with the ventral/intermediate layer), which was close to the expected depth of 0.24 mm. With this *in vitro* nail study system, Hui *et al.* examined antifungal drugs delivered into the dorsal/intermediate and ventral/intermediate nail layers, and into the support bed (cotton ball) to determine the flux and the efficacy coefficient (E) of drugs in nail (Table 1). This study shows both ciclopirox and econazole penetrate to the deep layer of the nail plate in

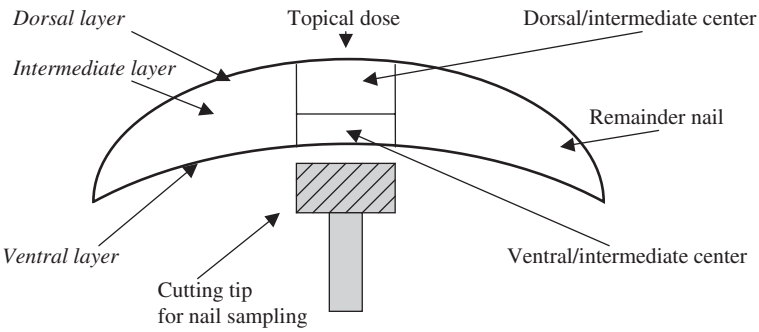

Fig. 1. Nail and nail drilling tip.

Table 1. Two antifungal drugs, econazole and ciclopirox, concentration and relative antifungal efficacy

Parameter	Nail Lacquer[a]	Control Formulation	*p* Value (*t* Test)
Econazole in the deeper layer ($\mu g/cm^3$)[b]	14,830 (341)	2,371 (426)	0.008
Efficacy coefficient E (MIC = 1 μg/mL)	14,830	2,371	0.008
Ciclopirox in the deeper layer ($\mu g/cm^3$)[b]	407 (106)	—	—
Efficacy coefficient E (MIC = 0.04 μg/mL)	10,175	—	—

Sources: econazole data is from reference [43]; ciclopirox data is from reference [44].

[a]The data represent the mean (SD) of each group ($n = 5$). The nail lacquer group of econazole contains 18% 2-*n*-nonyl-1,3-dioxolane and the control formulation contains no 2-*n*-nonyl-1,3-dioxolane.

[b]The deeper layer is the center of the ventral/intermediate layer of the nail plate. The data represent the amount drug in the sample after a 14-day dosing period.

concentrations above the MIC and that econazole penetration is significantly improved when formulated with a lacquer containing a penetration enhancer.

4. FACTORS AFFECTING DRUG PENETRATION

Using *in vitro* nail penetration models, studies have been performed to help understand the physicochemical properties that allow a molecule to penetrate into and through the human nail plate. External factors also have a major influence upon nail penetration and they are: nail thickness, the vehicle within which the drug is formulated, pH of the vehicle and addition of permeation enhancing agents to the vehicle. These factors have been recently reviewed [21,45].

4.1. Physicochemical properties of the drug

A recent study [46] investigated the relationship of molecular weight and lipophilicity of benzoic acid derivatives upon nail penetration. Using *p*-hydroxybenzoic esters ranging from methyl to hexyl, they found that permeation through the nail was mostly influenced by molecular weight and little, if any, by lipophilicity, which is in agreement with earlier studies [35,36]. This finding can be understood after consideration nail plate morphology. Because the nail plate is composed of many strands of keratin held together through disulfide bonds, the space between the strands must have a finite size causing the nail plate to act like a molecular sieve or size exclusion medium. Small molecules can weave through these spaces while larger molecules are unable to pass [21].

The molecular weight of most antifungal agents is > 300 Da. Accordingly, these drugs will have difficulty penetrating the nail plate, a likely reason for the low clinical efficacy observed [47].

4.2. External factors

4.2.1. Nail plate effect

Nails infected with onychomycosis are thicker than healthy nails due to the presence of the dermatophyte and the damage they have caused. The effect of nail thickness on penetration has been investigated and found to have an inverse relationship; as the nail increases in thickness drug penetration is reduced [46]. In the same study, the authors compared penetration of 5-fluorouracil though healthy nails versus fungal infected nails. They concluded that there was no significant difference between healthy and infect nails. However, they did not investigate heavily infected nail plates because the thickness of the uneven plate could not be accurately measured and that the uneven nail plate would collapse in water. They

speculated that penetration through this type of nail plate should increase due to the destruction of the plate caused by the fungi.

4.2.2. Excipients

The nail plate acts like a hydrogel and swells in the presence of water resulting in increased pore size [34]. This has the overall effect of increasing permeation [21]. A study into the penetration of ciclopirox **(4)** in three formulations concluded that when ciclopirox was formulated in an aqueous gel, its penetration was far superior than when formulated in a lacquer [44]. The effect of lipophilic vehicles on drug penetration through the nail plate has been investigated [40]. The authors concluded that providing the formulation does not affect the hydration level of the nail plate and the lipophilic vehicle does not penetrate the nail plate, then penetration of the drug is independent of the vehicle medium.

4.2.3. Vehicle pH

Antifungal agents have a range of pK_a values and so studies have been reported that compare the penetration of the ionic and non-ionic forms of the parent. These studies investigated the penetration of miconazole [48] ($pK_a = 6.7$), benzoic acid [35] ($pK_a = 4.2$), pyridine [35] ($pK_a = 5.3$) and 5-fluorouracil [49] ($pK_a = 7.9$) in vehicles over a pH range from ~2 to ~8.5. In the case of miconazole, it was reported that penetration was independent of the pH of the vehicle. However, in all the other cases, the ionic forms of the parent did not penetrate as well as the non-ionic forms. A recent study [46] investigating the penetration of ionic and non-ionic compounds and the relationship with molecular weight also found non-ionic compounds penetrate better. These authors speculated that the decrease in penetration of ionic drugs may be due to an apparent increase in molecular weight of around 100 Da from ion hydration.

4.2.4. Penetration enhancers

Efficacy rates of onychomycosis agents are widely believed to improve if penetration of antifungal agents through the nail plate is increased. Initial research has focused upon modifying environmental conditions, using penetration enhancers, to allow larger molecules to penetrate more easily through the nail plate. The most common method is to add a chemical enhancer to the vehicle. This enhancer can be a keratolytic agent [50], such as urea or salicylic acid, which break down the nail keratin; mercaptans [50], which break disulfide bonds in the nail keratin; solvent carrier, such as DMSO [42]; or 2-*n*-nonyl-1,3-dioxolane [43], which has been shown to fluidize stratum corneum lipids [51] (although its effect on nails is unknown). In most cases reported, an increase in penetration of the nail plate was observed. Mechanical methods to increase penetration have also been attempted. Lacquer formulations are the most common method to enhance penetration and are used in commercial preparations of ciclopirox and amorofine. After the lacquer dries, the drug impregnated film remaining creates a large drug gradient across the nail plate, which may force the drug through the nail plate. The lacquer also increases

hydration level in the nail plate, which will assist dissemination of the drug throughout the nail plate [21]. Another report described the use of pressure sensitive adhesives to enhance ciclopirox penetration [52].

5. INFECTION MODELS

Since a complete cure is highly improbable without good nail penetration, *in vitro* nail penetration studies are becoming one of the first screening tools used in the selection of compounds for treatment of onychomycosis. Once a compound has been identified that exhibits good penetration properties, the next step in lead optimization is to assess efficacy in a model of onychomycosis. This is a relatively new area of research and few models of onychomycosis exist. Several of these are summarized below.

5.1. *In vitro* infection models

One recent model of infection tested the minimum fungicidal activity (MFC) of antifungal agents against *T. rubrum* in a medium containing human nail powder, which the authors termed nail-MFC [53]. The medium by itself would not support the growth of *T. rubrum* but addition of the pulverized keratin allowed the dermatophytes to grow. The antifungal agents were incubated with *T. rubrum* in this nail medium for four weeks, after which the viability of *T. rubrum* was assessed. Terbinafine had a nail-MFC of 1 μg/mL, but other antifungal agents did not show efficacy at the highest concentrations tested (amorolfine 1 μg/mL, ciclopiroxolamine 128 μg/mL, clotrimazole 64 μg/mL, fluconazole 128 μg/mL, griseofulvin 64 μg/mL, itraconazole 4 μg/mL and naftifine 8 μg/mL).

Two groups have reported models of dermatophyte infection in human nail plates. In one study, nail clipping were applied to the top of an agar plate cultured with *T. mentagrophytes* and after a few days the infection spread onto the nail plate. Drugs were assessed by applying the formulation to the top of the nail and comparing the extent of dermatophyte infection that had spread to the nail plate with an untreated control [54]. In the second model, *T. mentagrophytes* was applied to the nail plate directly without a supporting medium and invasion occurred without addition of nutrients [55].

5.2. *In vivo* infection models

Two *in vivo* efficacy models in guinea pigs have been reported. In the first study, the authors infected the nails and toes of guinea pigs creating both onychomycosis and tinea pedis (fungal infection of the surrounding skin). They then used this model to show the efficacy of a topically applied triazole in comparison with amorolfine and terbinafine. All three were effective in clearing the tinea pedis, but only the experimental triazole showed efficacy against onychomycosis [27]. The second model was developed as an optimized *in vivo* model for dermatophytosis. The authors shaved and abraded the skin on the back of guinea pigs and infected the site with *T. mentagrophytes*. After the infection was established, the animals were treated with

oral and topical formulations of terbinafine and observed for improvement of the infection [56]. In this study, 1% topical terbinafine treatment had 100% clinical and mycological efficacy. Although this is a model for dermatophytosis, this model was used to determine the efficacy of a topical lacquer formulation of terbinafine.HCl, currently in clinical development for onychomycosis [57].

6. PRECLINICAL/CLINICAL PIPELINE

New treatments in development for onychomycosis are listed in Table 2. Currently, most treatments that are in clinical trials represent re-formulations of known antifungal agents combined with penetration enhancers in an effort to increase penetration through the nail plate. There are two treatments in clinical trials that contain novel antifungal agents. The first, in Phase 2, is a topical treatment that generates nitric oxide. The second, in Phase 3, is a topical treatment containing abafungin **(7)**, a membrane integrity antagonist that has antibacterial and antifungal activity. In addition to the treatments listed, azoline and ravuconazole, two oral triazole antifungal agents are in clinical trials for fungal infections and include onychomycosis as a potential indication.

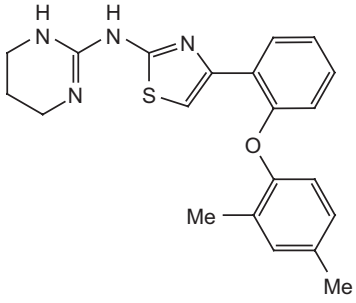

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Table 2. Onychomycosis treatments in preclinical and clinical development

Status	Drug	Further information
Discovery	Ciclopirox (**4**)	Formulated in a metered dose transdermal spray
	Ketoconazole	Formulation contains a topical carrier
Phase 1	Econazole	Formulation contains the penetration enhancer 2-*n*-nonyl-1,3-dioxolane
Phase 2	Clotrimazole	Formulation contains an absorption enhancer
	Topical NO donor	Novel topical treatment that generates nitric oxide
	Terbinafine	Formulation contains a penetration enhancer
Phase 3	Itraconazole	Oral treatment, once daily regime
	Abafungin (**7**)	A membrane integrity antagonist formulated as a topical treatment for bacterial and fungal skin infections

7. CONCLUSION

Onychomycosis is a common disease, especially in the older generation, that is difficult to treat using current medicines. In efforts to understand why current topical treatments work so poorly, research studies have focused on the nail plate and factors affecting movement of organic molecules through this barrier. The major problem that must be overcome is penetration and dissemination of the drug throughout the nail plate. Several models of nail penetration have been developed in order to assess this parameter. Data from these models suggest the optimal properties for effective penetration are: low molecular weight, low polarity, activity in the presence of keratin and optimal vehicle formulation (favorable excipients, pH and/or penetration enhancers). Infection models have also been developed that provide additional screening tools during lead optimization studies. These studies represent a major advancement in the search for new and effective treatments. However, as this remains a young field, it is unknown how success in these pre-clinical models will translate into clinical efficacy.

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Chemical Tools for Indications Discovery

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Contents

1. INTRODUCTION

The standard *modus operandi* of drug discovery is the identification of biologically active molecules via the high throughput screening (HTS) of large, diverse compound collections against specific biological targets, in low complexity assay systems. The specific biological targets of interest are selected from the results of basic scientific research on the aetiology of the disease of interest. Despite the *a priori* scientific appeal of the current standard approach, the rise in its popularity has also been concurrent with a reported decline in the productivity of the pharmaceutical industry, although of course this relationship is not necessarily causal and can be challenged (see the chapter on Pharmaceutical Innovation in this volume). Additionally, the HTS approach has often over-shadowed a wider variety of alternative and complementary drug discovery strategies that have been very successfully employed in the discovery of new medicines. Including HTS, the five grand strategies of drug discovery as outlined by Wermuth are [1]:

- Systematic screening of large numbers of diverse compounds in biological assays [2].
- Serendipitous observations and retroactive analysis of biological or clinical information in finding a new therapeutic use for a drug [3–5].

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- Selective optimization of off-target activities of known drugs on new pharmacological targets [1].
- Modification of an existing lead or drug to create an improved medicine (or new intellectual property). In terms of revenue generation and number of drugs approved, incremental innovations within an existing drug class are an important contribution to improving health [6–9].
- Rational design of drug from knowledge of the molecular mechanism and its role in disease [10,11].

Given the fundamental unpredictability of discovery and overall high attrition, it is wise for a research organization to mitigate risk by pursuing a portfolio of discovery strategies rather than being dependent on only one or two. The focus of this chapter is on how elements of the strategies outlined above can be combined and complemented with the use of annotated chemical tools.

2. NEW THERAPIES FROM EXISTING DRUGS

History is replete with examples of compounds that were originally developed for one disease and subsequently found to be beneficial in another. In contrast to the hypothesis-driven philosophy of modern drug discovery, many highly successful new treatments have been discovered by serendipity [3,5,12–14]. The phosphodiesterase-5 (PDE-5) inhibitor, sildenafil for example was originally developed as a potential anti-angina therapy but was observed during early clinical trials to be efficacious for male erectile dysfunction, for which it was subsequently first approved. Further studies on sildenafil have expanded its label to include approval for pulmonary arterial hypertension. The alpha-2 adrenergic agonist, brimonidine, was originally synthesized as an anti-hypertensive and later discovered and marketed as an anti-glaucoma agent. Further examples of drugs with unexpected benefits beyond their initially approved indications include: bupropion, which is approved as a smoking cessation drug, was originally developed and approved the treatment of depression [15]; gabapentin, which was originally launched for treatment of epilepsy but has also been extensively studied for several other indications including neuropathic pain [16]; eflornithine, an anti-trypanosomal drug, which was approved for the new indication of reducing unwanted facial hair [17,18]; finasteride, a type II 5-alpha-reductase inhibitor, which was originally approved as a treatment for benign prostate hyperplasia and then developed as a treatment for alopecia [19,20].

Far from being rare occurrences, additional indications for existing medicines are common. Indeed, additional indications can help contribute to the 'blockbuster' sales of a drug [21]. Two surveys of blockbuster drugs have revealed that up to 40% of revenue can be derived from secondary indications to that which the drug was originally invented. Gelijns *et al.* [22] examined the top 20 selling US blockbuster drugs of 1993 and found that 40% of the revenues came from sales for secondary/alternate indications. Ninety percent of the top 20 blockbusters were reported to have sales for secondary indications. In a similar analysis of the top 50 selling drugs

in the UK in 1999 Pritchard *et al*. [23] found overall that only 62% of revenues were for the original indication and in total 25% of sales were for new or unlicensed ('off-label') indications rather than the originally launched indication. The remaining 13% of prescriptions were classified as unknown but many of these may probably be for secondary indications. About half of the drugs examined in this survey had sales for additional indications.

The contributions to the medicine that are made by new indications is demonstrated by the number of new therapies approved for marketing. In recent years the number of therapies being marketed for new indications *per annum* is rising and is greater than the number of new therapies arising from first-in-class drugs reaching the market each year (Fig. 1). Around 10% of all new drugs launched each year target new mechanisms, about the same as the number of drugs approved with new indications.

3. PLEIOTROPIC EFFECTS OF DRUGS

A mixture of approaches that include sagacious insights and serendipitous observations of unexpected pharmacology in the clinic and the laboratory have led to the discovery of new applications for existing drugs [5,13]. The discovery of a new use for an existing drug has usually derived from one of four routes. The first route is the application of a drug to a closely related disorder, with the same underlying patho-physiological mechanism. For example, expanding the use of selective

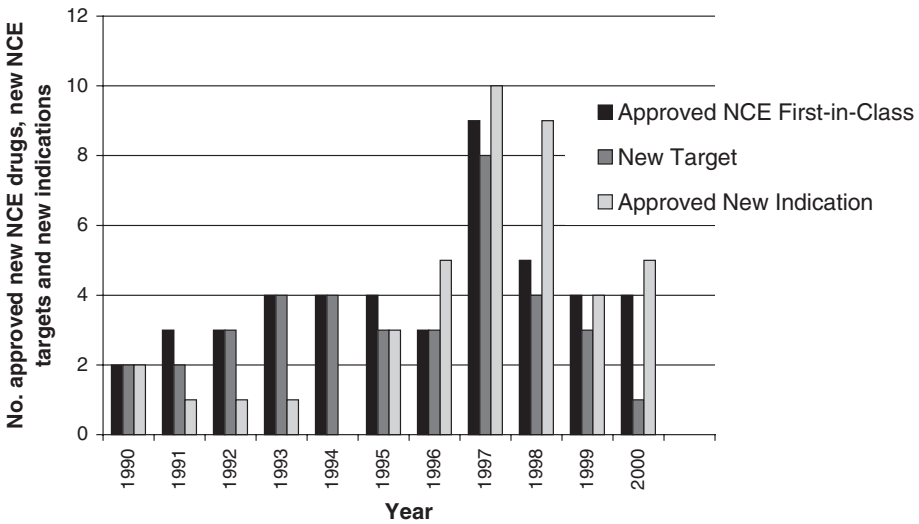

Fig. 1. Analysis of new drugs launched 1990–2000. The number of drugs launched for new indications each year rivals the number of 'first-in-class' drugs launched and associated new drug target of those 'first-in-class'drugs [24]. NCE, New Chemical Entity.

serotonin reuptake inhibitors (SSRIs) to include not only depression, but other related psychiatric indications such as obsessive-compulsive disorder, generalized anxiety disorder, panic disorder, social phobia and post-traumatic stress [25]. Such closely related disorders are usually covered within the same therapeutic area and are often considered in the normal drug development process or post-marketing by the product enhancement and ever-greening initiatives, several years after the launch of the drug.

The second route is through clinical serendipity. Many discoveries are the result of clinical observations of unexpected benefits of a drug or identification of the cause of the disease. Sildenafil was relatively unusual in that its use as a treatment for male erectile dysfunction was observed early in its clinical development. Most unexpected benefits for known medicines discovered this way are usually observed post approval, when a large and heterogeneous patient population with a range of underlying diseases are exposed to the new agent, such as the discovery or the uses of SSRIs for premenstrual dysphoric disorder [25], premature ejaculation and fibromyalgia [26].

The third route comes from changes in our understanding of aetiology that can modify ontological relationships between diseases [27]. Thus a drug developed for one disease may find applications in apparently unrelated diseases, which have similar underlying patho-physiological mechanism, but in different therapeutic areas. For example, the effect of sildenafil on the cGMP-nitric oxide axis in several diverse disease states, such as male erectile dysfunction and pulmonary hypertension.

The fourth route comes from the many discoveries that are the result of *in vivo* observations of unexpected benefits of a drug or the identification of the cause of the disease. Work of this nature is often carried out by academia and industry using the drugs as probes to explore the mechanism of a disease. The extension of the concept of drugs as *in vivo* probes to a wider range of annotated chemical tools and their application to the discovery of novel target-disease linkages is where we now focus our discussion.

4. INDICATIONS DISCOVERY

The common observation of the pleiotropic effects of many drugs indicate that it is not unusual for a drug to be involved in more than one disease. In recent years several organizations have explicitly attempted to discover and develop marketed drugs for new indications, either as monotherapies [28–31] or combination therapies [32,33]. This strategy has been called indications switching, therapeutic switching, drug re-positioning or drug reprofiling. However the concept of finding new applications can extend to beyond being limited to marketed drugs to biologically active compounds. Thus we have introduced the concept of indications discovery – a strategy to systematically search for new indications for existing drugs, leads and chemical tools as an orthogonal strategy to the systematic search for compounds for a specific disease.

A systematic approach to indications discovery combines clinical observations, detailed analysis of the biological information [34] and a variation of chemical genetics screening. Over the past decade there has been a revival in the empirical application of traditional small-molecule approaches, from pharmacology and physiology to the understanding of biological function for the genome, known as 'chemical genetics' and 'chemical genomics' [35–37]. Forward chemical genetics approaches screen a diverse chemical library against an *in vitro* phenotype screen (which may be an *in vitro* disease model) to identify chemical tools with a phenotypic effect. Commonly in phenotype screens the specific mode-of-action of the chemical tools may not be known so a subsequent stage of target identification and mechanistic study is required. Alternatively, reverse chemical genetic approaches begin with a biological target of interest and then attempt to discover a selective chemical tool to modulate the target, usually from screening a diverse chemical library. The target-selective chemical tool is then profiled against a range of phenotypic models to identify the function of the protein. Thus combining chemical tool and drug discovery approaches can enable alternative drug discovery strategies to the standard process, where specific annotated chemical tools are empirically screened across disease models to discover novel therapeutic utility.

5. WHAT ARE CHEMICAL TOOLS?

The phrase "chemical tools" has been used to describe selective pharmacology agents for the past 30 years. However the term has been repopularised since the post-genomics interest in the field of chemical genomics and chemical genetics [35,37,38] where selective chemical agents are usually used to perturb cellular systems to dissect the function of specific proteins or phenotypes [39,40]. The perturbation of biological systems using such tools was commonly employed to understand their behaviour before the advent of molecular biology. As the limitations of biological tools in terms of delivery, applicability and temporal dynamics are being appreciated, chemical tools are gaining resurgence in their application to perturbing and observing biological function. Compared to the relatively routine procedures, which can now be employed to generate a selective biological tool, targeting a specific gene or protein, the discovery of a new pharmacological tool was, and still is, a relatively rare event.

Traditional compound collections and modern chemical libraries generally consist of compounds where the chemical structures are known but the biological activity is generally unknown, until they are observed to bind to a target of interest in a screening campaign. In contrast, an annotated chemical tool [41] has at least one known biological activity, information of the relative selectivity of the compound against related targets and, most usefully, pharmacokinetic data. Annotated chemical tool libraries – chemical toolboxes – comprising a selection of chemical tools covering a wide range of biological activities [41] can therefore be very rich in diverse biological and chemical information compared to a traditional or combinatorial library of the same size.

The selection of the specific chemical tools, however, depends upon the application to which they will be employed. The tool properties required for *in vitro* and *in vivo* screening may differ markedly, thus the selection criteria of chemical tools for drug discovery and indications discovery differ from those required for chemical genetic studies [42]. Consideration of the drug-like properties of the tool, in terms of pharmacokinetics and the therapeutic index between efficacy and toxicity, are vital, if the goal is to utilize the chemical tool in *in vivo* animal models, which are likely to have far higher relevance to both normal and disease biology than *in vitro* systems [43–55]. Biologically active compounds that do not have the exacting properties required of a drug can be extremely useful tools for dissecting biological mechanisms and testing hypotheses in model *in vitro* systems. However if our goal is to expedite drug and indications discovery then the selection of drugs and drug-like tools shorten the path to the clinic, even if the phenotypic effect is due to polypharmacology.

6. SELECTIVITY AND POLY-PHARMACOLOGY

An important underlying assumption of the chemical genetics approach is that the tools will be selective enough to assign function to a specific protein and/or mechanism. Chemical tools and leads have long been important for target validation in drug discovery; that is, the testing of a hypothesis that a target is involved in a model system of a disease. However if a drug can modify more that one disease state does it follow that the molecular target it is acting on is also involved in more than one disease state? A challenge to this assumption is that the exquisite selectivity of small molecule drugs and chemical tools for only one biological target cannot be assumed [56–60]. Thus some of the new applications of drugs and chemical tools are due to new applications of the primary mode-of-action of the drug in a new disease phenotype, whilst others are due to the drug acting *via* an alternative target.

Inhibitors of HMG-CoA reductase and angiotensin I-converting enzyme (ACE) give examples of multiple indications deriving from modulation of one primary target. The myriad of pleiotropic effects of statins has been proposed to be dependent upon their effect on depletion of sphingolipid rafts and thus a reduction in the cell surface expression of many important immuno-signaling receptors caused by reduction in cellular production of cholesterol, by their primary mode-of-action: the inhibition of HMG-CoA reductase [61]. Likewise the beneficial effects of ACE inhibitors have been proposed to be due to the position of ACE at the rate-limiting nexus of multiple disease pathways. Epidemiologic results from genetic association studies and clinical outcomes experience indicate that of over-activity of ACE may underlie several age-related diseases [62,63].

An appreciation that many drugs may exert their phenotypic effects by acting on multiple targets has been grown in recent years due to transcript expression experiments [60] and large-scale assay profiling [57]. Indeed it is increasingly accepted that many kinase and GPCR agents are effective in disease states precisely because

they exhibit poly-pharmacology [64]. Arguably the initial and extremely successful drug discovery strategy employed by Paul Janssen can be described as a method of dissecting out specific targets and indications from drugs which exhibited multiple indications resulting from poly-pharmacology [65,66]. Exploitation of the poly-pharmacology behavior of drugs has been proposed as a deliberate drug discovery strategy [1]. The major challenge to exploiting and optimising off-target effects and poly-pharmacology is the identification of small molecule drug targets from the whole proteome [67]. Profiling of the biological activities of compounds against the largest available arrays of conventional assays only screens targets expressed by less than 1% of 24,000 genes encoded in the human genome [57]. Conventional experimental target identification methods often employ chemically modified ligands that bind sufficiently tightly to a target to enable it to be purified by affinity chromatography and identified by mass spectrometry. Recent developments in genome-wide target identification include phage display, protein microarrays, yeast three-hybrid systems [68], pathway mapping [69] and yeast fitness profiling [70,71]. Fitness profiling, which uses yeast heterozygotes to identify drug-specific growth defects, for example has demonstrated the cholesterol-lowering effects of the anti-angina drug, molsidomine, are due to inhibition of lanosterol synthase by its metabolite, although, of course, this technology is still limited to targets encoded in the yeast genome [71].

If chemical tools are to be screened in an *in vivo* disease model, one of the most important selectivity criteria in selecting chemical tools is the ability of the tool to bind to the homologous receptors in the model species. Most small molecule screening campaigns to discover leads and chemical tools are today directed against recombinant human proteins. The evolutionary distance between humans and the common model species such as mice, rats and zebrafish implies it cannot be assumed that a compound that binds to a target in one species will bind to the orthologous receptor in another species. If cross-species selectivity data is not available, careful sequence analysis comparing the structure-based sequence alignment of amino acids residues surrounding of the binding site can provide an excellent basis for inferring if a compound is likely to bind to an orthologous target [72]. However, in order to conduct an exercise in comparative binding site sequence analysis, a protein structure of the target protein (or homologue) is usually required. Advances in sequencing the genomes of models species, protein crystallography and large-scale comparative protein modeling are ensuring protein structure models are becoming available for many drug targets of interest and their model species' orthologues [73].

7. SCREENING CHEMICAL TOOLS IN DISEASES MODELS

The limits and relevance to human disease of current *in vivo* diseases models are well recognised. However, the practical problem in conducting drug discovery directly *in vivo* has been the difficulty in discovering new leads for new targets. It was to challenge this difficultly that led to the popular rise of current methods of screening

large numbers of diverse chemicals against recombinant proteins. An advantage of the use of phenotypic *in vivo* screens as primary assays are they require a compound to have suitable absorption, solubility and permeability characteristics, in addition to high potency at a given target and relatively low toxicity in order for activity to be detected. In contrast, good drug-like properties are commonly added in step-wise fashion to HTS-derived leads. Thus the selection of annotated chemical toolboxes, which contain compounds with known biological activities and pharmacokinetics, enables a much wider range of mechanism to be explored *in vivo* than was previously the case. The availability of a wide range of off-the-shelf chemical tools is likely to enhance the drug discovery endeavourby facilitating the probing of the function of particular drug targets in disease models.

An alternative approach to hypothesis-led experiments is the pursuit of a systematic, empirical method to the discovery of new indications for known drugs. A systematic empirical approach could be the routine testing of known drugs and chemical tools, representing new mechanisms, in relevant disease models. The aim of the systematic approach to testing known drugs in disease relevant models is to search for unexpected pharmacology and to discover new disease modifying pathways. Due to the richness of biological space covered by a careful selection of chemical tools, many drug target-diseases hypotheses can be tested in a disease model.

A number of groups have recently engaged in the systematic screening of approved drugs in a variety of disease models [28–33] with the logic that compounds which show activity can be quickly progressed to the clinic. The drawback of this approach is the relatively limited number of primary modes-of-action that are represented by approved drugs. For example the 1600 small molecule drugs approved by the FDA act on only 170 primary mechanisms for human targets [74]. Expanding chemical toolboxes to include compounds, which represent the targets modulated by investigational drugs and drug-like leads, can easily expand the available target space five-fold [75].

8. CONCLUSIONS

In recent years interest in annotated chemical tools has increased due to the potential application of assigning protein function in chemical genetic studies. Indeed, the Molecular Libraries Screening Center Network is being established as part of the recent National Institutes of Health ''Roadmap'' to facilitate the discovery of new chemical tools in the public sector by providing access to a large collection of compounds for high-throughput screening [76]. The easy availability of annotated drug-like chemical tools with associated molecular selectivity and pharmacokinetic data is expected to facilitate and accelerate target validation in *in vivo* disease models. Indeed annotating chemical tools from existing information sources is a cost-effective way of increasing the probes available to pharmacologists, requiring further consideration by industry and the public sector [42].

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Structural Genomics and Drug Discovery

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Contents

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1. STRUCTURAL GENOMICS

The concept of structural genomics, which aims to determine protein structures at a rapid rate, emerged in the mid-1990's from the coming together of genome information on one hand, and advances in the methods of structural biology on the other. At that time, the rationale for determining large numbers of structures ranged from "because we can" (or more correctly, "we think we can") to the fact that structural information provides among the most powerful means to annotate proteins of previously known or unknown function. Pilot projects were launched initially in the public sector (http://www.rcsb.org/pdb/strucgen.html), but within a short period of time the commercial applications of structural genomics were appreciated and, coincidental with the biotechnology boom of the late 1990's, several companies were formed to exploit this niche. Unfortunately, the heady pronouncements and predictions made at this time by many of the academic and commercial efforts were not in keeping with their ability to deliver, and the field was somewhat tarnished. However, in recent years, the investments in the area have begun to come to bear fruit. The public sector efforts around the globe have now contributed well over one thousand unique structures into the public databases (http://www.nigms.nih.gov/psi/). The cost per structure in the more successful projects has dropped ~5 fold over five years and, in the past year, structural genomics projects contributed over 25% of all new deposits into the Protein DataBank (http://www.rcsb.org) and nearly half of the "new" structures (defined as having less than 30% sequence identity to proteins whose structures had already been determined). In the commercial domain, the real impact of structural genomics is more difficult to assess, because of the long lag between discovery research and clinical impact. However, the ability to determine structures more quickly is certainly facilitating lead development for those cases in which the structure of the target is known.

This review will speak to three aspects of structural genomics. In the first section, we will provide some perspective into the origins of structural genomics and the advances made over the past decade in the science and technologies. In the second section, we will describe how the structures and technologies have and will impact the discovery and development of pharmaceuticals for human targets. In the last section, we will describe an application of the tools to the development of lead compounds in the anti-infective therapeutic area.

2. STRUCTURAL GENOMICS 1995–2000

The sequences of the first complete genomes were placed into the public domain in the mid 1990's [1]. This represented a crossroad of sorts for biologists, who were forced to realize that most of the proteins were uncharacterized and that hypothesis-driven research would be unlikely to make a timely impact on the annotation of these newly discovered genes/proteins. So began the –omics era, in which highly parallel experiments were designed to gather as much information for as many genes/proteins as rapidly as possible. The resulting approaches (which include DNA

microarrays, protein-protein interaction studies, yeast genetic analyses and comparative genomics) were made possible by associated improvements in engineering, computation and instrumentation. In essence, it is difficult to think of a molecular biology experiment that cannot be performed 100–1000 times faster than a decade ago.

Structural biologists, not to be left aside, were influenced by the –omics way of thinking. By 1995, the advances that had been made in molecular biology, instrumentation, synchrotron radiation, and computer hardware and software had already made the process of determining a crystal or NMR structure far simpler, faster and less risky than it had been even five years previously. It was a natural evolution of the field to apply these advances to the thousands of newly discovered proteins, in order to elucidate their structures and functions. Structural genomics was thus initiated in several pilot projects around the world. Interestingly, the specific aims of the various pilot projects differed. In Japan, among the first movers in the area, the aim was to determine the 3D structure of every protein in a single genome [2]. In Canada and at the Lawrence Berkeley Laboratories, the aim was to link 3D structural information and structural similarity to proteins of known function as a means to infer biochemical function for the new proteins [3,4]. In other laboratories around the United States, the aim was to discover new protein folds [5]. Other efforts were formed largely to develop enabling technology [6,7].

In the private sector, the concept of structural genomics met with welcoming ears. The successes of the genome sequencing efforts, the notion that gene sequences could be protected, and the conviction that the genomes contained thousands of new pharmaceutical targets spurred them to make significant investments in the area. Similarly, the push by the venture capital community to be part of the ''next big thing'' and the excitement of the late 1990's provided a ripe environment for investment in biotechnology companies in the area of structural genomics. Companies like Affinium, Astex, Structural GenomiX and Syrrx were quickly capitalized and launched programs in genome-scale structural biology. These companies initially invested significantly in technologies, but scientific and market events quickly forced them to focus on more direct applications to drug discovery. Pharmaceutical companies also launched internal, focused programs in structural genomics, largely as a means to assess its impact on their own drug discovery programs.

3. STRUCTURAL GENOMICS 2000–2005

The growing interest in structural genomics, and the successes of the pilot projects, has led to larger funding initiatives around the world and the building of a community of scientists in the area. The NIH announced the ''Protein Structure Initiative'', which provided funding for a period of five years to nine Centers to determine the structures of proteins with no known structural homologues (http://www.nigms.nih.gov/psi/). The Japanese efforts diversified to include eukaryotic proteins in addition to their existing focus on a single bacterium (http://www.gsc.riken.go.jp/eng/group/protein/index.html). The European Union funded SPINE, a collaborative effort to determine the structures of human and other disease relevant

proteins (http://www.spineurope.org). The Canadian and Ontario governments expanded their support for structural genomics as a genome-annotation tool (www.uhnresearch.ca/proteomics/). Finally, the Wellcome Trust, GlaxoSmithKline and the Canadian and Ontario governments funded the Structural Genomics Consortium, a charitable effort formed to place the 3D structures of human therapeutic targets into the public domain without restriction (http://www.thesgc.com). In retrospect, after five years, the individual structural genomics projects have met with some success, with some initiatives now able to generate 3D structures at far greater efficiency than previously possible. The Midwest Consortium for Structural Genomics (http://www.mcsg.anl.gov), out of Argonne National Laboratories in Chicago, deposited and released over 80 new structures of prokaryote proteins into the Protein DataBank from July 2004–April 2005. The Structural Genomics Consortium over the same period and in its first year of its operation deposited and released over 50 new structures of human therapeutic targets into the Protein DataBank.

4. STRUCTURAL GENOMICS TECHNOLOGIES

The combination of public and private investment in the technologies of structural genomics may have the most significant impact on the drug discovery process. The following section describes an assortment of technologies that have been developed or improved in associated with structural genomics (SG).

4.1. cDNA availability and cloning

The availability of a cDNA is obviously a prerequisite for any structural project. Access to entry clones for human (or any) genes normally requires significant dedicated resources and thus often constitutes a rate-limiting step for SG initiatives. As human genes cannot be directly derived from PCR of genomic DNA (due to the frequent presence of introns) the source needs to be cDNA collections/libraries. Most projects in the SG research area either rely on in-house cloning from genomic DNA (prokaryotic genomes) or clone access through collaborations.

Fortunately, most of the human genome is now available, as sequence-verified cDNA in the public domain. The Mammalian Gene Collection from the MRC gene service, for example contains ~19,000 unique human genes (http://www.hgmp.mrc.ac.uk/geneservice/index.shtml). About 90% of the genes targeted by the Structural Genomics Consortium could be acquired from public sources over the first 9 months of operation. For cDNAs that are not available, any of a number of suppliers (e.g. from the gene synthesis service company GenScript http://www.genscript.com) can provide gene synthesis services. In weeks, for a few dollars per codon, the complete, sequence-verified clone can be delivered in an "expression-ready" form without imposing any scientific or administrative burden.

Once cDNAs are in hand, the methods used to transfer or adapt the cDNA to generate clones suitable for structural biology or protein expression are quite standard. Implementation of traditional directional cloning methods, or

ligation-independent cloning, provides more throughput than any structural biology lab could possibly match. It is possible for one molecular biologist, with normal throughput, to deliver and characterize several hundred expression constructs per month. There has been much debate about the recombination systems that enable scientists to transfer cDNAs among different vectors. In our experience, the use of these systems expedites the shuttling of cDNA into different vectors, but has little impact on the rate with which groups determine 3D structures or produce proteins, largely because the optimal target for structural biology is usually a fragment/domain of a protein.

4.2. Protein expression

Most laboratories around the world continue to use traditional methods to produce proteins for structural biology. The bacterium *Escherichia coli* remains the workhorse of any protein expression laboratory. With a team of two people, it is now routine to grow hundreds of different cultures per week, in liter quantities, with traditional shakers. There are also variations on the method, for example to use 2L polyethylene "soft drink" bottles (http://www.mcsg.anl.gov/), or more complex 96 channel fermentors (http://www.jcsg.org/), but for the foreseeable future, these methods will not have significant impact on the economics or efficiency with which protein structures are produced, since this part of the process is not rate determining or tremendously costly.

The alternate expression systems that are more relevant to proteins from higher eukaryotes, such as insect, yeast and human cells, are more difficult and more expensive to process in high throughput. Recently, there have been reports that recombinant baculoviruses, for expression in insect cells, can be generated at rates of 96 viruses per person per week in smaller volumes. This capacity should enable scientists to screen many possible baculovirus constructs for suitable expression and solubility properties. However, the process has not been implemented yet in the high throughput laboratory scale, so it is not yet possible to judge its impact on the numbers of structures determined.

4.3. Protein purification

The high-throughput purification of proteins without exception requires that the proteins are engineered to contain protein or peptide tags to facilitate purification. There is a wide selection of tags that are available and over the past five years many groups have explored the properties of most of them in the context of structural genomics.

Polyhistidines have been added to the N-terminus of the recombinant proteins, with a recognition site for a highly specific protease between the tag and the protein, so that the tag can be removed after the purification. The factors underlying the

choice of histidine tags were: first, the analyses of the properties of the various tags has shown that the choice of tag (gluatathione transferase (GST), maltose-binding protein (MBP), polyhistidine, *etc.*) can influence the expression of the fusion protein, but once the tag is removed, the behaviour of the proteins are most often unaffected. In other words, a largely insoluble protein with one tag may have improved properties when fused to a sizeable tag, but once it is removed, the protein is likely to remain insoluble. Second, the SG Laboratories at the University of Toronto have shown that the histidine tag, which is among the shortest of the various tags, can often promote protein crystallization. These data showed that the probability of generating diffraction-quality crystals increases by 30% by testing both the tagged and untagged protein in crystal trials (unpublished). The combination of the ease and efficiency of purification with the crystallization enhancing properties of the histidine tag make it the best choice for structural genomics.

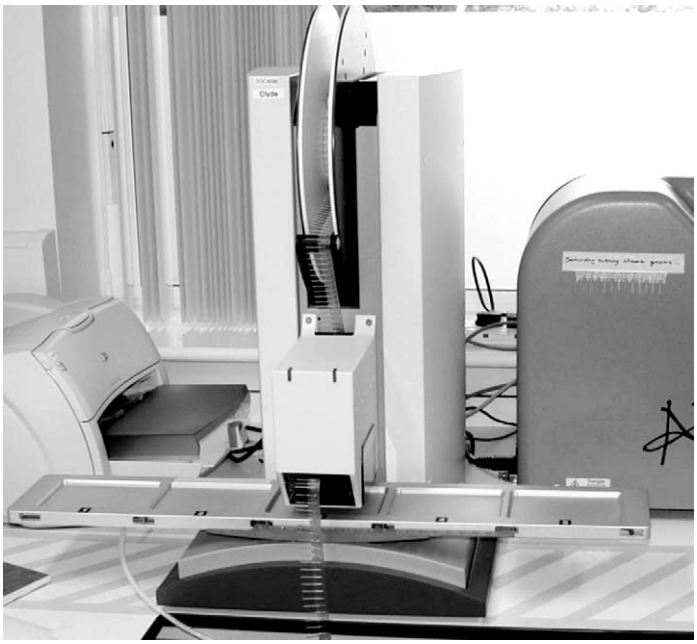
The recently launched Äkta Express (Amersham/GE Healthcare) was designed specifically for the high throughput laboratory. The machine can perform parallel purification of four proteins through three different procedures. The use of the Äkta Express is most favourable for proteins that follow the same purification protocol, but this is not unusual in the structural genomics laboratory. It is also possible to achieve high throughput using traditional FPLC or HPLC systems. The use of these systems has the advantage that they provide greater versatility in experimental design.

4.4. Crystallization robots

Protein crystallization is, at its heart, simply a matter of liquid handling. Hence it has not been difficult to develop crystallization robots. However, there are complexities that have impeded the adoption of any single system. First, protein crystallizers have particular preferences as to how they wish to perform the experiments (sitting versus hanging drops, for example) and this adds engineering complexities, and cost, to the design. Second, crystallizers are increasingly keen to use smaller amount of material, which brings with it the challenge of dispensing small amounts of viscous solutions.

There are many robotic crystallization systems on the market [8]. The throughput of any of the systems is quite reasonable and all provide sufficient throughput to process thousands of protein samples for crystallization per year. In selecting a robot, in our opinion, most attention should be focused on ease of use, software, the ease of maintenance and the speed/accuracy of dispensation.

The increase in the throughput of the crystallization process places burdens upstream on the preparation of crystallization solutions. There are many commercial sources of initial crystallization screens; however, most scientists prefer to prepare the subsequent crystal optimization screens in their own laboratories often using liquid handling robots. Although attention must be paid to the viscosity of the liquids, in practice, liquid handlers can be integrated immediately into the process.



The Mosquito Crystallization Robot (TTP Labtech, Cambridge, UK) as installed at the Structural Genomics Consortium.

4.5. Databases and protein crystallization

Based on the scale of protein crystallization and the efforts of scientists to store and mine the experimental results, it has been possible to correlate what is required to more efficiently achieve crystals from protein samples. Such studies include systematic analyses of protein properties. In one such study, comparisons were made on ~500 proteins that were successfully expressed, purified and crystallized versus the rest of the 'pursued proteome' [9]. This analysis showed that parameters, such as isoelectric point, sequence length, average hydropathy, low complexity regions and combinations thereof could be used to extract rules to improve target selection and improve the output of structural genomics in subsequent efforts. In other studies, it has been possible to derive more productive crystallization screens [10–12]. For example, Kimber and colleagues reported that 6 conditions alone would have yielded starting points for crystal optimization for about 60% of the proteins pursued and 94% if using 24 conditions. Thus, a common strategy for protein crystallization has emerged from these analyses in which small (typically 96–192 conditions) primary screens are used to investigate the crystallizability of the protein sample followed by broader crystal optimization approaches.

Unfortunately, most of these comparative studies on crystallization conditions are based on results from proteins of model organisms and thus similar results from

a consistent and large set of human proteins are not currently available. However, it is not expected that the crystallization properties of soluble human proteins will differ significantly.

4.6. Crystal visualization

In structural genomics or large structure-based drug discovery efforts, it is not uncommon to set up hundreds of crystallization plates per week, imposing significant burden on the crystallizer to inspect the plates. Although there is a great desire to have the inspection and assessment of crystallization trials done automatically, to date there is no system that adequately accomplishes this task. There are many visualization systems, but no software recognition algorithm that can unambiguously identify crystals or even promising conditions. The current state of the art is to have crystallization trials imaged by a vision system and to have the scientists inspect the trials remotely on a computer.

4.7. Synchrotron science

Over the past decade, the use of synchrotron radiation has become prevalent, even *de rigeur*, in the structural biology community [13]. The X-ray radiation is brilliant enough to allow the determination of complete structures in minutes to hours and the software and hardware for data acquisition and processing are now 'fully' developed. There are large numbers of excellent beamlines around the world, which can be accessed either on a peer-review or fee-for-service basis. The ease of data collection has enabled "FedEx" crystallography, in which scientists ship frozen crystals to the beamline scientists, who collect the data and deliver them back to the sender.

Prompted by developments at Abbott Laboratories, the community now has access to a number of robotic systems to mount crystals from liquid nitrogen storage to the X-ray unit, and back again [14]. This process enables scientists to screen large numbers of crystals in order to select those that are most suitable for data collection. These robotic capabilities are being used to screen for conditions with which to cryo-protect crystals, as well as to select the best diffracting crystals from large numbers of visibly similar crystals. The robotic mounting capabilities also offer structural biologists effective means to screen small molecule co-crystallization or soaking trials.

4.8. Software

The ability to process and refine diffraction data has improved dramatically in the past five years. For derivatives of known structures, for example small molecule/protein complexes, with reasonable data, such as to a diffraction limit of 2.4 Å resolution, it is possible to determine most of the protein structure with little human

intervention. This is the case even for protein/small molecule complexes that crystallize in different space groups.

The improvements in the programs that perform phasing and automated map building have been remarkable. Using any of a number of software packages (http://www.hkl-X-ray.com; www.ccp4.ac.uk/main.html), it is possible to process data, locate the phasing atoms, calculate phases and build the map within a short time frame, often within a few hours.

5. TECHNICAL ISSUES IN EXPERIMENTAL STRUCTURAL GENOMICS

The suite of structural genomics technologies is now being applied to the analysis of human therapeutic targets in the pharmaceutical and biotechnology sectors. Far from being a straightforward endeavor, the generation of large numbers of human protein structures is plagued with many technical challenges, which are briefly outlined below.

5.1. Expression of human genes

The cost efficient protein expression for Structural Genomics/Biology is usually carried out in the bacterium *E. coli*. Most often the proteins are linked to an expression and affinity tag that can be cleaved off by a specific protease if desired. The most common tag is hexahistidine as it is small and can be kept throughout the procedure from expression to crystallization (alternatively NMR data collection). However, full-length human proteins are rarely expressed in soluble form in *E. coli* due to a number of reasons, which include:

a. Human proteins are encoded by codons that are rarely used in *E. coli*.
b. Human proteins (like all eukaryotic proteins) are not adapted to the *E. coli* folding system, often leading to precipitation as inclusion bodies, especially if they contain more than one domain.
c. Human proteins are often misfolded and sensitive to proteases/degradation.
d. Eukaryotic proteins often require glycosylation or other post-translational modifications.
e. Human proteins have more frequent requirements for co-factors, interacting proteins or other natural ligands for appropriate function and structural integrity. The absence of these ligands in bacteria often causes the protein not to be expressed or to be expressed in insoluble form.
f. Human membrane proteins are rarely expressed in bacterial cells in a biologically relevant form.

As a result of these problems, the general consensus is that full-length human (and indeed eukaryotic genes in general) are more difficult to express and purify in a

soluble and biologically relevant form in *E. coli* and must be expressed in systems like baculovirus, the yeast *Pichia pastoris* and in cell-free translation systems [15,16]. Baculovirus is the most common eukaryotic protein expression system for structural biology/genomics and has been quite successful even in more difficult target classes, such as the protein kinases [17].

5.2. Tricks to express human proteins in *E. coli*

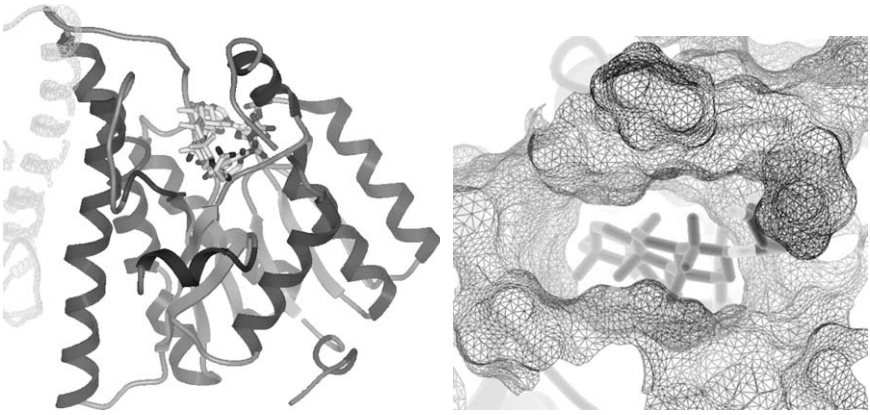
Because *E. coli* is the most cost-effective and versatile system for protein expression, great effort is being focused to improve the system for human proteins. For membrane proteins, it has recently been reported that eukaryotic membrane proteins were successfully expressed in *E. coli* using an autonomously folding membrane protein Mistic (from *Bacillus subtilis*) as a fusion partner. Mistic was shown to form a helical bundle with a polar lipid-facing surface that could be used to promote high-level production of other membrane proteins in their native conformations, including eukaryotic proteins previously not tractable in *E. coli* [18]. Further developments and the use of Mistic will be monitored with interest.

A number of 'rescue routes' are being developed to increase the success rates in *E. coli* for non-membrane bound human proteins. These include co-expression with general and periplasmic folding modulators and suppression of protease activity [19]. One method that may lead to improvements in the yield of biologically relevant proteins from *E. coli* is the co-expression with natural ligands (chaperones, interacting proteins or co-factors) and/or chemical inhibitors. In one specific study, the pharmaceutical target, human 11β-hydroxysteroid dehydrogenase I (Fig. 1) was co-expressed with a tight binding chemical inhibitor as well as chaperones, allowing the protein to be detectably expressed and purified presumably by assisting the protein folding as well as stabilizing the protein and thus protecting it from being degraded by proteases [20].

5.3. Purification and characterization of human proteins

As stated above, structural genomics efforts more or less exclusively rely on the presence of a purification tag as the initial (or only) step, allowing the protein to bind to the affinity chromatography column and to be eluted with a competitive agent, often giving a high degree of enrichment. This is a highly efficient initial purification and often is sufficient for a single step process to pure protein. However, low expressing proteins as a rule require additional protein biochemistry and chromatography approaches. In most standard approaches, affinity purification is followed by either ion-exchange chromatography or gel filtration or both and for the vast majority of proteins a two or three step procedure will be adequate.

It is often also critical to undertake extensive biophysical analysis and *in vitro* functional characterization to guide the optimization of the environmental parameters (such as pH, buffer, salt condition, presence of metal ions, etc) to establish conditions that are most favourable with respect to ensuring that the protein



(A) (B)

Fig. 1. An example of a pharmaceutically relevant target structure determined by the SGC is the short chain dehydrogenase 11β-HSD1 in complex with the prototype inhibitor carbenoxolone (A) which has been widely used to inhibit isozymes of 11β-HSD. The protein could only be produced in sufficient quantities when expressed in the presence of chaperones and carbenoxolone. It binds with high affinity to human 11β-HSD1, effectively blocking the enzymatic reaction. The structure revealed that carbenoxolone (B) is bound in a large hydrophobic cleft (PDB ID: 2BEL). Images were generated using the software ICM from Molsoft LLC (www.molsoft.com).

physical properties are optimal (such as homogeneity, stability and solubility) allowing crystallization. Further, it is important to ensure that the protein sample has a 'biological relevance' e.g. ensuring that the protein at least binds to its co-factor (if it is an enzyme depending on it for function) or that other measures of its activity can be reconstituted (e.g. catalytic activity for enzymes).

6. INDUSTRIAL APPLICATION OF STRUCTURAL GENOMICS

Structural biology is now an integral part of the drug discovery process. From identification of "druggable" targets to lead optimization, having protein structures improves the timeliness and effectiveness of the search for new therapeutic agents. What does structural genomics offer above the traditional uses of structural biology?

First, the application of structural genomics in the pharmaceutical sector provides structural information for different targets at an unprecedented rate, providing the ability to compare targets with highly related active sites. In the area of human therapeutics, this ability provides knowledge of the chemical landscape of the active sites across gene families (e.g. protein kinases) and facilitates the design of both specific and selective compounds. In the anti-infective area, structural

genomics efforts to determine the structures of many orthologous proteins from multiple bacterial species allows medicinal chemists to design broad spectrum inhibitors able to target the same protein from different bacteria.

6.1. Structural biology and the medicinal chemist

6.1.1. Target characterization

Quite simply, having access to high-resolution structures prevents one from working blindly [21]. The widespread and growing availability of protein apo, holo or ligand-bound structures (*vide infra*), increasingly through structural genomics efforts, allows the characteristics of active sites of potential targets to become criteria for target prioritization [22]. Given that active sites vary substantially in their sizes, shapes, surface accessibility and amino acid compositions, structure guided rules for target prioritization provide an important opportunity to improve the efficiency of drug discovery by steering project teams toward the *selection of targets* whose active site characteristics possess features that increase the likelihood of the discovery of inhibitors having drug-like properties (being 'druggable').

6.1.2. Facilitate virtual screening

The availability of high quality proteins protein structures of sufficiently high resolution (<2.5Å) can guide the initial identification of inhibitors using computational and/or experimental approaches. Current high throughput docking and scoring methods can screen large virtual compound libraries; the success of these methods is estimated at 10–50%, based on comparisons with experimental results [23,24]. Improvements in the accuracy and efficiency of virtual screening algorithms should be anticipated as computational methods improve, as the number of protein structures on relevant drug targets grows, and as the availability of protein structures motivates drug discovery teams to explore virtual screening as a complimentary or alternative to experimental high throughput screening.

The use of virtual screening is expected to increase significantly as the numbers of structures in the public domain increase (e.g. www.thesgc.com), and compound collections are made publicly available (for examples of recently released web sites of compounds see http://pubchem.ncbi.nlm.nih.gov/, http://blaster.docking.org/zinc/ and http://iccb.med.harvard.edu/screening/compound_libraries/index.htm). Here the analysis of target classes (*i.e.* kinases and proteases) and ligand pharmacophore motifs has proven to be quite successful [25,26], including examples of cross-over designs [27]. Additionally, as the number of differing high-resolution structures grows, the attractiveness and reliability of various homology models (especially of closely related proteins) can serve as starting points for target selection and selectivity [28].

Recent approaches to hit generation are beginning to take advantage of so-called ''fragment screening'' approaches as conceptualized in [29]. Here, utilizing either bioassay, NMR [30,31], or X-ray crystallography [32–35], relatively high

concentrations of low molecular weight (generally less than 300 M_r) compounds are examined for relatively weak binding interactions and subsequently expanded into higher affinity compounds. A combination of virtual ligand screening, protein structural data and biophysical and/or structural verification of binding properties have facilitated the speed and efficiency of the process from initial hit (fragment) to potent compounds [36]. The field looks forward to the first entry into the clinic of a compound derived from the fragment based approach.

6.1.3. Lead optimization

The knowledge gained by detailed examination of target structures and target-inhibitor co-structures provides a greatly advantaged basis from which to optimize the various properties of a compound that are important to make a drug. Determination of structure activity relationships (SAR) – which parts of the ligand are necessary for activity – has historically been a trial and error process. Designing compounds, synthesizing them, and testing them can take weeks to months just to obtain the results for a single compound. Thousands of person-hours can be spent determining even the initial SAR for a lead series, and for well-studied molecules such as the fluoroquinolones (which inhibit the bacterial DNA Gyrase & Topoisomerase IV enzymes), hundreds of person-years can be spent expanding the detailed SAR [37].

Lead optimization is more efficient with protein structures in hand, as regions of the lead that are involved in binding to the target protein are visualized and ideas to alter properties of a compound become readily apparent. Beginning a drug discovery program with knowledge of the tertiary structure of target proteins, binding sites, and even better, with bound small molecule ligands can short-circuit many of the dead ends that "blind" SAR (structure activity relationships) generates. The utility of protein structure in contributing to the lead optimization process has proven effective in the design of many compounds, notably antiviral agents (HIV protease inhibitors and influenza virus neuraminidase inhibitors). Moreover, a defined understanding of the interactions between small molecule and target proteins provides a "roadmap" to alternative templates or scaffolds to interact with the target proteins. These alternatives are particularly important when searching for backup compounds and appropriate intellectual property coverage. In the context of structural genomics, multiple structures for different family members will provide the ability to understand the commonalities and differences among the druggable sites, allowing medicinal chemists to focus their synthetic efforts with a greater appreciation of the determinants of selectivity and specificity.

7. CURRENT STATUS OF STRUCTURAL GENOMICS AND DRUG DISCOVERY IN HUMAN THERAPEUTIC AREAS

The protein data bank contains ~28,000 three-dimensional protein structures (May 2005) and ~15 new protein structures are added daily. Approximately 6000 of these structures are from human protein chains originating from ~2000 unique human

structures (when their sequences are clustered at the 90% sequence identity level). For the most part, these structures only cover part of the full-length protein chains. Thus, the PDB contains direct experimental structural information for less than 10% of the human proteome and much less if one considers all possible variants of a protein that can arise from the translation process (e.g. splice variants) and post-translational modifications (e.g. glycosylation). The coverage of the human proteome is also rather biased away from certain proteins. For example, there is almost a complete absence of structural information of human integral membrane proteins.

7.1. The use of human protein structures in drug discovery

Focused efforts on structure determination of human proteins have increased over the last decade to a large degree, carried out in the pharmaceutical and biotech industries to allow structure guided drug design. A large fraction of these results have remained proprietary. Experimental structures (complexes with hit and lead compounds) as well as indirect data (active site models based on comparative modelling approaches) are being used by medicinal chemistry in industry in order to more optimally use resources for rational hit expansion and lead optimization. This process has enabled potent, selective compounds to be designed and synthesized. Prominent examples of the use of direct structure based drug design include the development of protein kinase, farnesyl transferase and MMP inhibitors [38–40].

7.2. The use of human protein structures in drug discovery: All in the family

Many human proteins of therapeutic importance are members of protein ''families'' or functionally related classes (proteases, kinases, *etc.*). Viewing ligand discovery from a family-centric perspective provides substantial advantages in solving protein structures as well as efficiencies in assembling downstream methods, such as assay development and small molecule template designs [41]. This approach adds a new and valuable dimension to traditional disease-centric single target approaches. Drug discovery efforts aim to identify ligands with highly selective effects on the therapeutic target, but not other members of the protein family [42].

It is fair to say that structural information in the context of gene families will play a central role in the drug discovery paradigm only if the structures are available for many members and the turn-around time of structure determination rivals the medicinal chemistry cycle. Indeed, most structural information on medically relevant human targets is retained in proprietary form by commercial operations, as it is considered a competitive advantage not to reveal this information to the public. However, if the industry is to reap maximal benefits of the availability of protein structures, then publicly available structural information is needed for many members of all important pharmaceutical targets, and their structural and sequence homologues. This is a major goal of industrially relevant structural genomics.

7.3. Large scale structural studies on human proteins in the public sector

There are four major initiatives focusing on the structure determination of human proteins: the Protein Structure Factory; the **S**tructural **P**roteomics **in** **E**urope (SPINE) group; the Riken Structural Genomics Initiative and the Structural Genomics Consortium (SGC). The SGC is a recently funded Anglo-Canadian initiative whose objective is to determine the structure of ~350 novel human and malarial drug discovery targets before July 2007. By June 2005, the SGC determined the structures of over 60 novel proteins of which ~85% were novel human targets with the remainder being malaria/plasmodium proteins (http://www.thesgc.com).

7.4. Large scale structural studies on human proteins in the private sector

In the end of the 1990's, companies were formed to provide novel protein structures of drug targets to pharmaceutical companies. Examples are: Syrxx, recently acquired by Takeda Inc. (http://www.syrrx.com), which was focused on various human targets of therapeutic interest, e.g. Dipeptidyl Peptidase IV, protein kinases and 11β-hydroxysteroid dehydrogenase type 1); Structural GenomiX (http://www.stromix.com focused on e.g. protein kinases); Integrative Proteomics (now Affinium Pharmaceuticals, http://www.afnm.com); Plexxicon (http://www.plexxicon.com focused on e.g. protein kinases and phosphodiesterases) and Astex Technology (http://www.astex-technology.com focused on e.g. protein kinases as well as drug metabolism in relation to structural data on cytochrome P450's). Although initially set out as structural genomics (or high throughput structural chemistry) operations, these companies have now developed into more classical drug discovery companies with focus on structure-based drug design (Affinium, Syrxx/Takeda, Plexxicon) and fragment-based drug discovery approaches (SGX, Astex). Thus, it is clear that structural genomics today to a very large degree is a publicly funded research effort, with the exception of the investment made by GlaxoSmithKline in the SGC and the pharmaceutical consortium supporting part of the RSGI operations.

8. INTEGRATING STRUCTURAL GENOMICS WITH ANTIMICROBIAL DRUG DISCOVERY

8.1. Challenges of anti-infectives and recent examples in the antibacterial area

Structural genomics technologies can also impact the discovery of anti-infectives, which pose special challenges in the discovery of new drugs. The most efficient design of full spectrum anti-infectives requires insight into the binding sites of orthologous proteins. Strong binding between a small molecule inhibitor and each

member of the related orthologous proteins must occur, despite differences in molecular details of their binding sites. In contrast, the design of selective agents for mammalian targets requires the ability to discriminate between close structural variants. Although genomics provides us with a genome-scale view of the similarities among microbial species and viral isolates, subtle differences in the binding pocket's shape and amino acid composition can determine whether a small molecule will productively interact with a protein. When viewed at the resolution of a small molecule interacting with a specific collection of orthologous proteins, the differences between these proteins may become more apparent and more significant for the discovery of full spectrum inhibitors and compounds targeted against differing isolates.

In the antibacterial field, in spite of significant progress and success based predominately on natural products work, the number of targets that existing antibiotics actually utilize is very limited. As previously mentioned, fluoroquinolones act *via* inhibition of bacterial DNA Gyrase and Topoisomerase IV targets, aminoglycosides, oxazolidinones (the first new class of chemistry introduced in the antibacterial arena in over three decades), macrolides, ketolides and tetracyclines all *via* inhibition of the ribosome, rifamycin *via* bacterial RNA polymerase, glycopeptides *via* peptidoglycan cell wall biosynthesis and β-lactams, cephalosporins, penicillins and carbapenems *via* penicillin-binding proteins. Given that the average bacterial genome contains over 1500 open reading frames, there exists a rich reserve of potential antibacterial targets to reap the rewards afforded by structure-guided drug discovery.

A recent example of the application of structure-guided antibacterial discovery is the area of peptide deformylase inhibitors where a search of the PDB (http://www.rcsb.org/pdb/) reveals that 34 different structures (and co-structures) are deposited and an additional 11 structures are on hold awaiting release (n.b. protein structure initiative centers submit their structures for immediate release) [43]. Importantly, Novartis reports LBM-415, a peptide deformylase inhibitor, is in the clinic (http://www.nibr.novartis.com/DiseaseAreas/InfectiousDiseases/) [44]. Work is emerging describing structure-guided efforts on several additional bacterial targets, among these LpxC [45,46] and TyrRS [47]. Numerous other antibacterial targets are poised for structure-guided discovery efforts (for example, the enzymes involved in the Mur, fatty acid biosynthesis and Isoprenoid pathways), even work on the ribosome itself is progressing [48] with erythromycin, azithromycin, clindamycin, virginiamycin S, and telithromycin bound [49].

Within the fatty acid biosynthesis cycle, emerging reports have highlighted opportunities to apply structure-guided principles. In the FabH area, an initial report [50] describing efforts which began from available crystal structures and a knowledge survey of substrates, inhibitors and active site topology, made use of substructure, 2D similarity and pharmacophore constraints to dock a filtered set of commercially-available compounds. One of the initial compounds prepared was found to have an IC_{50} of 1.6 μM and iterative design and synthesis, utilizing additional crystal structures, quickly led to compounds with IC_{50} values less than 50 nM. In the FabI arena, starting from a weak μM screening compound (Fig. 2, compound A), iterative synthesis, solution of co-structures and design has led to

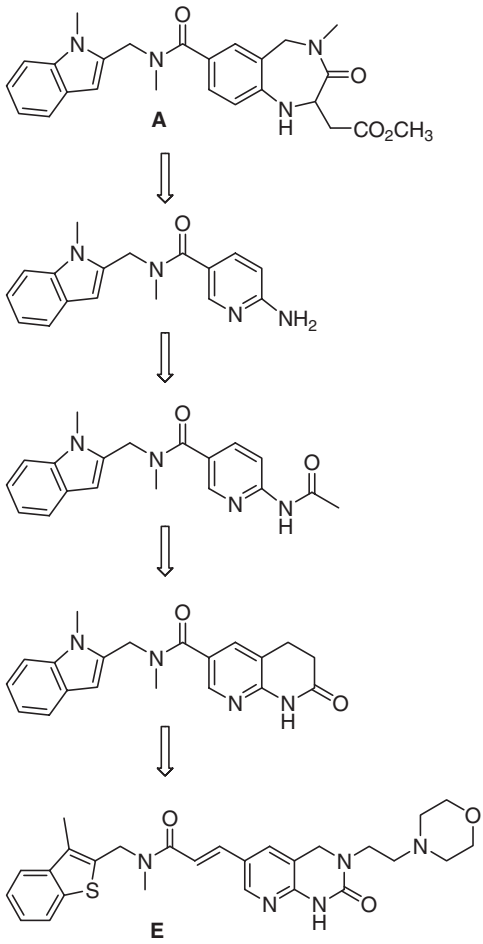


Fig. 2. From initial screening compound (A), through iterative design to a nM soluble derivative.

low nM inhibitors with enhanced solubility properties (Fig. 2, compound E) [51–53]. Here, a key insight was afforded by the knowledge of how the inhibitors bound into the enzyme active site and which portions of the molecule projected towards bulk solvent (Fig. 3). The emerging ability of high resolution structure determination to keep pace with lead optimisation now offers significant opportunities to design compounds simultaneously, optimizing potency and selectivity whilst reducing metabolic and physicochemical liabilities.

8.2. Adaptive inhibitors and beyond

Recently, the design and synthesis of *Plasmodium falciparum* protease inhibitors (for the potential treatment of malaria) has provided an interesting case [54]. Here,

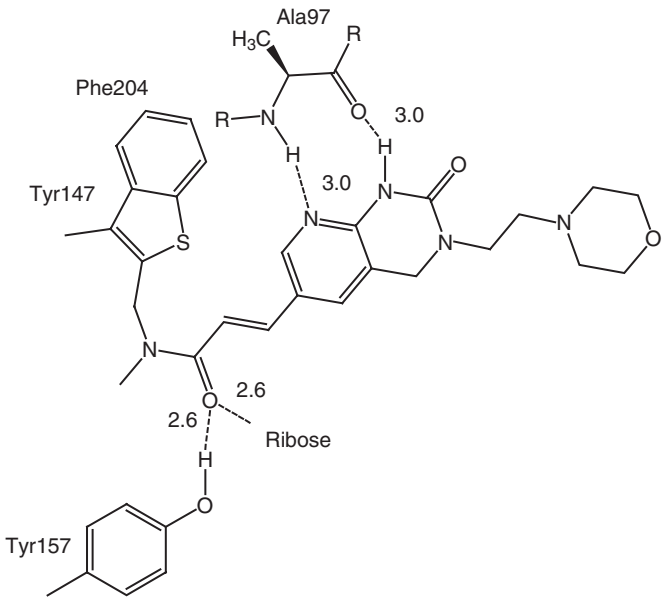


Fig. 3. Road-kill view of the binding pocket of *S. aureus* FabI with compound **E** from Fig. 2. Note the morpholine projects towards bulk solvent.

a fundamental design principle sought ligands containing flexible parts for the variable regions of the target binding site while establishing strong H-bonding and polar interactions with evolutionarily conserved target structural elements. These insights, garnered from a structure-guided drug discovery program, may provide new opportunities for controlling the selectivity and spectrum of anti-infective agents. In addition, for anti-infective medicines, the agent should provoke minimal resistance, so that the clinician can be assured of the agent's efficacy for current and future patients. The combination of protein structure and genetic methods may make it possible to predict and document potential resistance mutation steps and to design molecules that will anticipate the evolutionary moves available to the microbe. A design process that effectively anticipates the likelihood of emergence of resistance to inhibitors (or other modulators of function) would be a fundamental advance in the discovery of new anti-infective therapeutic agents.

8.3. Integrating structural genomics into the drug discovery process

In the past, the pace of structural biology has often lagged behind the drug discovery project team's pace of new compound discovery. Expertise in structural genomics provides structural biology and computational chemistry with the speed and success rates required to become integrated partners with medicinal chemistry and biology in the drug discovery process. Improvements in the rates of success, reproducibility, and speed of obtaining diffraction-quality protein-ligand co-crystals have provided substantial advantages for a project team to use structural and

binding modes to design new compounds in real time. Such capabilities can save significant time typically spent by project team members in synthesizing and testing new compounds that fail to bind the target protein. Scientists can spend a greater fraction of their efforts on the most attractive new compounds. Reaping the best of these integrated processes allows structural biologists to determine the structures of protein–ligand complexes well within the typical 7–10 day medicinal chemistry process cycle time.

9. THE USE OF CHEMICAL PROBES: THE FUTURE OF STRUCTURAL GENOMICS

Most human proteins interact with a ligand as part of their physiology. In most cases, the interacting ligand confers stability to the proteins, and this property is likely to promote protein crystallization. Thus, it is a practical advantage in human structural genomics to obtain stabilizing ligands for each protein target as a method to increase the success rate from clone to structure.

Chemistry and biology are commonly viewed as distinct and separated scientific disciplines in academia; of course, they are highly integrated. Scientists have for long used chemical tools to study biological processes at the gene, cellular and organism level; often with the aims to understand and to treat disease. Chemical Biology, which extends these concepts to the genome scale, aims to systematically develop tools and methods to explore biology using small molecule compounds (chemical bioprobes) to modulate the functions (activation, specificity, inhibition) of all proteins and biomolecules *in vitro* or *in vivo*.

Biology and chemistry are well integrated in the pharmaceutical sector when scientists undertake (high-throughput) screening of chemical compound collections and the identified compounds are validated for activity, safety/toxicity and pharmacokinetics/metabolism in cells and in animals. The implementation of screening on a genome scale would provide great insight into selectivity and specificity but would also be prohibitively inefficient and expensive using current technologies. It is here that structural genomics will play a central role. The molecular definition of the interactions of many members of protein families with large numbers of chemical compounds will allow medicinal chemists to develop rational hypotheses for the generation of novel compounds with the desired selectivity and specificity. It is likely that the interaction of structural genomics and (medicinal) chemists will provide the basis for better communication between the chemistry and biology disciplines in the academic sector.

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G-Protein Coupled Receptor Inverse Agonists: Identification, Pharmacological Relevance and Functional Assays

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Contents

1. INTRODUCTION

The notion that pharmacological activation of molecular receptors can occur in the absence of agonist binding is contrary to classical receptor theory wherein a stimulus is necessary to bring about a response [1,2]. With the generation of multiple pharmacological reagents and the molecular cloning, functional expression, and biochemical characterization of multiple G-protein coupled receptors (GPCRs), classical receptor theory has advanced to include an understanding of ligand-dependent and ligand-independent receptor activation mechanisms. In addition, a deeper understanding of cellular receptor internalization and desensitization mechanisms, and an emergence of biochemical descriptions of orthosteric and allosteric inhibition has furthered our understanding of GPCR functionality [3–5]. Each of these conceptual advances has relevance for the selection of GPCR targets, as well as for the small molecule pursuit of such drug discovery targets, regardless of whether agonists or antagonists are being sought.

Since the pioneering works on δ opioid [6] and β_2 adrenergic receptors [7], there has been a growing appreciation that GPCRs can be activated in the absence of agonist ligand binding. This phenomenon, known as constitutive activation, has expanded our understanding of ligand-GPCR activation mechanisms, and has become more fully appreciated as a consequence of identifying distinct molecular entities that display activities ranging from full agonism, to neutral antagonism, to inverse agonism. Lingering concern that residual endogenous agonist may account for constitutive activity has been eliminated for the class 3 GPCR mGlu5, where

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constitutive activity was retained in a truncated receptor that did not contain the N-terminal agonist binding domain. In this case, constitutive activity was blocked with an inverse agonist [7].

A two-state model of receptor function has been proposed to account for this broader range of ligand activity [8], however multistate models have also been suggested to account for more complex receptor functioning [7,9]. As receptor activation theory has evolved more slowly than GPCR ligand identification, many antagonist drugs have reached the market only to be redefined as inverse agonists (e.g. risperidone, losartan [10]). Thus, inverse agonists can be developed as useful drugs. But how are inverse agonists discovered and are there advantages or different utilities for inverse agonists over neutral antagonists? It is the goal of this report to summarize key molecular pharmacological approaches available that can be used to discover initial leads and define the functional mechanisms of ligands, and to define constitutive activity as being relevant to *in vivo* GPCR function. A second objective is to review constitutive activity as it relates to human disease and altered CNS states. Important reviews have appeared that provide additional breadth to this important topic [3,11–13] and the reader is also referred to discussions on the nomenclature used for quantitative pharmacology [14].

2. GPCR Gα COUPLING PARTNERS

GPCR signal transduction is effected through several second messenger systems, largely determined by the particular Gα protein associated with each receptor. In order to understand the utilities and limitations of functional assays, an appreciation for this Gα coupling specificity is necessary. The broad classification of GPCRs into families based on primary amino acid sequence homology (class 1, 2 and 3 GPCRs) is independent of Gα coupling partner and does not clarify or describe second messenger coupling pathways [15,16]. Representative GPCRs that couple to three of the four main classes of Gα subunits, and their primary second messenger signaling pathways, are illustrated in Table 1. A fourth class of Gα proteins, the Gα12 and 13 class, largely functions *via* cytoskeletal rearrangement, cell-cell interactions, and oncogenic transformation, and does not appear to couple with many GPCRs that are extensively being pursued as discovery targets, and will not be discussed further. It is important to appreciate that at least some GPCRs have the ability to couple to several Gα subunits to support multiple signaling

Table 1. Selected examples of GPCR signal transduction pathways

Gα subunit	Primary second messenger	Representative GPCR
Gαi/o	Adenylyl cyclase inhibition	CB_1, CB_2, D_2, $GABA_B$, H_3, $5HT_{1A}$, $5HT_{1B}$
Gαq	Inositol phosphate accumulation	α_{1a}AR, H_1, $5HT_{2A}$, $5HT_{2C}$, mGlu1a, mGlu5
Gαs	Adenylyl cyclase stimulation	β_2AR, CRF_1, D_1, H_2, MC4R

pathways including ion channels and enzymes [17], and that this is dictated by GPCR-G-protein kinetic and affinity considerations. Importantly, for some GPCRs coupling promiscuity occurs, particularly in transfected cell systems where the Gα protein expression levels can be manipulated, which can blur the simplistic perspective provided herein. Moreover, βγ G-protein complexes have signaling properties relevant to GPCR function. For further information on these important areas of GPCR function the reader is referred to other relevant literature [18,19].

3. GENERAL CONSIDERATIONS AND APPROACHES TO FUNCTIONAL GPCR ASSAYS

The GPCR signal transduction process allows for significant signal amplification through the involvement of a cascade of downstream messengers. As a consequence, there are multiple biological events and/or biochemical endpoints that can be used to observe receptor activity. A schematic illustration of the varying degrees of agonism, from full agonism to inverse agonism, observed in GPCR functional assays is shown in Figure 1.

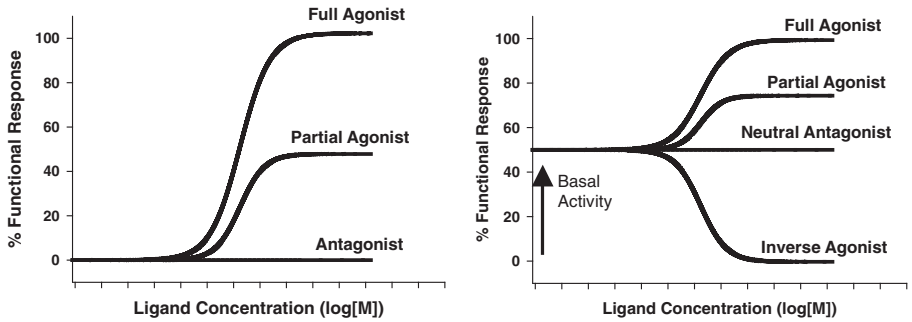


Fig. 1. Hypothetical degrees of agonism observed in two modes of GTPγS functional assays. The data on the left illustrate the types of responses observed when the basal exchange of GTP for GDP is low. On the right, note that when the basal level of response is elevated, discrimination occurs between agents that display neutral antagonism and inverse agonism. Depending on the basal state of receptor activity, similar data can be generated in a variety of assay modes. If the basal state of a response is too low to provide a sufficient signal for the definition of inverse agonism, the basal state response can be elevated by a variety of empirical factors including cellular growth conditions, neurotransmitter or hormone treatment, or manipulation of substratum and intercellular interactions.

It has been shown for several GPCRs that constitutive activity can be reduced by sodium ions (Na^+) [12]. One of the first observations that Na^+ plays a modulatory role at GPCRs was made with the Gαi/o-linked delta opioid receptor [20] and muscarinic acetylcholine receptor [21]. While the earliest observations of the Na^+ effect were made for Gαi/o-linked GPCRs, Gαq-linked receptors such as

bradykinin-2 have also demonstrated the allosteric inverse agonist effect [22]. All GPCRs, however, are not modulated by Na^+. Na^+-induced stabilization of the inactive (R) state is not observed with the neurokinin-1 receptor [23]. The data indicate that pharmacologically, Na^+ acts as an allosteric inverse agonist, stabilizing the R state and reducing basal G-protein activity [3,46]. The precise molecular target of Na^+ has been shown to be the carboxylate group on the highly conserved aspartate residue in TM II of the GPCR [24]. Accordingly, raising the concentration of Na^+ may have the effect of increasing the binding affinity of inverse agonists as well as decreasing the binding affinity for full agonists [3]. Therefore, Na^+ concentrations between 10 mM and 150 mM may serve to affect the degree of constitutive activity at relevant GPCRs *in vivo* [12].

Following agonist stimulation of most GPCRs, phosphorylation and internalization events occur, and the receptor enters the endocytic pathway. This process underlies GPCR desensitization, and usually lasts for 30 minutes to an hour upon intense ligand stimulation. Immunocytochemical methods best define internalized GPCRs *in situ* when coupled with confocal, light or electron microscopy approaches, and can provide evidence for the extent of tonic receptor cycling in the steady-state and under conditions of significant stimulation. After GPCR internalization, trafficking can occur back to the plasma membrane or to lysosomes for degradation. In fact, inverse agonists have in certain cases been shown to elevate the numbers of cell surface receptors of either wild-type or constitutively active GPCRs [25,26]. Concern has been raised about this, as the associated risks of receptor upregulation, drug tolerance and withdrawal symptoms may be a consequence [5].

The point at which an assay intersects with the transduction process will determine how broadly useful the assay will be in detecting GPCR activity. For example, assays that detect inositol phosphate turnover will generally find utility for the Gαq subset of GPCRs. High throughput assays have been developed for several steps of the GPCR activation cascade including transient redistribution of arrestin proteins involved in desensitization, and receptor dimerization [27]. However, assays that operate at the level of GPCR-G protein interactions may find much broader utility.

4. FUNCTIONAL ASSAYS THAT DETECT CONSTITUTIVE ACTIVITY

The two-state model of GPCR activation assumes that the receptor isomerizes from an inactive (R) state to an active (R^*) state. While the conformational changes associated with R to R^* isomerization have been directly assessed in fluorescence spectroscopy studies [28], the fluorescence changes observed with inverse agonists are rather small. Due to the relatively small dynamic range of the fluorescence spectroscopy assay, limiting its use in detailed pharmacological studies, most investigators study constitutive R to R^* isomerization of GPCR's indirectly, measuring either GDP/GTP exchange at the G-protein or changes in downstream effector system activity, either directly or indirectly through the coupling to a reporter gene. As a consequence, there are multiple biological events and/or

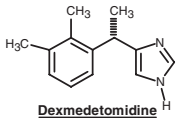
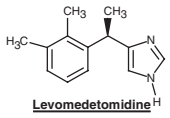
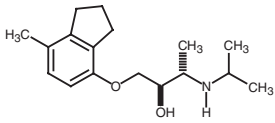
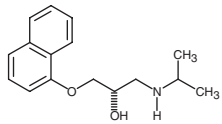
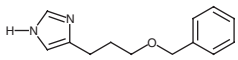
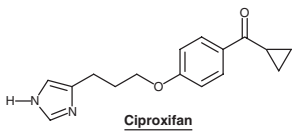
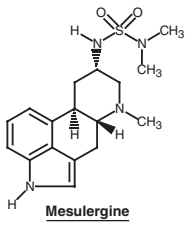
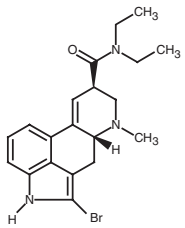
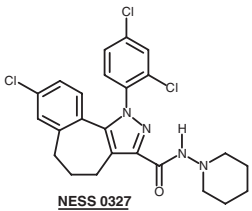
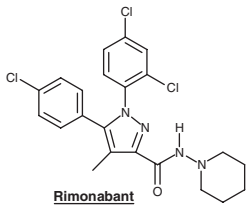
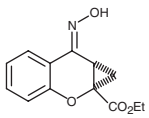
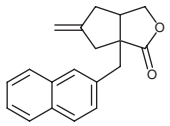
biochemical endpoints that can be used to observe receptor activity. While in some cases signals measured far downstream of the GPCR have significantly improved sensitivity, one must balance this against the possibility that many more factors apart from the GPCR activity can affect the actual read-out.

Depending on the G-proteins that a GPCR couples to the signal transduction pathway through, the types of assays typically used to study receptor activation can vary. For Gαs-coupled GPCRs, constitutive activity is typically measured by an increase in cAMP accumulation in intact cells or as adenylyl cyclase (AC) activity in membranes. The sensitivity of traditional nucleotide exchange assays (GTPγS binding and GTPase) is usually too low to monitor constitutive activity of Gαs-coupled GPCRs directly at the G-protein level [29]. The nucleotide exchange may have broader utility if a fusion protein is engineered (GPCR-Gαs) ensuring close proximity and efficient coupling of the signaling partners [30,31]. For Gαi/o-coupled receptors, GTP hydrolysis and GTPγS binding in membranes are widely considered the standard methods for assessing constitutive activity. The basal activity of the Gαi/o-coupled receptor can be enhanced in this membrane-based assay by one of at least two ways: by decreasing the GDP concentration in the assay or increasing the temperature. One can also monitor reductions in basal or forskolin stimulated cAMP in intact cells or AC activity in membranes [32], although with Gαi/o-coupled receptors, assay sensitivity is superior in nucleotide exchange assays. Constitutive activity of Gαi/o-coupled receptors has also been measured by assessing cAMP-dependent reporter gene expression [33], release of [^{3}H]arachadonic acid from whole cells [16], and mitogen-activated protein kinase (MAPK) activity [34]. For Gαq-coupled GPCRs the standard assay is the analysis of [^{3}H]inositol phosphate accumulation in myo-[2-^{3}H]inositol-labeled intact cells. While phospholipase C (PLC) increases in reporter gene expression have been used to assess constitutive activity of Gαq-coupled GPCRs [35], the fact that it measures signals far downstream of the GPCR allows for many factors apart from the GPCR activity to influence the results [36]. Measuring Gαq-coupled GPCRs constitutive activity at the G-protein level is challenging. However, co-expression of a regulator of G-protein signaling (RGS protein) along with the Gαq-coupled GPCR greatly enhances steady-state GTP hydrolysis, thereby increasing the sensitivity of the GTPase assay for Gαq-coupled receptors [37].

5. INVERSE AGONISTS VERSUS NEUTRAL ANTAGONISTS

The advent of constitutive GPCR activity has necessitated a reclassification of many antagonists. Some ligands may simply block the agonist-activated state of the receptor, i.e., neutral antagonists, and others prevent the basal signal transduction that can be induced in the absence of an agonist, i.e., inverse agonists. Just as partial agonists produce sub-maximal receptor activation, partial inverse agonists can reduce, but not completely eliminate, constitutive GPCR activity. Thus, GPCR ligands may form a continuum with full agonists and full inverse agonists defining the functional limits. As discussed above, assays that can be configured to detect basal

Table 2. Representative GPCR neutral antagonists and inverse agonists*

GPCR	Assay	Neutral Antagonist	Inverse Agonist	Ref.
α_{2A}AR	cAMP	 Dexmedetomidine	 Levomedetomidine	[39]
β_2AR	cAMP, cardiac contractility	 ICI 118,551	 (-)Propranolol	[40]
H_3	[^{3}H] arachidonic acid release	 Proxyfan	 Ciproxifan	[41]
$5HT_{2C}$	IP	 Mesulergine	 Bromo-LSD	[42]
CB_1	cAMP	 NESS 0327	 Rimonabant	[43,44]
mGlu1	IP	 CPCCOET	 BAY36-7620	[45,46]

*Some examples may display different profiles depending on the particular assay.

receptor signaling are well suited to distinguish between neutral and inverse agonists. Table 2 provides a comparative selection of inverse agonists and neutral antagonists at a variety of GPCRs. A recent evaluation of the inverse agonist-neutral antagonist literature concluded that inverse agonists predominate in accord with theoretical predictions [13].

6. CONSTITUTIVE GPCR ACTIVATION, DISEASE, AND INVERSE AGONIST UTILITY

There exists a growing literature on constitutive activation of GPCRs, and the reader is referred to several recent reviews on the topic [12,26,38]. It is important to distinguish GPCR systems that are persistently activated as a consequence of mutation, termed a constitutively active GPCR mutant from that of a wild type GPCR system that displays a high level of constitutive activity. Both types of systems are relevant for the utility of inverse agonists assuming the need to tone down the excessive GPCR activity, and, as discussed below, the efficacy of the blockade mechanism of inverse agonists for tonic compared to ligand stimulated situations may differ substantially. Perhaps it is not surprising that several gain of function constitutively active GPCR mutations have been observed in endocrine systems since the endpoints of organ system activation are typically strong, easily observed physiologically, and monitored by the robust alterations in plasma hormone levels. Though such gain of function mutations have not yet been described physiologically for CNS GPCR systems in man, systems that are typically modulatory in nature, it is likely that such mutations exist but are difficult to phenotype due to the incomplete understanding to date of the roles that GPCRs play in neuronal circuits. There are many more examples of loss of function GPCR mutations than gain of function mutations presumably due to the fact that it is easier to lose function than to gain function. These situations will not be described here as our focus is on inverse agonists as compared to neutral antagonists.

Perhaps the best studied system in terms of constitutively active mutant GPCRs is the luteinizing hormone receptor (LHR), also known as the human chorionic gonadotropin receptor, which is a class 3 GPCR. There exists a gonadotropin-independent dominant autosomal familial form of precocious puberty, and this relatively rare disorder is limited to males. Greater than a dozen single germline amino acid mutations have been discovered, mostly in the third intracellular loop and in helix 6, which cause constitutive activation of the LHR in the absence of ligand [47,48]. This constitutive activity of the germline mutant receptors appears to predominantly affect cAMP levels. The LHR is expressed in Leydig cells of the testes, and in this situation of constitutive activation there is abnormal Leydig cell growth, termed hyperplasia, and precocious production of testosterone. In one patient where there was a constitutively active mutation identified in helix 1, a gonadotropin releasing hormone agonist, which can be used to cause cellular desensitization of the Leydig cell and loss of androgen secretion in normal patients, was shown to completely suppress testosterone secretion. It is also of interest that a somatic cell mutation of the LHR has also been identified in Leydig cell tumors and this mutant form results in the constitutive activation of both Gαq and Gαs signaling pathways [49].

A somewhat similar situation is seen with the related human thyroid stimulating hormone receptor (TSHR) system. Both sporadic and familial non-autoimmune hyperthyroidism are quite rare in occurrence, though in the past few years several examples of constitutive TSHR activation have been identified to account for at least some cases of familial and sporadic hyperthyroidism. It is important in these

studies to rule out autoimmunity, as this is generally responsible for Grave's disease, a situation where autoantibodies have been shown to act as agonists of the TSHR. Moreover, transient disease can be caused by the passage of such antibodies from the milk of mother to child. Persistent hyperthyroidism in non-autoimmune patients is one of the key findings that points to a possible TSHR mutation. The age of onset in such disorders is variable and not fully explained at present. As with LH in the Leydig cell, the Gαs- and Gαq-coupled TSHR stimulates thyroid hormone production and thyroid cell growth. Several germline mutations in the TSHR have been identified that result in constitutive activation of the cAMP pathway [50,51], and somatic mutations have been identified in hyperfunctioning thyroid adenomas whereas nearby non-adenomatous thyroid tissue contained the wild-type TSHR [52]. Interestingly, a similar phenomenon has been shown to occur in so-called hot thyroid nodules from multinodular goiter [53].

An extreme and unique example of constitutive GPCR activity comes from the Kaposi's sarcoma-associated herpes virus (KSHV), which upon activation expresses a viral oncogene that is an activator of local angiogenesis. KSHV encodes an orphan GPCR (KSHV-vGPCR) that displays homology to the human chemokine receptors CXCR1 and CXCR2, and to another orphan GPCR expressed in the saimiri herpes virus. The current view is that the virus co-opted the GPCR from the human genome and utilized this in viral pathogenesis (see [54] for a comprehensive review). KSHV-vGPCR signals constitutively *via* the phospholipase C pathway and *via* activation of jun kinase and MAPK pathways, causing oncogenic transformation [55]. In fact, certain human angiogenic chemokines such as GROα act as agonists, whereas angiostatic chemokines such as IP-10 act as inverse agonists [56]. When the KSHV-vGPCR is transgenically expressed in mouse hematopoetic cells, induction of angioproliferative lesions in multiple organ systems occurs that resemble the vascular tumors in Kaposi's sarcoma [57]. Importantly, when various mutant forms of KSHV-vGPCR that are deficient in *in vitro* constitutive activity, or in chemokine regulation, are transgenically expressed in mice, the mice fail to develop the KS-like pathology [58]. Thus, these data provide strong evidence that that KSHV-vGPCR constitutive activity underlies viral pathogenesis.

For GPCR mutations that cause ailments due to excessively high constitutive activity, the potential utility of inverse agonists is clear. Beyond these orphan indications, the benefit of inverse agonists over neutral antagonists is more speculative. However evidence is beginning to emerge, particularly where the GPCR is presynaptic and broadly controls transmitter release (auto- and heteroreceptors). Constitutive activation of presynaptic GPCRs can reduce synaptic neurotransmitter concentrations and blunt afferent signaling. In situations where neurotransmission is already impaired such as Alzheimer's disease and other cognitive disorders, this constitutive activity may exacerbate disease.

There is substantial evidence supporting high constitutive activation of presynaptic histamine H_3 receptors *in vivo* [41,59]. H_3 is a Gαi-coupled GPCR that serves as an autoreceptor for release of histamine and a heteroreceptor modulating release of a variety of other neurotransmitters including norepinerphrine, dopamine, 5-hydroxytryptamine, acetylcholine and γ-aminobutric acid from histaminergic nerve endings [60,61]. H_3 inverse agonists are hypothesized to be useful in the

treatment of a number of disorders including cognitive dysfunction associated with attention deficit hyperactivity disorder, obesity and sleep disorders [62–65].

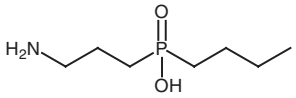
Physiologically relevant constitutive activity of H_3 receptors is suggested by several lines of evidence. For example, a significant level of GTPγS binding activity has been reported for H_3 receptors in rodent cortical synaptosomes in the absence of activating concentrations of histamine. Application of FUB 465, ciproxifan or thioperamide, which act as H_3 receptor inverse agonists, reduced GTPγS binding and increased basal histamine release. Further, proxyfan was found to be a neutral antagonist in this test system: blocking the decrease in GTPγS binding produced by inverse agonists and the increase in binding elicited by the synthetic H_3 receptor agonist imetit without affecting GTPγS binding when applied alone. Consistent with their function as in inverse agonists, FUB 465 and ciproxifan were found to increase *in vivo* release of histamine as evidenced by increases in levels of the histamine metabolite *tele*-methylhistamine (t-MeHA) whereas the agonist imetit decreased t-MeHA levels. Proxyfan acted as an antagonist to the increase in t-MeHA elicited by inverse agonists and the decrease produced by agonist [41].

In accord with native H_3 receptors possessing a high level of constitutive activity, preclinical studies suggest that H_3 inverse agonists sometimes elicit distinct *in vivo* responses versus antagonists. For example, it has been reported that GT-2394, identified as a selective H_3 inverse agonist, increased histamine release and suppressed feeding in rats whereas the neutral antagonist GT-2331 and other compounds identified as neutral antagonists failed to affect feeding despite achieving exposure estimated to provide up to 100% receptor occupancy if one assumes limited *in vivo* metabolism [63]. In contrast, both a neutral antagonist GT-2331 and a potent inverse agonist ABT-239 have been reported to be effective in enhancing cognitive performance in rat models [66,67]. As a caveat to the evidence for H_3 receptor inverse agonist activity as a driver of *in vivo* efficacy, it has been reported that H_3 ligand functional effects may vary with numerous factors such as receptor expression level, second messenger measured and species. For example, proxyfan has been shown to act as an H_3 inverse agonist, antagonist or agonist depending on the system studied [68].

CB_1 receptors regulate release of multiple central and peripheral transmitters [69]. High constitutive activity of CB_1 receptors has been reported in both rat and human brain [42], a finding that is recapitulated in native receptor bearing cell systems [70]. Thus, a therapeutic utility for CB_1 inverse agonists is possible for a variety of indications. The most advanced CB_1 inverse agonist, rimonabant (SR141716A) produced sustained weight loss in phase III clinical trials [71–74]. Though there are multiple reports that characterize the inverse agonist profile of SR141716A [75,76 and references therein], the perspective is not universally supported. It has been suggested that cross talk between CB_1 and adenosine A_1 receptors is responsible for the apparent capacity of SR141716A to block basal CB_1 function. Thus, in GTPγS binding studies in rat cerebellar membranes, SR141716A could not prevent constitutive activity when tonic A_1 signaling was blocked. In guinea pig small intestine, endocannabinoids such as anandamide or constitutive receptor activity limit acetylcholine release and thus reduce contractile responses [77]. Inhibition of both acetylcholine release and the resulting twitch response by CB_1 agonists (+) WIN

55212-2 or CP 55940 was relieved by SR141716A. The inverse agonist also increased acetylcholine levels in the absence of exogenous CB_1 agonist. In the CNS, endogenous CB_1 ligands anandamide and arachidonylglycerol act as retrograde messengers to reduce ACh release after electrical stimulation. In human neocortical tissue, WIN 55212-2 further reduced [^{3}H]ACh release by about 30% during electrical stimulation, the modest effect was presumably additive with the actions of endogenous agonists. Again, SR141716A completely abolished the agonist-induced inhibition though the inverse agonist profile was not observed [78]. Presynaptic CB_1 activation also reduces glutamate release in the hippocampus [79]. These observations provide a possible explanation for the impairment of cognitive function associated with recreational cannabanoid use and suggest a therapeutic role for CB1 antagonists/inverse agonists in the treatment of dementia.

Presynaptic $GABA_B$ receptors also control neurotransmitter release and a similar role in cognitive function may be postulated. Competitive antagonist SGS742 improved several measures of cognitive function in a phase II study in patients with mild cognitive impairment (MCI) [80]. If presynaptic $GABA_B$ receptor constitutive activity can be inhibited, greater efficacy could be uncovered. However, $GABA_B$ inverse agonists have not been described.



SGS742

Insufficient neurotransmission through serotonin receptors plays a role in depression as demonstrated by the proven therapeutic utility of serotonin reuptake inhibitors. Presynaptic $5HT_{1B/1D}$ autoreceptor activation can contribute to reduced synaptic serotonin levels by blunting serotonin release. When coadministered with sertraline, the $5HT_{1D}$ antagonist GR127935 increased synaptic serotonin levels more than sertraline alone as measured in *in vivo* rat dialysis experiments [81]. This supports a role for $5HT_{1D}$ autoreceptors in depression and invites speculation that selective inverse agonists could show greater efficacy [82]. Unfortunately, the lack of selective $5HT_{1B/1D}$ antagonists and inverse agonists complicates evaluation of the true potential utility of such a compound.

Modulation of GPCR constitutive activity *via* endogenous mechanisms can be important in normal physiological function and is sometimes the mechanism for expression of genetic differences. For example, melanocortin MC4 receptors (MC4Rs) regulate feeding and energy homeostasis. Activation of the MC4Rs by endogenous agonist α-melanocyte stimulating hormone (α-MSH) results in a reduction of feeding whereas agouti-related peptide (AgRP) acts as an antagonist to the effects of α-MSH and stimulates feeding. Further, it has been shown that AgRP acts as an endogenous MC4R inverse agonist [83,84]. MC4, a Gαs-coupled receptor, maintains significant adenylyl cyclase activity in the absence of agonist. AgRP concentration-dependently inhibits MC4R basal adenylyl cyclase activity and this effect is blocked by the neutral antagonist SHU9119 [84]. The interplay between constitutively active receptor, agonist and inverse agonist provides a mechanism for extensive modulation of MC4R signaling *in vivo* [85].

Mutations that reduce constitutive activity of the MC4R are associated with human obesity. Such mutations can reduce signaling by various mechanisms including lower cell surface expression, reduced α-MSH affinity but unchanged AgRP affinity, and normal receptor expression and affinity for ligands but lower inherent constitutive activity [86–88]. Some rare obesity-related mutations of the MC4R involve mutations in the N-terminal domain [88]. This region contributes to constitutive activity by acting as a tethered agonist. Point mutations in this domain found in obese individuals reduce MC4R constitutive activity by 50% or more. These N-terminal mutations do not alter α-MSH responses or AgRP affinity. Thus, the MC4R system provides an interesting example of how constitutive activity can be important in normal physiology.

7. CONCLUSIONS

The understanding of GPCR constitutive activation and the utilities of neutral antagonists compared to inverse agonists is coming of age. Experimental animal studies coupled with *in vitro* molecular pharmacologic investigations have demonstrated important differences in the efficacy of inverse agonists compared to neutral antagonists. The challenge to discovery scientists, however, is to implement the assay technologies that are relevant and predictive to the identification and differentiation of such agents, and to evaluate the utility of inverse agonists and neutral antagonists in predictive animal models of human disease. Considering the extensive *in vitro* and emerging *in vivo* data demonstrating the relevance of constitutive activity of many GPCR systems, it will be necessary to tailor the pharmacological profile of future GPCR ligands to best match the particular needs of a specific disease state. Looking forward, new inverse agonist drugs will now be brought forward by design.

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The Utility of Metabonomics for Drug Safety Assessment

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Contents

1. INTRODUCTION

In any organism, the complexity of its biochemical cascade is dynamically influenced by environmental parameters, genetic disposition, etiologies, and/or drug administration, to name but a few factors. The ability to monitor the multiparametric changes that an organism will experience is of great diagnostic and prognostic value to better understand the metabolic status of the organism and its relationship with induced stimuli [1]. In recent years, the field of metabonomics has evolved to effectively derive information from a combination of data-rich analytical techniques (NMR and more recently, mass spectrometry) and statistical multivariate analysis [2] for studying *in vivo* metabolic profiles. Metabonomics as a profiling technology was pioneered by Nicholson and colleagues. It can be defined as an approach to investigate complex metabolic consequences of patho-physiological or genetic modification in a multivariate space [3]. Metabonomics offers the advantage of being applicable to samples collected in non-invasive (urine), or minimally invasive ways (serum, tissues). The data provides qualitative and/or quantitative assessments of small endogenous molecules (~50 to 1000 amu) and follows qualitative changes of unique biological macromolecules, *i.e.* lipoproteins [4,5]. The development of these information-rich techniques has prompted the scientific community to evolve from the original concept of a biomarker representing a single molecule, to a complex biomarker represented by a panel of molecules emanating from

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multi-parametric analysis. It has been demonstrated that the accuracy and predictability of a panel of biomarkers is of greater value than that of a single entity [6].

The holistic approach that systems biology (*i.e.* genomics, transcriptomics, proteomics, and metabonomics) brings to the study of biology assumes that each tier of the system depends on the other, and alterations in one tier may affect another. Compared to the other ''omics'', metabonomics focuses on the assessment of small endogenous metabolites. Since these cellular components of the metabolome represent the end products of gene expression and define the phenotype of a cell, tissue, or organism, metabonomics is well positioned to provide the most functional information amongst the ''omics'' technologies [7]. Biomarkers that change either in pattern or concentration, can relate to both site and mechanism of toxicity [8]. However, because of technical challenges to measure all metabolites present in biomatrices, metabonomics as a metabolic profiling tool has been delayed as a developing technology when compared to genomics, or proteomics. Rapid growth in the use of metabonomics is underway, and positive impacts to the study of biological systems are now being demonstrated [7].

A strategy aggressively pursued by the pharmaceutical industry is the discovery of specific, selective and robust biomarkers, as illustrated by the amount of work done with ''omics'' technologies. These efforts, driven by drug discovery needs, are intended to enhance the quality of lead prioritization decisions, decrease attrition by yielding better candidate selection, provide toxicity screening for better selection of backups, and provide a tool for continuous safety assessments that can be translated from early development to the clinical arena [9]. In cases where a particular toxicity has been observed in pre-clinical studies without a clear understanding of the relevance of these findings to humans, it is critical to possess the analytical tools that can be equally applied to pre-clinical and clinical situations. As our knowledge of data processing expands, allowing pattern recognition to evolve towards mathematical modeling, predictive assessments and metabolite identification/quantitation, scientists are starting to seek biomarkers that not only enable the elucidation of complex disease pathways, but also provide predictive safety assessments. This critical bridge is often the key to successful risk management strategies for the continuation of compounds in development.

Metabonomics, although not a panacea for safety assessment or biomarker discovery, has been shown to provide an unbiased ability to differentiate genotypes based on metabolite levels that may, or may not produce visible phenotypes [10,11]. Numerous examples show the application of metabonomics to finding biomarkers of disease and efficacy, however these are out-of-scope for this chapter. It is our intent to focus on the latest applications of metabonomics to toxicity and safety assessments, while taking a cursory look at the analytical instrumentation and chemometric methods that are needed for producing and evaluating the vast amounts of generated data.

2. TECHNOLOGIES

The analytical challenge of metabonomics is to provide a comprehensive investigation of biomatrices, with qualitative and quantitative information. The

two principal techniques used to generate metabolic profiles of biomatrices are proton nuclear magnetic resonance (^{1}H-NMR) and mass spectrometry (MS). Excellent reviews have recently been published, which appraise the current applications, developments, and strategic directions for metabonomics [12–14]. The strengths and limitations of each technique will be briefly summarized.

2.1. NMR

Early in the development of metabonomics, the majority of work in this field was generated using NMR as the method of choice. NMR, as a non-destructive technique, is very information-rich with regard to molecular structure and is capable of generating a comprehensive profile of low molecular weight metabolites from biomatrices if concentrations are sufficiently high [15]. Relative quantitation by NMR is intrinsically accurate and precise, as has been demonstrated through the COMET consortium [16], but caution must be exercised in areas of high spectral overlap (*e.g.* sugar area). The primary limitations of NMR are its relatively low sensitivity compared to MS. Current detection limits for NMR spectroscopy are approximately 100 μM in a tissue extract, or 10–50 μM in biofluid [12]. Increasing field strengths for spectrometer design (400 MHz to 800 MHz) has resulted in considerable improvements in spectral quality (sensitivity and resolution). Parallel developments in cryogenic NMR probes have provided significant improvements in spectral signal-to-noise ratio, with increases as high as 5–10 fold for samples with low salt content, and as low as 2–2.5 fold for samples with high salt content (human urine) [17]. Sample volume requirements range from 500–700 μl for acquisition in 5 mm tubes, to 20 μl with miniaturized probes [1]. The ideal design and equipment of a metabonomics laboratory may vary based on the expected results. While high throughput would be important for toxicity screening, it may become secondary when focusing on biomarker identification, or when handling particularly biohazardous fluids. Recent arrangements have included custom-designed automated liquid handlers to minimize sample manipulation (TECAN) and automatic sample insertion systems to standardize timing and preparation (Bruker BioSpin GmbH, Germany). An example of a useful customized module is an automated pH measurement station integrated in a liquid handler to measure the sample pH following acquisition. Because pH can have a major impact on peak chemical shift of certain molecules, this feature has proven to be a very valuable tool to check the data integrity and identify outliers due to pH variations. Although pH is not the only parameter that may influence the spectral profile observed, its measurement provides an additional level of quality control on generated data, since application of multivariate analysis requires spectra to be acquired in the same physico-chemical conditions, notably at the same pH.

With the development of high-resolution magic angle spinning (HR-MAS), intact tissues can now be measured directly with spectral resolution comparable to that of biofluids. The broadening effects of the chemical shift anisotropy and dipolar

coupling are considerably reduced by spinning the solid-like sample at an angle of 54.7° with respect to the static magnetic field [18].

2.2. MS

Investigators that have published metabonomics studies with MS, have typically utilized an initial chromatographic stage (gas or liquid chromatography) prior to mass spectral analysis [19]. By separating each component prior to detection, chromatography provides inherent value to the MS analysis. The development of multiple soft ionization sources for mass spectrometers (electrospray ionization, atmospheric pressure chemical ionization, photo-ionization, matrix assisted laser desorption ionization, etc.) provides the metabonomics researcher with multiple tools to cover the chemical diversity of the metabolome. Yet, the primary limitation of these ionization sources is that not all metabolites can be ionized with one particular source because the structural characteristics (functional groups, polarity) of the molecule determine the optimal mode of ionization. Therefore, multiple analyses of a sample set are needed for complete detection of different compound classes. The extent of ionization may also be impaired by ionization suppression parameters, such as high salt content, lipids, or other polar moieties [1], underscoring the need for chromatographic separations to minimize or eliminate suppression effects [19,20].

The strengths that MS brings to metabonomics far outweigh the limitations. MS-based approaches can be up to two orders of magnitude more sensitive than NMR when operating in a full scan mode [1,21]. The added ability to determine exact masses for metabolites is critical for molecular formulae determination and identification, which is needed to complete metabonomics studies [12,21]. Two platforms capable of delivering accurate and reproducible mass information are Fourier Transform Mass Spectrometry (FTMS) and Quadrupole-time-of-flight platforms (Q-TOF's). FTMS can provide extremely high mass resolution ($R = 500{,}000$ to 1×10^6) and high mass accuracy (average errors < 1 ppm) that allows for mass separation and differentiation of complex mixtures without resorting to chromatography [22]. Unfortunately, the duty cycles of FTMS operated in high resolution and broad band mode usually limit the incorporation of a chromatographic system to the spectrometer, and the high cost of FTMS is prohibitive for widespread use [7]. Conversely, Q-TOFs offers an attractive alternative to FTMS in that their are significantly less expensive, yet provide very good mass accuracy (typically within 5 ppm), sensitivity, and high scan rates that will support narrow chromatographic bandwidths [19]. Although exact mass instruments can be critical for elucidating unknown metabolites (via derivation of molecular formulae), none of the MS approaches can differentiate chemical isomers such as the common hexoses. By coupling a chromatographic system to an exact mass instrument one can easily remove confounding markers (*e.g.* xenobiotic and its metabolites) to clearly assess endogenous metabolic profile changes. Removal of these signals is easily accomplished due to the specificity of mass and retention time

selectivity. This cannot be easily achieved with NMR spectra where removal of confounding peaks may also affect the evaluation of overlapping endogenous metabolites [20].

3. DATA ANALYSIS

Both techniques presented above generate large amounts of data per sample, making it impossible to manually organize, interpret and draw conclusions from multidimensional datasets. To simplify the task at hand, chemometrics have come to the rescue of the scientist by providing multivariate statistical algorithms [20]. Unsupervised methods such as principal component analysis (PCA) and hierarchical cluster analysis (HCA), are typically suited for datasets with limited numbers of well-defined classes. They also present the advantage of segregating the data points without *a priori* decision on which class they belong to. PCA is commonly used to look at time trends, outliers, and trajectories for toxic events and recovery. However, more often than not, the metabolic variations between classes are very subtle and the inherent complexity of the biological system studied requires utilization of more sophisticated supervised algorithms. Utilizing the additional knowledge of classes (toxicity, disease) these supervised methods (soft independent modeling of class analogy: SIMCA, partial least square: PLS, partial least square with discriminate analysis: PLS-DA) are designed to effectively "tune out" the variations unrelated to the classes that one wishes to characterize. Supervised statistical methods present the unique advantage of yielding a model that can be tested, validated and ultimately used for predictive purposes. Indeed, the model building step is the foundation for testing the predictive ability of a model on samples with known classes, and for assessing samples of unknown classes. Predictability is the main objective for applying metabonomics in the pharmaceutical industry as it may help screen out toxic compounds earlier, monitor and diagnose diseases at earlier timepoints, and make better overall informed decisions regarding compound development.

To maximize the use of metabonomics datasets, pre-processing methods are commonly applied for scaling and/or filtering data. Scaling options include mean-centering (centers the data without scaling to preserve peak height information and enhances changes in intense peak), autoscaling (centers the data and scales it to mean variance), pareto scaling (enhances small features without inflating baseline). Orthogonal signal correction (OSC) is a filter used to maximize segregation between classes by eliminating changes that are uncorrelated to the classes studied. It provides a way to focus on a certain toxicity, while ignoring changes induced by diet, age, gender, etc. Additional examples of application of OSC to metabonomic data can be found in recent publications [23–25]. To generate biomarkers with metabonomics, multivariate analysis is typically followed by detailed analyses of individual spectra to confirm the identity of metabolites. This identification is guided by the loading values calculated by the algorithms. Ideally, identified components need to be correlated to biochemical pathways, thereby establishing a more

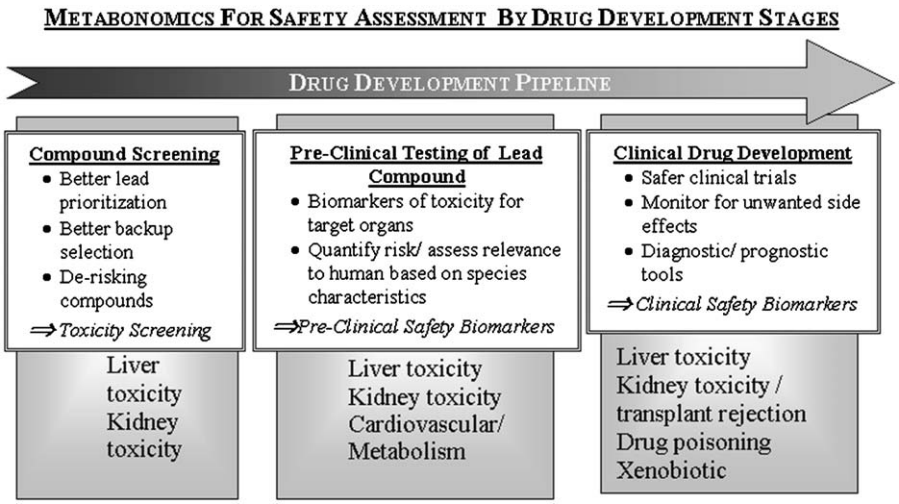

Figure 1.

complete understanding of the therapeutic phenomena, gene function, or biological response [7].

4. TOXICITY AND SAFETY ASSESSMENTS

Metabonomics has been applied to a wide range of scientific inquiries. Literature pertaining to its applications to safety assessment can be categorized by stages of drug development. Fig. 1 summarizes the ground covered by the published literature in the past couple of years and a few examples will be discussed below to illustrate the current state-of-the-art of metabonomics.

4.1. Toxicity screening

By focusing on fast NMR spectroscopic acquisition, automated chemometric batch analysis, and relatively high throughput of samples (up to 300/day) with flow probes, scientists developed an efficient tool that uses pattern recognition (PR) analysis to effectively perform toxicity screening. PR looks for unique profile changes linked to a certain targeted toxin using supervised multivariate analysis. For the past decade, in an effort to optimize efficiency of lead compound selection and decrease attrition in early drug discovery, metabonomics and PR have been used to define unique spectral profiles that correspond to organ specific toxicities [26].

Toxicity screening using metabonomics is primarily based on the assumption that compounds with similar toxicological targets will yield similar profiles. By building a large spectral database describing several hundred toxins with well-characterized pathologies, scientists have built a ''reference library'', against which unknown

samples can be compared to predict potential target toxicity [26]. Profiles are, however rarely simple and each toxin induces its characteristic lesions with its own time-dependence, recovery rate, excretion rate and effects on other tissues. Therefore, assignment of a particular toxicity to an unknown compound, based on spectral profiles is not always straightforward. However, some types of toxicity have been identified as having specific spectral profiles that can be used to predict lesions. The target tissues deemed best suited for screening by metabonomics are renal papillary, renal cortex regions (S1 and S3) [26], renal glomerula, testes and liver [27]. Early on, urine appeared as the biofluid of choice for toxicity screening because of the adequate volume available for repeated sampling and the non-invasive collection in refrigerated metabolism cages [28].

Because a substantial amount of data analysis needs to be done to transfer the acquired spectral knowledge from a large xenobiotic database into a true classification of samples according to their spectral similarities, the pharmaceutical industry answered that challenge in 2002 by forming a Consortium for Metabonomics Toxicology (COMET) between (then) six pharmaceutical companies (Bristol-Myers-Squibb, Elli Lilly, Hoffman-La Roche, NovoNordisk, Pfizer Inc., and Pharmacia) and Imperial College (London, UK). COMET's objective was to build study protocols and methodologies to apply metabonomics to preclinical toxicity screening of drug candidates. Eighty model toxins with well-known toxicological effects were administered using common protocols across companies. COMET focused on building a database for predicting kidney and liver toxicity in rat and mouse [16]. A subset of the COMET work (19 model toxins) more recently reported a predictability of 86% for the control group, 85% for the liver toxicity group, 91% for the kidney toxicity group and 88% for the "other toxicity" group, thereby illustrating the potential value of this application [29]. To truly show added value for toxicity screening, metabonomics will have to show measurable impact on portfolio and "real life" cost savings to the industry.

With toxicity screening, the goal is to gain knowledge and confidence in the ability to spot candidates with potential toxicities early in the process, in hope of decreasing if not eliminating the instances of drug adverse events that occasionally appear later in clinical development, at tremendous cost in time and money to the industry. One common assumption is that a pre-clinical model is indeed representative of how the drug would behave in humans. This may arguably be the hardest and least examined aspect in this line of work. The other assumption is that animal models will also provide a predictive assessment of idiosyncratic responses in humans. Because idiosyncratic toxicity is by definition a physiological response peculiar to an individual who does not respond as the majority, seeking to build a model of idiosyncratic toxicity is a challenging venture that requires understanding of underlying mechanisms by which idiosyncratic events may occur (genetic predisposition, genotypic diversity, concurrent diseases, relevance to human). An alternative to approach idiosyncratic toxicity may be to design well-targeted clinical protocols with multiple clinical sites to gather biofluid samples from individuals who display these adverse events.

A targeted search for unique biomarkers associated with specific toxicological events is the focus of the two following sections.

4.2. Pre-clinical safety biomarkers

4.2.1. Assessing baseline physiological reference ranges

Accurate monitoring of metabolic profile changes between two groups is tightly linked to a characterization of the reference range one can expect for a control group. Profile variations can be attributed to gender, species, oestrus cycle, diurnal rhythm, strains, or dietary intake. Several studies have documented the impact of these physiological parameters, showing minor impact of oestrus cycle in female rats compared to inter-rat variation [30], or characterizing the bimodal distribution of a genetically homogenous population of Sprague-Dawley rats based on low urinary hippurate with concomitant increase in hydroxyphenyl propionic acid (3-HPPA) and 3-hydroxycinnamic acid (3-HCA) [31]. By taking this investigation further, antibiotic treatment was used to demonstrate that differences in urinary excretion of 3-HPPA was due to intestinal microflora [32]. Strain differences were also reported for urine spectra of Han Wistar and Sprague Dawley rats, highlighting the impact of polymorphism on a urinary profile [33]. The complex mixture found in plasma (lipoproteins, proteins, low molecular weight metabolites and electrolytes) has always presented a challenge for quantitation by NMR. This can be overcome by using spectral simplification by NMR diffusion. Metabolite quantitation correlated with ultra centrifugation results, can also bring a new alternative for biomarker work in plasma [34]. Most physiological variations are superseded by toxicicological events, giving metabonomics a large dynamic range to document various types of toxicities. When reviewing the literature by target organ, pre-clinical toxicity biomarker studies are heavily skewed towards liver and kidney toxicity, with less biomarker work devoted to other organs (CNS, cardiovascular, ocular, etc.). Studies summarized below represent the various applications of metabonomics, the type of results that may be expected, and how far the data can be pushed toward mechanism elucidation, or metabolic pathway interpretations.

4.2.2. Drug-induced hepatic toxicity

Detection of drug-induced phospholipidosis is very difficult by conventional biochemical procedures. Using metabonomics, authors have shown that administration of two cationic amphiphilic drugs (CAD) induced foamy alveolar macrophages (lung phospholipidosis) in rats, which resulted in urinary increase of phenylacetylglycine (PAG) with decreases in citrate and 2-oxoglutarate. The mechanism by which PAG is formed as a result of phospholipidosis is unclear [35]. The association of increased urinary PAG with phospholipidosis was later confirmed for three other CADs administered in rats, one targeting the lungs (amiodarone), one targeting the liver (chloroquine) and one targeting both (DMP 777). Because histopathology reported minimal phospholipidosis in these cases, hence early onset of the toxicity, monitoring PAG as a biomarker of phospholipidosis in rat urine appears to be a promising development [36].

Taurine elevation has often been proposed as a biomarker of liver toxicity, as recently corroborated by urine spectra of rats dosed with CCl_4, known to induce centrilobular necrosis with steatosis (fatty liver) [37]. Liver steatosis is also a major toxicity after hydrazine administration, for which serum NMR revealed increases of citrulline and 2-amino adipic acid, decrease of lipoproteins, lipids, glucose and choline, and increases of alanine, valine and tyrosine [38]. These results corroborate the mechanistic hypothesis that steatosis perturbs the following pathways: lipid transport from the liver, lipoprotein regulation and β-oxidation of fatty acids.

Peroxisome proliferation (PP) is another case for which metabonomics yielded two potential novel biomarkers. Because many lipid-lowering drugs can induce PP, the pharmaceutical industry has looked for early biomarkers that can detect PP in rats and mice. Although there is little evidence of significant PP in humans, the assessment of human hepatic responses to drugs like PPARα agonists is hampered by the lack of PP biomarkers, as well as a limited understanding of the mechanism of PP in rodents. The two biomarkers of PP identified in plasma and urine of Wistar Han rats were N-methylnicotinamide (NMN) and N-methyl-4-pyridone-3-carboxamide (4PY), both end products of the tryptophan-nicotinamide adenine dinucleotide (NAD+) pathway. Urinary NMN and 4PY were increased 24- and 3-fold, respectively, with a correlation between NMN and peroxisome count of $r = 0.87$ ($r^2 = 0.76$) [39]. Metabonomics data was further exploited to build a PLS predictive model (*see Section 3*) for PP and successfully applied to classify samples from another strain, Sprague-Dawley, dosed with a PP model drug, fenofibrate [40].

Combination of technologies and techniques can provide complementary data for well-known therapeutic agents, such as acetaminophen. By dosing mice with the drug, and acquiring data on intact liver tissues by HR-MAS (*see Section 2.1*), plasma by ^{1}H-NMR and lipid-soluble tissue extracts by 2D-NMR, it was then possible to more completely understand metabolic changes, including decreases in hepatic glucose and glycogen, increase in lipid content, increases in glucose, pyruvate, acetate and lactate in plasma, and increases in alanine and lactate in tissue extracts. Additional gene chip array data also provided context to better understand metabolic pathway changes [41]. Other biomarkers of liver toxicity are included in the Biomarker Summary Table 1.

4.2.3. Drug-induced nephrotoxicity

Metabonomics has also provided biomarkers associated with drug-induced nephrotoxicity. It is now well documented that one can locate the renal lesions by following the urinary profile of treated animals. As summarized in Table 1, it is possible to differentiate lesions to the S1 and S3 segments of the renal cortex, renal proximal tubules, the renal papilla, renal medular damage, and renal glomerular region.

Ischemic reperfusion injury associated with kidney retrieval, cold storage and graft survival has also been studied by metabonomics because it not only induces proximal tubular lesions, but it may be prevented by trimetazidine. This drug acts by protecting the mitochondrial function. A kidney transplant study in pigs showed that the most relevant NMR metabolites for evaluation of renal function after

Table 1. Summary of metabonomics biomakers

Species	Metabonomics Biomarkers	Associated Toxicity	Ref.
Rat	↑ urinary excretion of taurine and creatine	Liver lesions	[50]
Rat	↑ urinary excretion of taurine & creatine associated with bile aciduria	Liver lesions with cholestatic lesions	[23,51]
Rat	↑ serum creatine	Liver (hepatocellular necrosis, steatosis, cholestasis)/Testicular lesions/ Reduced food intake/fasting	[52]
Rat	Altered serum/plasma lipoproteins (changes in broad alkyl features)	Liver	[52]
Rat	↑ N-methylnicotinamide (NMN) & N-methyl-4-pyridone-3-carboxamide (4PY)	Peroxisome proliferation	[39,40]
Rat	↑ urinary glucose and organic acids	Damage to S3 segment of kidney renal cortex	[53]
Rat	↑ phenylacetylglycine (PAG) and DMG	Phospholipidosis, liver necrosis	[35,36]
Rat	TMAO perturbation, dimethyl glycine (DMG), Dimethyl amine (DMA, succinate)	Renal papillary lesions	[52]
Rat	Early ↑ of urinary TMAO, DMA, methyl amine, betaine. ↓ dimethylglycine, citrate, β-hydroxy butyrate, α-ketoglutarate (ketone bodies). Sometimes aceto acetate & acetone	Renal papillary necrosis	[53,54]
Rat	↑ glucose, amino acids. Sometimes ↑ in organic acids, or lactate, or ↓ citrate, succinate.	Renal proximal tubule	[44,55]
Pig	↑ urine TMAO, DMA, acetate	Renal medullar damage by ischemic reperfusion injury	[42]
Rat	↑ glucose, acetate, TMA, succinate. ↓ TMAO, kynuric acid, xanthurenic acid, citric acid, riboflavine	Cyclosporine A nephrotoxicity	[43]
Rat	Lower hippurate & ↑ 3-HPPA	Gut microflora	[31,32]

Table 1. Continued

Species	Metabonomics Biomarkers	Associated Toxicity	Ref.
Rat	Appearance of ketone bodies in the plasma: 3 hydroxybutyrate & acetoacetate	Fasting/reduced feeding /ketosis	[52]
Rat	↓ alanine & lactate concomitant w/ ketone bodies in plasma	Gluconeogenesis	
Rat	Appearance in urine of medium chain dicarboxylic acids (suberic acid, sebacic acid, pimelic acid). ↑ taurine & ↓ citrate, succinate, 2-oxoglutarate & hippurate.	Fatty acid metabolism impairment/liver toxicity	[56]
Human	Appearance of urinary salicylic acid (~75%), salicyluric acid (~20%) and gentisic acid (~5%)	Lysine acetylsalicylate poisoning	[49]
Human	Appearance of urinary valproyl-O-glucuronide	Valproic acid poisoning	[49]
Human	Appearance of paraquat (dimethyl bypyridylium ion) in urine	Paraquat poisoning	[49]
Human	Appearance of THF and 4-hydroxybutyric acid, and lactate in urine and plasma	Tetrahydrofuran (THF) poisoning	[49]
Human	Appearance of ethylene glycol and glycolic acid in urine	Ethylene glycol poisoning	[49]

transplantation were citrate, dimethylamine, lactate, and acetate in urine, as well as TMAO in both urine and plasma [42]. Early detection of impaired renal function to assess graft viability will increase chances of graft success by providing decision-making results to the clinician. Cyclosporin A induced-nephrotoxicity [43] has also been studied in rat urine by NMR and MS. From day 6 onward, urinary changes observed by NMR data included a pronounced glucosuria with high concentrations of acetate, succinate, and TMA, and reduced concentrations of citrate, α-keto-glutarate, and TMAO. Urine was analyzed by positive and negative ion modes and revealed a reduction in kynurenic acid, xanthurenic acid, riboflavin, and large variations of 3-hydroxyphenylpropionic acid sulfate, hippuric acid, and indican during the course of the study. The information derived from these complementary techniques (NMR and MS) is evidenced by the limited overlap between the two sets of biomarkers, thus increasing the quantity of information obtained.

In another study, HPLC-MS demonstrated that exposure of rats to D-serine resulted in significant damage to the renal proximal tubules. Urinary changes

included increases in numerous amino acids (proline, betaine, methionine, leucine, isoleucine, phenylalanine, tryptophan), glycerate, lactate, and decreased levels of creatinine, xanthurenic acid, methylsuccinic acid, and sebacic acid [44]. MS identified several metabolites not previously detected by NMR.

4.2.4. Drug-induced cardiovascular injury

Drug-induced vascular lesions have represented a great area of interest for the pharmaceutical industry because of the unknown significance of animal findings to humans. Vascular injury is currently a cause of attrition for phosphodiesterase inhibitors (PDEs) and has recently generated a gathering of FDA representatives, pharmaceutical and academic scientists (Vascular Injury Symposium, Washington D.C., Dec 2004).

The aim was to better understand the mechanisms involved in animal vascular injury, assess the corresponding relevance to humans and the usefulness of new technologies for finding biomarkers. Administration of a PDE4 inhibitor (CI-1018) with or without dexamethazone (anti inflammatory) in rats was performed with the intent to separate inflammation markers from vascular injury markers. Results showed that the separation observed between the control group and the PDE4 group was not due to inflammation, since the dexamethazone group clustered with the PDE4 group. Changes in the PDE4 group included ketone bodies (Table 1), and changes in Krebs cycle intermediates (citrate, 2-oxoglutarate, succinate) as well as taurine, hippurate and TMAO [45].

4.3. Clinical safety biomarkers

Metabonomics has been used for many years to investigate inborn errors of metabolism, disease biomarkers, and to some extent to diagnose drug toxicity. Because drug safety is mainly assessed and tested in pre-clinical studies, the body of work related to toxicity studies in human subjects is not as extensive. The technology has been used in humans to diagnose drug poisoning, to follow kidney transplant survival, evaluate liver and kidney toxicities, and document xenobiotic metabolism. Consequently, the first step is to assess the variability in human populations.

4.3.1. Assessing baseline physiological reference ranges

To assess the feasibility of metabonomics in clinical trials a study was performed by sampling urine and plasma of healthy individuals on two separate days. The plasma data revealed little variability between subjects and study days. Urinary data showed considerable inter-subject variability but little intra-subject variation [46]. Similarly, to assess the feasibility of clinical trials across different countries, scientists analyzed urine from males and females in two groups of British subjects and Swedish subjects. Data revealed high TMAO excretion in Swedish subjects and high taurine excretion, likely due to the Atkins diet, documenting that urinary profile is

clearly subject to cultural and dietary influences. Great care needs to be taken in interpretation of biomarkers of disease and response to drug therapy [47].

4.3.2. Xenobiotic-induced toxicity in humans

Scientists have recently studied ibuprofen in human plasma to characterize its interactions with plasma lipoproteins. Analysis revealed interactions of the drug with phospholipids and lipoproteins (phosphatidylserine, phosphatidylcholine, sphingomyelin), olefinic chains, and $(CH_2)_n$ and CH_3 groups from unsaturated lipids and lipoproteins [48]. Metabonomics can be applied to a large variety of xenobiotics in acute case poisoning. A recent human study reviews the urinary markers of toxicity characterisitic of acetylsalicylic acid, valproic acid (used in the treatment of epilepsy), paraquat (pesticide), tetrahydrofuran (solvent), alcohol and glycols (solvents, antifreeze) (*see Table 1 for biomarkers*) [49].

5. CONCLUSIONS AND FUTURE DIRECTIONS

Both MS and NMR techniques have demonstrated value towards metabonomics investigations and an integrated approach will provide a holistic metabolic profile of biomatrices. Though individual efforts are currently ongoing, future developments will demand construction of merged databases of metabolites and associated pathways, containing both NMR- and MS-derived data that will rapidly identify the thousands of metabolites present in biomatrices. By correlating metabolic profiles with metabolic pathways, these metabolite databases will provide a deeper understanding of the associated toxicology/pathology. These correlations are currently being manually conducted and represent a time consuming task. Ideally, vendors still need to develop automated processing software that will deliver identification and quantitation of all metabolites present in a biomatrix.

Metabonomics best reflects a phenotypic metabolic status and therefore has great potential to generate valuable data for a ''systems biology'' approach. However, the integrated approach of analyzing data from each technology is still being developed. The best chance of success for building a ''systems biology'' approach may very well reside in the laboratories having simultaneous access to all the technologies (metabonomics, proteomics, genomics) and expertise (chemometrics, biostatisitics, software development, biochemistry, …). In the meantime, metabonomics still aims at reliably providing biomarker panels that can be translated into diagnostic tools. Because most drug safety assessment is still being done in animal studies, an obvious future orientation should be to build a robust translational link between the identified pre-clinical biomarkers and the corresponding human biomarkers. This parallel approach to biomarker development would also answer the recurrent question of relevance of pre-clinical toxicity findings to humans. However, because metabonomics looks at a phenotypic profile in biofluids, one cannot assume that similar profile changes in animals and humans, mean similar etiologies, or pathways. Additional steps should be taken to document and

validate pathway hypothesis before translating markers to the clinic. There is an unquestionable need for more clinical biomarkers that can be utilized widely as diagnostic tools.

In the pharmaceutical industry, the true utility of metabonomics will continue to be evaluated on its ability to clearly impact drug development programs. This will be accomplished by facilitating lead prioritization, provide toxicity screening for better selection of backups, generate novel biomarkers that can return a stalled compound, or advance a new one to development. From a business perspective, successful impact of metabonomics on the pharmaceutical portfolio will determine future investment, support and sponsorship for this rapidly evolving field.

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Computational Prediction of Blood-brain Barrier Permeation

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Contents

1. INTRODUCTION

At the turn of the century, there were an estimated 35 million people aged 65 or over in the USA. This figure is expected to increase to 71.5 million by 2030 as the ''baby boom'' generation joins this segment of the population [1]. Similar trends are anticipated elsewhere in the developed world. This demographic shift is having profound implications for healthcare, given that disorders of the central nervous system (CNS) increase markedly in frequency after the age of 65. Consequently, CNS drugs currently represent the fastest growing segment of the pharmaceutical market and are predicted to account for 20% of blockbuster sales by 2007. There is thus a clear incentive and challenge for the pharmaceutical industry to discover and develop novel therapeutics to meet this burgeoning medical need.

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2. THE BLOOD-BRAIN BARRIER

For a drug to exert a therapeutic effect at a CNS target, it must be able to cross from the systemic circulation into the CNS. There are two interfaces at which this may occur: the blood-brain barrier (BBB) and the blood-cerebrospinal fluid barrier. Given that the surface area of the former (some $20\,m^2$ [2]) is approximately 1000 times greater than that of the latter, the BBB represents the primary interface for solute exchange between the CNS and the systemic circulation [3]. There are two principal aspects of the BBB that make it a formidable hurdle for prospective CNS drugs. First, in terms of its morphology, the BBB consists of endothelial cells that are connected by complex tight junctions that effectively eliminate the possibility of paracellular transport. Second, the same cells express a multitude of metabolic enzymes and efflux transporters whose roles are to transform and remove compounds from the brain [4–6]. This combination of physiological and biochemical barriers prevents the brain uptake of over 98% of all potential neurotherapeutics [2].

In an increasingly time- and cost-conscious industry, the early estimation of the BBB permeation of drug candidates is vital in prioritising compounds for further development. In the case of CNS-targeted drugs, signs of good BBB permeation will be sought; conversely, for systemically targeted drugs, minimal BBB permeation will help reduce the likelihood of CNS side-effects, such as the sedative effect observed in early generation anti-histamines [7]. For this reason, there has been great interest in recent years in the computational prediction of BBB permeation, which offers the possibility of assessing compounds even prior to synthesis. In this chapter, we survey the state-of-the-art in BBB permeation prediction and outline some of the current issues facing practitioners in this field. Other recent reviews may also be of interest [8–12].

3. EXPERIMENTAL BLOOD-BRAIN BARRIER PERMEATION DATA

In this section, a brief overview will be given of the main types of experimental blood-brain permeation data that are available for predictive modeling. A more detailed review of the *in vitro* and *in vivo* methods used to generate such data has been published elsewhere [13].

3.1. *In vivo* data

To date, the type of data most commonly used for generating BBB permeation models has been logBB, where logBB = log ([brain]/[blood]). There are logBB data for approximately 150 compounds in the public domain [14]. LogBB is determined at steady-state or by calculating the area under the brain and blood concentration curve [15]. However, recently, logBB has been severely criticised as a measure of brain permeation, particularly because the brain concentration it employs is the sum of the bound and free drug concentrations [10,15–17]. Thus, it is quite possible

that, due to extensive binding to brain tissue, a compound with a high logBB value may in fact have a lower free concentration in the brain than a compound with a lower logBB value. For this reason, it has been strongly recommended that future BBB permeation models be based not on logBB, but on logPS [17]. LogPS is measured using a short-duration vascular perfusion method from which a permeability-surface area product is calculated (hence logPS), which is a measure of the rate of transfer of the compound from the blood to the brain. Unlike logBB, which measures partitioning into whole brain, logPS is a true permeability measure and, in principle, is not confounded by binding to plasma and brain tissue [15]. It may thus be expected that in the coming years, a new generation of BBB permeation models will emerge that is based on logPS, rather than logBB, data.

3.2. *In vitro* data

The quest for an *in vitro* model of the BBB has been likened to that for the Holy Grail [18]. Although several different culture cell systems have been developed to assess brain permeation – the most well-known being the BBMEC (Bovine Brain Microvessel Endothelial Cell) system – all of these suffer from the fact that cultured cells exhibit a severe downregulation of transport functions and/or that they express different subtypes of multi-drug resistance proteins compared to the *in vivo* situation [19]. Despite this, it has been shown that co-culturing brain endothelial cells with astrocytes can produce a model that has many of the characteristics of the *in vivo* BBB and can provide permeability data that correlate well with *in vivo* data [20]. For the future, the hope remains that conditionally immortalized cell lines may yield models with a better reproduction of the *in vivo* characteristics of the BBB [19].

In the meantime, other *in vitro* systems that are not reliant on cerebral cells are being employed to give an early, if approximate, indication of the brain penetrating properties of discovery compounds. MDCK (Madin Darby Canine Kidney) cell monolayers have been proposed as a potential model for the BBB based on their high TEER (Transendothelial Electrical Resistance) value and low permeability to sucrose. However, significant concerns remain over their non-cerebral origin, *inter alia*, and these are likely to inhibit the use of this cell-line for estimation of BBB permeation [13,19]. Notable among the alternatives is PAMPA-BBB [21], a modification of the original PAMPA (Parallel Artificial Membrane Permeability Assay) screen developed for predicting intestinal permeability [22]. Other workers have experimented with surface-activity profiling of compounds and shown that the measurements correlate well with passive permeation of the BBB [23]. In summary, the development of a reliable and realistic *in vitro* BBB permeation model is still ongoing. As a consequence, to date, there are very few *in vitro* BBB permeation data in the public domain for modeling and no models of BBB permeation have been published based on such data.

3.3. Surrogate measures

Owing to the paucity of *in vivo* and *in vitro* BBB permeation data available upon which to base predictive modeling, some workers have turned to the use of a

surrogate measure of brain permeation: CNS activity. Typically, this involves the mining of databases such as the CMC (Comprehensive Medicinal Chemistry) and/or MDDR (MDL Drug Data Report) and selecting compounds that are active at a CNS target to form a CNS^+ (brain permeating) class and using the remainder to form a CNS^- (non-brain permeating) class. In one report, such an approach yielded CNS^+ and CNS^- sets comprising over 15,000 and over 50,000 compounds respectively [24]. The primary concern with the use of CNS activity as a surrogate for brain permeation is that while the CNS^+ class manifestly cross the BBB, the converse is not necessarily true (''the absence of evidence is not evidence of absence''). In other words, a compound may permeate the BBB but not show activity at any monitored CNS target.

4. COMPUTATIONAL ESTIMATION OF BLOOD-BRAIN BARRIER PERMEATION

The computational models for BBB permeation that have been developed in recent years can be grouped into three classes. First, there are simple ''rules of thumb'' that have been derived by examining the molecular properties of compounds that do and do not cross the BBB. Second are classification models that typically predict whether or not a compound is a BBB permeator. The final class comprises models predicting continuous values of BBB permeation based on either logBB or logPS data.

4.1. Rules of thumb

The great popularity of the ''rule-of-5'' for predicting poor permeability and solubility demonstrates the appetite of medicinal chemists for mnemonics based on simple, interpretable molecular properties [25]. Thus, it is not surprising that similar ''rules'' for predicting BBB permeation have emerged in recent years. These are summarised below.

- If the sum of nitrogen and oxygen atoms (N + O) in a molecule is five or fewer, then the molecule has a high chance of permeating the BBB [8].
- If ClogP − (N + O) > 0 for a molecule, then the molecule's logBB is likely to be positive [8]. Here, ClogP is logP computed using the Biobyte software distributed by Daylight Chemical Information Systems Inc., Mission Viejo, CA.
- The polar surface area (PSA) of a molecule has been shown to be a key determinant of BBB permeation [26]. Upper limits for PSA of 60–70 $Å^2$ and 90 $Å^2$ have been suggested if good BBB permeation is to be achieved [26,27].
- Molecular weight (MW) should be kept below 450 Da. [27].
- A logD value in the range 1–3 is recommended [27]. One comparative study found the mean calculated logD for 48 CNS drugs to be 2.08 compared to 1.07 for 45 non-CNS drugs [28].

- A modification of the rule-of-5 for CNS permeation has also been reported: MW < 400, ClogP < 5 (MlogP < 4, where MlogP is logP computed using the method of Moriguchi [29]), number of hydrogen-bond donors < 3, number of hydrogen-bond acceptors < 7 [30].

Overall, compared to their non-CNS counterparts, CNS drugs tend to be more lipophilic, more rigid, have lower molecular weights, fewer hydrogen-bond donors and acceptors, fewer formal charges (especially negative charges) and lower PSA values [28,31].

4.2. Classification models

A recent review of classification models for BBB permeation concluded that, typically, the CNS^+ class could be predicted with greater than 80% accuracy, while the CNS^- class was generally less accurately predicted, possibly because of the uncertainties in its definition [32]. Some papers published subsequently have borne out these observations. In the first, a logBB model was used to predict the CNS activity classes of two test sets of compounds using logBB < -1 as the definition of the CNS^- class and logBB $\geqslant -1$ as the CNS^+ class [33]. Across the two test sets, the prediction accuracies for CNS^+ were 85.7% and 71.7% respectively. For CNS^-, the corresponding results were 46.7% and 64.0%. Raising the logBB cutoff value to -0.75 or -0.4 led to an increase in the accuracy of prediction for the CNS^- class but only at the expense of the accuracy of predicting the CNS^+ class. In general for this kind of application, the accurate prediction of CNS^+ is most important to avoid losing potentially useful compounds through ''false negative'' predictions.

In other work, a linear discrimination analysis model was derived from a training set of 302 compounds, 150 of which were classed as CNS^+ and 152 as CNS^- [34]. The model comprised TOPS-MODE topological descriptors encoding hydrophobicity, polar surface area and dipole moment. In the discriminant function, increasing hydrophobicity was positively correlated with CNS activity, while an increase in either of the polarity-related descriptors was correlated with CNS inactivity. For the training set, the derived model was able to classify 247/302 compounds (81.79%) correctly. When applied to an external test set of 78 compounds (39 CNS^+, 39 CNS^-), the overall classification accuracy was 80.77%. In both cases, the prediction accuracy was greater for the CNS^+ class than the CNS^- class, in keeping with previous observations.

Finally, a large and carefully chosen set of 1696 compounds has been assembled based on activity classes in the World Drug Index database. The set comprised 1336 BBB-crossing (BBB^+) and 259 non-BBB-crossing (BBB^-) molecules together with 91 substrates for P-glycoprotein (PGP^+, also classed as BBB^-) [35]. From these data, a variety of discriminant analysis models for BBB permeation was derived. It was shown that a simple consideration of the number of heteroatoms in a molecule led to a 92% correct classification rate. Most BBB^- compounds have more than eight heteroatoms while most BBB^+ compounds have fewer than nine. It should be

noted that in this work, phosphorus, sulfur and the halogens were included as heteroatoms, which is why this "rule" differs from the rule mentioned earlier [8], which included only nitrogen and oxygen atoms. A model for separating P-glycoprotein substrates and non-substrates was also derived and can be used in conjunction with the BBB prediction model to give additional information to aid CNS drug design.

4.3. logBB models

A host of logBB prediction models has been published in the last decade and many of these have been reviewed previously [8–11,32]. It is apparent that given the publicly available data, the current generation of models seems to be converging, with models exhibiting similar statistical quality, even when derived using quite different descriptors and model building techniques. In this section, some of the more recent work in the area of logBB prediction will be summarised.

The TOPS-MODE descriptors mentioned above have also been applied to the modeling of logBB data [36]. From a training set of 114 compounds and associated logBB data, the following equation was obtained by linear regression:

$$\log \mathrm{BB} = -0.032 - 0.046 \times 10^{-3}\, \mu^{\mathrm{PS}} \cdot \mu^{\mathrm{AM}} + 0.227 \times \mu^{\mathrm{H}}$$

$$N = 114,\ \ r^2 = 0.71,\ \ s = 0.42,\ \ F = 127.8$$

where the variables μ^{PS}, μ^{AM} and μ^{H} are related to PSA, molecular weight and hydrophobicity, respectively. N is the number of compounds used to derive the model, r^2 is the fraction of the variance explained by the model, s is the standard deviation of the regression and F is the Fisher value, a measure of statistical significance. The negative dependence of logBB on hydrogen-bonding capacity (represented by PSA) and the positive dependence on lipophilicity are commonly observed in logBB models [32]. The paper also includes a useful table comparing a number of logBB models published previously.

Two recent papers have reported the application of neural networks to logBB modeling. A Bayesian neural network was used to model the relationship between an 85-compound logBB data set and a set of computed molecular property descriptors including counts of hydrogen-bond acceptors and donors, hydrophobes, rotatable bonds, logP, MW and PSA [37]. The model with the best statistics ($r^2 = 0.81$, $s = 0.37$) was obtained using four nodes in the hidden layer. Applying this model to a 21-compound test set gave rise to a q^2 (cross-validated r^2) value of 0.65 (standard error of prediction = 0.54). The statistics for the training and test sets are typical of what is observed in high-quality logBB models [32]. Applying automatic relevance determination to the whole data set showed that the most important descriptor in the model was logP, closely followed by the count of rotatable bonds and PSA. Notably, the model showed that hydrogen-bond donors had a greater influence on brain permeation than hydrogen-bond acceptors. This has been observed in various absorption/permeability-related models and it has been hypothesised that this is due to the favourable interactions that can occur

between hydrogen-bond donors and the ester moieties located within lipid headgroups [38]. In other work, a neural network of the backpropagation variety was used in conjunction with CODES topological descriptors to create a logBB model [39]. However, this model was based on a rather small training set (36 compounds) and was less extensively validated and interpreted than the model derived using the Bayesian neural network.

Finally, a novel and interesting approach using 4-D molecular similarity measures and cluster analysis to partition a logBB data set into subsets has been reported [40]. Each subset of compounds was then further divided into training and test sets from which QSAR models were derived using a large pool of molecular descriptors together with the genetic function approximation approach for model building [41]. The idea underlying this approach is that it can be difficult to construct an optimal QSAR model from a very heterogeneous training set. Thus by splitting the training set into smaller, more homogeneous clusters, better models can be obtained. The prediction of previously unseen compounds then requires the additional step of discovering which cluster it should belong to and then using the appropriate QSAR model to predict its logBB value. In this case, the data set was divided into three subsets and it was shown that the r^2 and q^2 values for the subset models were superior to those for a model derived from the whole data set.

4.4. logPS models

With the recently expressed concern over the utility of logBB data for brain permeation modeling, it is timely that two papers have appeared recently describing logPS models. The first of these used a training set of logPS data for 30 neutral compounds and derived the following model using Abraham's solute descriptors [42]:

$$\log PS = -0.639 + 0.312\,E - 1.009\,S - 1.895\,A - 1.636\,B + 1.709\,V$$

$$N = 30,\ r^2 = 0.87,\ s = 0.52,\ F = 32.2$$

where E is an excess molar refraction, S is dipolarity/polarisability, A is the hydrogen-bond acidity of the compound, B is the hydrogen-bond basicity of the compound, and V is its characteristic (McGowan) volume. The small size of the available data set did not allow for the creation of an independent test set, so there is no measure of the predictive power of this equation. However, what is reassuring is that the equation has many similarities with an analogous model developed from logBB data [14]:

$$\log BB = 0.021 + 0.463\,E - 0.864\,S - 0.564\,A - 0.731\,B + 0.933\,V - 0.567\,I$$

$$N = 148,\ r^2 = 0.75,\ s = 0.34,\ F = 69$$

With the exception of the indicator variable for carboxylic acids (I), the two equations show a similar dependence on the various descriptors. This suggests that what has been learned about brain permeation from logBB modeling is still useful, at least in a qualitative sense.

The second logPS model was developed from data for 23 compounds presumed to permeate the BBB by passive transport [15]:

$$\log PS = -2.19 + 0.262 \log D + 0.0683 \text{ vsa\_base} - 0.009 \text{ TPSA}$$

$$N = 23, \quad r^2 = 0.74, \quad s = 0.5, \quad F = 18.2$$

where log D (at pH 7.4) was calculated by ACD/LogD software (Advanced Chemistry Development, Inc., Toronto, ON, Canada), vsa_base is the van der Waals' surface area due to basic atoms and TPSA is the topological polar surface area – the latter descriptors being computed by MOE (Chemical Computing Group, Montreal, QB, Canada). Here again, there is a reassuring concordance with previous logBB models that have included a positive dependence on lipophilicity and a negative dependence on hydrogen-bonding capacity [32]. This model was evaluated by applying it to the prediction of two small external test sets each comprising 12 compounds. For one set, a good agreement between the absolute values of predicted and experimental logPS was observed. For the other, the model was able to rank the compounds successfully, although there was poorer agreement between the absolute values. As well as being useful for the prediction of passive BBB permeation, the model may also assist in the early identification of compounds that are substrates for active transporters (experimental logPS $\gg$ predicted logPS) or efflux systems (experimental logPS $\ll$ predicted logPS).

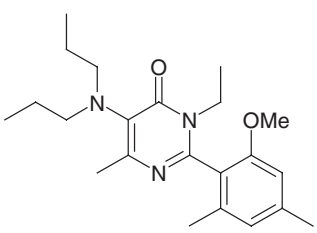
These early models show promise for the prediction of logPS and suggest that currently available descriptors are able to capture the information in the data successfully. Larger data sets will enable the development of more generally applicable and robust models with increased applicability for drug discovery.

4.5. Case studies

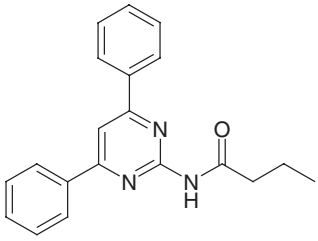
Given the inevitable time lag between medicinal chemistry projects being carried out within the pharmaceutical industry and their reaching the literature, it is not surprising that there are not yet many published examples of the application of computational BBB permeation models. However, some are beginning to emerge suggesting that such calculations are being used quite widely within drug discovery projects.

The search for non-BBB-permeating kappa opioid agonists was guided, at least in part, by reference to computed parameters [43]. In particular, the aim was to reduce lipophilicity by incorporating polar substituents while still maintaining efficacy at the desired receptor. Another project aiming to develop corticotrophin-releasing factor-1 antagonists used PSA and MlogP to suggest that compounds should be membrane permeable and brain penetrating [44]. Compound **1** (PSA $= 40.4\,\text{Å}^2$, MlogP $= 3.52$) was shown to have a brain-to-plasma ratio of 0.9 ± 0.4 mL/g indicating approximately equal partitioning between brain and plasma (in this instance the brain/plasma ratio was obtained by quantifying the amount

of compound in the brain (in ng/g of tissue) and in plasma (in ng/mL) giving a ratio units of mL/g [45]). A computational model embodied in the VolSurf software (Molecular Discovery, Ponte San Giovanni, Italy) was used to predict favorable BBB permeation for a series of prodrugs of non-steroidal anti-inflammatory agents targeted against Alzheimer's disease [46]. Most recently, a set of adenosine A_1 receptor antagonists has been designed explicitly making use of one of the rules of thumb mentioned earlier: PSA < 60–70 Å^2 [47]. One particular compound (**2**) emerging from this project combined high affinity for the hA_1 receptor ($K_i = 4nM$) with good selectivity over the hA_{2A}, hA_{2B} and hA_3 receptors (7%, 54% and 38% inhibition respectively at 1μM) and a PSA value of 53 Å^2.



1



2

5. MOLECULAR DETERMINANTS OF BLOOD-BRAIN BARRIER PERMEATION

From the modeling studies reported in this paper and others conducted over the years, a picture is gradually emerging of the molecular determinants of BBB permeation. Here, a brief overview will be given – more details can be found in [32].

5.1. Lipophilicity

The influence of lipophilicity in BBB permeation has been appreciated for many years. For instance, it was suggested in the early 1970s that the optimum logP value for diffusion into the CNS was around 2 [48]. Many BBB permeation models contain terms relating to lipophilicity and invariably, these suggest that increasing lipophilicity is favorable for BBB permeation [9].

5.2. Hydrogen bonding capacity

One of the earliest indications of the role of hydrogen-bonding in BBB permeation came in a seminal paper that suggested a linear relationship between logBB and $\Delta\log P$ ($= \log P_{oct} - \log P_{cyc}$, considered to be an approximate measure of the hydrogen-bonding capacity of a molecule) for 20 histamine H_2 receptor antagonists

[49]. Since then, the impact of hydrogen-bonding on brain penetration has been widely recognised and many BBB permeation models now contain terms showing the detrimental effect of increasing hydrogen-bonding capacity.

5.3. Molecular charge

It has been observed that compounds with a pKa value <4 or >10 are poor permeators of the BBB [50]. The particularly poor BBB permeating behavior of carboxylic acids has also been noted in a QSAR model in which an indicator variable with a negative coefficient was included [14]. Conversely, the possession of a positive charge at pH 7–8 has been shown to favor brain permeation [51].

5.4. Molecular size

It has been suggested that MW should be kept below 450 Da. to facilitate brain permeation [27] and certainly, as a class, CNS drugs tend to be of lower molecular weight than compounds from other therapeutic areas [31]. Two early logBB models built from relatively small training sets contained molecular volume or mass terms with negative coefficients, suggesting that increasing molecular size is detrimental to BBB permeation [52,53]. However, the opposite has been suggested more recently by two independent BBB permeation models containing molecular volume terms with positive coefficients [14,54].

5.5. Molecular shape

Two BBB models have suggested that a spherical, rather than rod, shape is favorable for BBB permeation [51,55]. However, it has also been reported that increased molecular branching hinders brain penetration [56], which would seem to be contradictory.

5.6. Molecular flexibility

Two studies comparing CNS and non-CNS drugs have concluded that the former tend to be less flexible than the latter [28,31]. The count of rotatable bonds was found to be an important descriptor in a recent neural network logBB model [37], but it was not stated whether its contribution was positive or negative. A different study concluded that increasing solute flexibility was favorable for BBB permeation [57].

Overall, then, it would seem that clear guidance for molecular design can be given in terms of lipophilicity, hydrogen-bonding capacity and molecular charge but that the influence of molecular size, shape and flexibility on BBB permeation is still somewhat unclear.

6. CURRENT ISSUES AND FUTURE DIRECTIONS

Probably the largest obstacle in the path to better predictions of BBB permeation is the paucity of *in vivo* brain permeation data, especially logPS data. For robust and reliable models, data will be required for a large number of drug-like compounds that are diverse in structure and property space and that span a wide range of experimental response. It is unlikely that such a compound collection will come into being by chance – what is required is the bespoke generation of data for modeling. In addition to this, it has to be conceded that we do not yet fully understand the complexities of BBB permeation and there are almost certainly still unknowns to be discovered, particularly active transport systems.

Given the small data sets that are available for BBB modeling, one of the key developments to be expected in the near future is the incorporation of better estimates of the errors accompanying predictions and warnings when a test compound falls outside the model's training set space [58,59]. Work is ongoing in this area [60,61]. Improvements here should lead to more confidence among the user community. More robust predictions should also be facilitated by the increasing use of consensus models, in which multiple predictors are combined often in a "jury-like" fashion. Finally, as more data become available for active transport and efflux systems at the BBB, models incorporating these phenomena in addition to passive BBB permeation should be anticipated.

7. CONCLUSIONS

The prediction of BBB permeation has developed over the last decade into a fascinating area. Over that time, there has been gradual progress in the accuracy of prediction and growing insight into the molecular determinants of BBB permeation that should aid in drug design. Early signs suggest that the current generation of models is being applied to medicinal chemistry projects with some success. In the immediate future, the acceptance of such models by medicinal chemists and other users will be enhanced by better reporting of the errors and limitations associated with predictions. In the longer term, the availability of more logPS data will lead to models of greater accuracy and wider applicability.

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Pharmacogenetics and Drug Development

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Contents

1. INTRODUCTION

A number of factors contribute to variability in drug responses, including pathogenesis and severity of the disease being treated, concomitant illness, age, gender, renal and liver function, and genetic differences among patients. The genetic basis of drug responses emerged in the 1950s through clinical observations. The term ''pharmacogenetics'' was first introduced by Vogel in 1959 [1], two years after this field of study had been conceptualized by Motulsky [2]. During the past decade, scientific efforts have increased to elucidate the genetic basis of interindividual variability in drug responses. Through these efforts, many genetic polymorphisms have been identified that affect drug responses in terms of efficacy and/or safety. Pharmacogenetic studies may focus on particular genes or single-nucleotide polymorphisms (SNPs). Alternatively, pharmacogenetic studies may require a genome-wide analysis, for example by SNP and haplotype mapping [3,4].

2. SCIENTIFIC RATIONALE AND RECENT EXAMPLES

During the past decade, many genetic polymorphisms have been identified, which affect drug responsiveness. Amongst those are polymorphisms in the metabolism and disposition of drugs, polymorphisms in drug transporters, targets of drug therapy, and those predisposing to toxicity. However, several points should be noted before discussing specific examples: (i) A unique feature of DNA, necessitated by its function as the keeper of genetic information, is its biological and chemical stability. In most cell types – important exceptions include tumor cells and the antigen receptor genes in lymphocytes – genetic information is not altered postnatally. Since the information is identical in most cell types, DNA sampling for analysis of specific genes or whole genomes can often be done by harvesting peripheral blood mononuclear cells. However, in cancer patients testing the tumor

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DNA for somatic alterations is often required, rather than testing germ line DNA from peripheral blood mononuclear cells. (ii) While it is possible to study the functional genome by analyzing levels of DNA methylation, epigenetic information is contained in a cell type-specific manner. Similar restrictions apply to the study of RNA or proteins. Since the transcriptomes and proteomes are expressed in a cell type-specific manner, the relevant tissue(s) may not be easily accessible. Patients are also subject to strong non-genetic, environmental influences, which modulate data sets both qualitatively and quantitatively. While metabolomics is a interesting new approach to study interindividual variability of drug responsiveness, the degree to which it can be applied is still uncertain. (iii) Some genetic polymorphisms may result in structural diversity of a gene-encoded protein, affecting its function; other polymorphisms can affect protein levels by modulating its expression and/or stability, and some modulate drug responsiveness through unidentified mechanisms. (iv) Information gathered from pharmacogenetic studies can be correlated with clinical data, including clinical endpoints, surrogate markers, and biomarkers. Example of biomarkers include clinical chemistry, imaging data, and data from electrophysiology, histology, gene array, proteomics and metabolomics studies. (v) Recent experiments suggest that animal models may also prove useful in identifying pharmacogenetic loci. For example, mutations in the fatty acid transporter CD36 influence the insulin-sensitizing action of pioglitazone [5]. Finally, it is likely that humanized animal models will play an increasingly important role in dissecting the role of pharmacogenetic loci and in compound screening [6].

An important and perhaps the most common group of pharmacogenetic traits involves the metabolism of drugs, affecting both Phase I and Phase II enzymes. There are more than 50 human cytochrome P450 enzymes, but only 10 of these isoforms contribute to drug metabolism, with the major contribution coming from 3 isoforms, CYP3A4, CYP2D6 and CYP2C9. Since CYP-450 proteins are the most important drug-metabolizing enzymes, it is not surprising that the metabolism of many drugs is influenced by genetic variation in particular CYP-450 genes [7]. In fact, most cytochrome P450 genes are subject to genetic polymorphism, with certain alleles impacting gene expression or resulting in the production of enzymes with altered catalytic activity or substrate specificity. For example, genotypic variations in CYP2D6 can either increase or decrease the metabolism of debrisoquine. Indeed, individuals may be phenotyped for their CYP2D6 metabolism capacity by administration of debrisoquine and assaying its metabolism. Some of the extensive metabolizers have CYP2D6 gene duplications; not surprisingly, the frequency of gene duplications is distinct in different ethnic populations [8].

Inherited variation in N-acetylation, for example due to genetic variation in the *N*-acetyltransferase-2 (NAT2) gene, accounts for the phenotypic variability in the pharmacokinetics of several structurally diverse drugs, including the antituberculosis drug isoniazide [9], the antiarrythmic drug procainamide [10], and the antihypertensive drug hydralazine [11]. The effects of genetic variation on the molecular properties of the NAT-2 enzyme have been studied in detail [12].

The enzyme Thiopurine *S*-methyltransferase (TPMT) catalyzes the S-methylation of thiopurine drugs such as 6-mercaptopurine, which is used for the treatment of

childhood leukemia. The most common allele of TPMT in Caucasians is *TPMT*3A*, a variant rarely detected in East Asians [13]. The enzyme encoded by *TPMT*3A* is degraded rapidly by the proteasome through the ubiquitin pathway, possibly involving heat-shock proteins [14,15]. Since thiopurines have a narrow therapeutic window, expression of the *TPMT*3A* allele can lead to overdosing, which in turn can cause life-threatening myelosuppression.

It has been estimated that approximately 500–1200 genes code for drug transporters [16]. The best characterized drug transporter is the multidrug resistant transporter MDR1 (also known as ABCB1 and P-glycoprotein), encoded by the *MDR1* gene. MDR1 is a glycosylated membrane protein of 170 kDa and belongs to the ATP-binding cassette superfamily [17]. A number of structurally unrelated drugs are substrates for MDR1, and their intestinal absorption, hepatobiliary secretion, renal secretion and blood-brain barrier permeability are regulated by MDR1. Multiple MDR1 polymorphisms have been described, the frequency of which differ depending on ethnic background. For example, the C3435 T polymorphism in exon 26 has a frequency of 73–84% in individuals of African origin and frequencies of 34–59% in individuals of European and Asian origin [18]. A recent report indicates that MDR1 polymorphisms may play a role in determining the pharmacokinetic and clinical toxicity profile of the HIV protease inhibitor nelfinavir [19].

Many prescribed medications or their metabolites exist as organic anions at physiological pH. These compounds are transported by organic anion transport systems, the best studied of which is the organic anion secretory pathway of the renal proximal tubule. Substrates of this secretory pathway are highly diverse and include such clinically important pharmaceuticals as β-lactam antibiotics, probenecid, diuretics, angiotensin converting enzyme inhibitors, non-steroidal anti-inflammatory drugs, and antiviral nucleosides/nucleotides [20]. The family of organic anion transporters (OATs) currently comprises 8 members, including OAT1-4, URAT1, UST1 and UST3 [20]. In addition to the OATs, other, phylogenetically unrelated, organic anion-transporting proteins, such as the OATPs/OAT-Ks, and the MRP efflux pumps, also contribute to the apical secretion of some small organic anion substrates. Given the importance of organic anion transport, it is not surprising that allelic variants have been identified that lead to interindividual differences in drug responses. For example, certain commonly occurring single-nucleotide polymorphisms have been identified in OATP-C, such as T521C (Val 174Ala), which may affect the pharmacokinetics of pravastatin [21]. Alternatively, the importance of particular residues to OAT function may be estimated from their degree of evolutionary conservation [20]. The applicability of this principle has recently been demonstrated by a study of polymorphisms in OCT2, a member of the OAT-related family of organic cation transporters, wherein the only variants that resulted in decreased function were those that altered conserved residues [22].

It is now generally accepted that genetic variation in drug targets can account for variability in drug responses. Examples include drug targets for asthma, haematological and solid tumor malignancies, diseases of the CNS and cardiovascular

diseases, especially hypertension. The influence of allelic variation in drug targets on the treatment of cardiovascular diseases [23,24] and of diseases of the CNS [25,26] has been recently reviewed. This review will focus on drug targets relevant to asthma and oncology, which may have been the most fruitful areas of pharmacogenetic investigation.

Considerable efforts have been made to elucidate the pharmacogenentics of asthma. The most investigated aspect of this problem has been the response to β agonists, the most commonly prescribed class of asthma medications [27,28]. Efforts to explain differences in responses to β agonists have centered on the gene encoding the β2 adrenergic receptor. The coding variant at position 16 within the *β2AR* gene have been shown to be functionally important. The β2 adrenergic receptor expressing glycine at position 16 exhibits down regulation after agonist exposure *in vitro*, whilst receptors expressing arginine at this position are more refractory to ligand-induced down regulation [29]. In a large multi-center, placebo-controlled, double-blinded trial involving 255 subjects with mild asthma, regular albuterol use was associated with a decline in peak expiratory flow in patients with the Arg/Arg genotype at position 16 [30]. In a recent study on Puerto Ricans with asthma with baseline FEV1 $<$ 80% of predicted, there was a strong association between the Arg/Arg genotype at position 16 and greater bronchodilator responsiveness [31].

ALOX5 (5-lipoxygenase) is another asthma drug target that has been the subject of extensive pharmacogenetic studies. ALOX5 is the enzyme required for the production of both the cysteinyl leukotrienes (LTC_4, LTD_4, and LTE_4) and LTB_4. Individuals with variants of the *ALOX5* promoter, which negatively affect the expression of 5-lipoxygenase, are less susceptible to therapy with 5-lipoxygenase inhibitors [32,33].

The most exciting therapeutic area for pharmacogenetic and diagnostic biomarker exploration has been oncology. An early example of targeted therapy in cancer therapy was the use of tamoxifen in breast cancer patients whose tumors were estrogen receptor-positive. Since then, the regulatory approvals of trastuzumab for patients with HER-2/neu overexpressing breast cancer, imatinib mesylate for bcr/abl translocation-positive chronic myelogenous leukemia and cetuximab for epidermal growth factor receptor (EGFR)-overexpressing colorectal cancer have highlighted the potential for novel, more targeted anticancer therapies. In addition, pharmacogenetic studies have identified mutations in non-small cell lung cancer that correlate with response to gefitinib therapy [34].

The discovery of the HER-2/neu oncogene overexpressing breast cancer was followed by the demonstration that HER-2/neu is a relevant diagnostic biomarker that could be used to guide therapy. The successful development of trastuzumab, a humanized anti-HER-2/neu monoclonal antibody, highlights the potential of pharmacogenetic studies in pharmaceutical R&D. Trastuzumab is a true example of a targeted anticancer therapy since it is only indicated for HER-2/neu-positive tumors; the therapeutic use of trastuzumab thus requires prior testing of the tumor, which is carried out by immunostaining for HER-2 protein or by fluorescence *in situ* hybridization [35].

Treatment with imatinib mesylate, an ATP-competitive inhibitor of the ABL kinase, has become the standard of care for chronic myelogenous leukemia patients with *BCR/ABL* translocation [35]. In addition, imatinib mesylate is used for the treatment of gastrointestinal stromal tumors that feature particular mutations in c-KIT. Patients whose tumors express c-KIT with mutations in exon 11 have a significantly better response rate to therapy with imatinib mesylate (83.5%) than patients whose tumors express mutations in exon 9 (47.8%) [36].

Although imatinib mesylate is effective in treating cancer, some patients relapse with resistant disease. Resistance may develop through several mechanisms, including but not limited to (i) point mutations in the drug target, which lead to higher IC_{50} values for imatinib mesylate, (ii) amplification of the target gene, which upregulates the expression of the target protein, and (iii) increased expression of MDR1 [37]. Mutations in the ABL kinase domain are found in the majority of patients with secondary resistance to imatinib [37]. Such mutations, which cluster in four distinct regions of the ABL kinase domain (the ATP binding loop, T315, M351, and the activation loop), interfere with binding of imatinib to ABL [38–40]. Crystallographic studies of various ABL mutants predict that most should remain sensitive to inhibitors that bind ABL with less stringent structural requirements. Using this insight, new small-molecule inhibitors have been identified that retain activity against the majority of imatinib-resistant BCR-ABL mutants [41–43].

The EGFR plays an important role in the growth of multiple cancers; consequently, it has been for some time an oncology drug target of considerable interest. Cetuximab is a chimeric monoclonal antibody that blocks ligand binding and EGFR-mediated signal transduction [35]. The antibody was approved in 2004 for the treatment of colorectal cancer. Similar to trastuzumab, an expression test was used to define patient eligibility during the development of cetuximab. However, the predictive value of EGFR expression for cetuximab response is still subject to debate [44,45].

The EGFR is also an important target for small molecule drug discovery. Gefitinib, an EGFR kinase inhibitor, was approved in 2003 for the treatment of non-small cell lung cancer (NSCLC). However, in late 2004, the manufacturer announced the results from a recent clinical trial, IRESSA Survival Evaluation in Lung cancer (ISEL), a double blind, placebo controlled, randomized Phase III survival study comparing gefitinib (250 mg) and best supportive care *versus* placebo and best supportive care in patients with advanced NSCLC, who had received and either failed or were intolerant to prior chemotherapy. The results from the ISEL trial were discouraging. The analysis of the primary endpoint of the study showed that gefitinib did not significantly prolong survival in the overall patient population. Based on the results of the study, the manufacturer suspended promotion of gefitinib pending further molecular analysis. A second EGFR kinase inhibitor, erlotinib, was approved more recently. Erlotinib is indicated for the treatment of patients with locally advanced or metastatic NSCLC after failure of at least one prior chemotherapy regimen [46].

The EGFR has become a landmark example of the potential of pharmacogenetic studies. Somatic gain-of-function mutations in exons encoding the EGFR tyrosine

kinase domain are found in about 10% of NSCLCs from the United States. Some 90% of NSCLC-associated mutations occur as either multi-nucleotide in-frame deletions in exon 19, involving elimination of four amino acids, Leu-Arg-Glu-Ala, or as a single nucleotide substitution at nucleotide 2573 (T→G) in exon 21, resulting in substitution of arginine for leucine at position 858 (Leu858Arg). Both of these mutations are associated with sensitivity to either gefitinib or erlotinib [47–49]. Unfortunately, nearly all patients who experience marked improvement on these drugs eventually develop progression of disease. In a recent study, it was shown that tumor cells from three of six patients with acquired resistance to gefitinib or erlotinib, contained, in addition to a primary drug-sensitive mutation in *EGFR*, a secondary mutation in exon 20, which leads to substitution of methionine for threonine at position 790 (Thr790 Met) in the kinase domain [50]. This mutation, which confers resistance to EGFR mutants usually sensitive to either gefitinib or erlotinib *in vitro*, was not detected in untreated tumor samples [50]. It remains to be determined whether other EGFR kinase inhibitors can be identified that are active against the Thr790 Met mutation. Recently, the crystal structure of the EGFR inhibitor lapatinib (GW572016) bound to EGFR was solved [51]. This study revealed that the quinazoline rings of erlotinib and lapatinib interact differently with the EGFR kinase domain, raising the possibility that the Thr790 Met mutation may not affect inhibition of EGFR by compounds similar to lapatinib [50]. Finally, and most importantly, it remains to be determined whether the very recent molecular studies on gefitinib can explain the results obtained in the ISEL and other clinical studies.

Patient safety is a major concern – both during the development and after the launch of a newly approved medication. It is now generally accepted that there are genetic polymorphisms in off-targets, i.e. unrelated to the intended drug target and to proteins affecting the metabolism and transport of compounds, which can result in drug-induced toxicity. Earlier studies had linked dopamine D3 receptor polymorphism with dopamine D2-like receptor antagonist-induced tardive dyskinesia [52] and potassium channel mutations with clarithromycin-induced dysrhythmias [53]. Several recent studies have revealed the association of polymorphic repeats within the UDP-glucuronyltransferase I (UGT1A1) gene with the occurrence of hyperbilirubinemia after administration of the experimental drug tranilast [54]; and the association of the polymorphic variant HLA-B57 with hypersensitivity reaction after administration of abacavir, an approved nucleoside reverse transcriptase inhibitor for the treatment of HIV [55,56]. Together, these studies demonstrate the feasibility of using pharmacogenetic approaches to predict drug-related adverse events [57].

3. PHARMACOGENETIC TRIALS: METHODOLOGICAL AND REGULATORY CONCERNS

There are many practical issues associated with pharmacogenetic trials: Firstly, the *CYP2D6*, *NAT2*, and *TPMT* polymorphisms are monogenic Mendelian traits.

However, as discussed above, multiple proteins – Phase I and Phase II enzymes, drug transporters – participate in the drug uptake, metabolism and excretion of drugs, and the targets of drug discovery are also subject to genetic variation. Moreover, drug responses are influenced not only by genetic, but also by environmental factors. Therefore, simple Mendelian models cannot be applied generally. Secondly, while the application of DNA-based assays promises to make sequence information rapidly available to a treating physician, in practice and in clinical development it may be necessary to study multiple genes simultaneously. It is obvious that the physician is interested in the phenotype rather than the genotype. Since the currently available DNA-based tests fail to reflect the full extent of phenotypic variation, comprehensive genotype-phenotype correlations are still outstanding [34]. Thirdly, not all clinical trials will benefit from a retrospective analysis: The distribution of the phenotypic trait within the patient population must allow for ''binning'' of the patient population, e.g., in a bimodal distribution. Also, the phenotypic trait must be represented in the total sample size at a frequency sufficient to justify a genetic analysis. Therefore, a pharmacogenetic analysis of a clinical trial may or may not be useful. Although this needs to be examined on a case-by-case basis, suitable statistical methods have been developed and will be facilitated by the refinement of halpotype maps [57]. Finally, pharmacogenetics trials also pose logistical questions related to the collection, handling and storage of samples, and to the anonymization and analysis of data.

The regulatory environment is complex and varies between countries. IRBs respond differently to pharmacogenetics studies from country to country. A planned pharmacogenetics element may negatively impact patient enrollment into the trial. Moreover, the positions of regulatory agencies are still evolving. The FDA has a long-standing commitment to ''individualization factors'' and supports pharmacogenetic studies. However, the FDA has also raised several important questions about the genotypic and phenotypic information that should be included in the label of a drug product, and about the guidance that needs to be given to both the doctor and the patient regarding the genetic information [58].

4. PHARMACOGENETICS – POTENTIAL AND RISK

At a time when the pharmaceutical industry is plagued by soaring costs of R&D and by low drug approval rates, the knowledge gained through pharmacogenetic experimentation provides tremendous opportunities for improved decision making along the R&D value chain: Firstly, pharmacogenetics is being applied today, to optimize drug selection and dosing in development [34,57]. Some large pharmaceutical companies now store patient DNA in every new Phase-IIA study so that, if trials suggest that a molecule is efficacious for part of the patient population, pharmacogenetic analyses can be conducted [57]. Similarly, pharmacogenetic data have the potential to rescue development compounds that show toxicological findings in a small number of patients. Again, the concept is that the pharmacogenetic analysis leads to the design of subsequent studies that will include/exclude specific

patient populations. Although the pharmacogenetic information would likely not alleviate the need for patient monitoring, a reduction in adverse event rate may make a drug become acceptable for a particular disease indication. It might also facilitate the marketing of products by providing predictive toxicological information. Secondly, pharmacogenetic studies may lead to the identification of new targets [57]. Finally, pharmacogenetics has the potential to revolutionize the market for diagnostics. This will obviously hold true for chronic diseases; however, it may also come to fruition for acute diseases, either if novel high-speed technologies become implemented (''bed-side chips''), or if prospective haplotype mapping becomes a reality on the population scale.

There are several important risks associated with the implementation of a pharmacogenetics strategy. Firstly, the introduction of additional tests has led to an increase in development costs; however, there may be differences between first-in-class compounds and follow-up compounds. Secondly, the personalization of medicine may lead to reduced sales [59]. Finally, there are important ethical concerns: While the current focus of the pharmaceutical industry has been on the characterization of candidate genes, the availability of haplotype maps together with innovations in DNA sequencing technology will allow for genome-wide scanning on a routine basis. The generated data will facilitate pharmacogenetic analyses by the pharmaceutical industry and will enable the generation of well-characterized patient cohorts for clinical trials. However, the availability of such extensive genetic information raises important ethical questions: As genetic information becomes more widely available, it is likely that a particular haplotype will predict not only pharmacological responsiveness, but also predisposition to particular diseases. These concerns may be offset by the need and desire of patients for safer and more effective drugs; however, safeguards need to be put into place to protect both the patients and the pharmaceutical industry.

5. CONCLUSION

The promise of pharmacogenetics lies in its potential to identify sources of inter-individual variability in drug responses, which affect drug efficacy and safety. Recent success stories in oncology demonstrate that the field of pharmacogenetics has progressed substantially. The knowledge created through pharmacogentics trials can contribute to the development of patient-specific medicines as well as to improved decision making along the R&D value chain.

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Trends in Pharmaceutical Innovation

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Contents

1. INTRODUCTION

In 2004, 31 new molecular entities were approved by the Food and Drug Administration (FDA), ten more than in 2003 [1]. At the same time, more than half the scientists from the top 20 pharmaceutical companies surveyed by Kline believed R&D productivity had declined, despite also stating that scientists worked harder and completed more projects [2]. This survey reflects the current lack of confidence by internal and external stakeholders in the innovation capabilities of the pharmaceutical industry. Yet, within a long-term, six-decade context, the current innovation rates are the highest ever achieved by the pharmaceutical industry, with a record 307 new chemical entities approved by the FDA in the last ten years, compared to an average of 183 new chemical entities for the five preceding decades. There was also a clear upward trend for drug approvals that had received priority FDA review over this time period.

The lack of confidence in the innovation capabilities of the pharmaceutical industry was mirrored by the lack of confidence in sustaining innovations on the market in 2004. The second in class COX-2 inhibitor Vioxx (rofecoxib) was withdrawn for cardiovascular safety reasons [3], followed by an FDA review of other COX-2 inhibitor drugs. Some support for innovation and benefit/risk came with the recommendation to allow Vioxx to continue to be marketed along with other COX-2 drugs including the first in class, market leader, Celebrex (celecoxib). Earlier in the year, the selective serotonin reuptake inhibitors as a class experienced a label change to warn against potential suicide risk [4] after Seroxat (paroxetine) was linked to some cases of paediatric suicidal thoughts.

More than ever before, the value of pharmaceutical innovations was challenged in 2004. Newer drugs in established drug classes, or in non-lifethreatening indications

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were particularly affected. For instance, Crestor (rosuvastatin) came under attack by Public Citizen [5] for its safety profile compared to older statins.

Despite these negative perceptions, 2004 was a year of great successes and innovation. Avastin (bevacizumab) increased survival in colon cancer patients [6] and lung cancer [7] after it had failed previously in a Phase 3 metastatic breast cancer trial. Oncology phase 1 studies demonstrated higher benefits than previously reported [8].

New medicines had a dramatic and positive impact on life expectancy, economics and quality of life [9].

2. THE VALUE OF NEW MEDICINES

Cancer, Cardiovascular Disease or HIV are currently probably amongst the areas of highest public interest, and can therefore be regarded as a test case for public perception of the value of new medicines and the innovation capabilities of pharmaceutical companies. If pharmaceutical advances are not recognized in these areas, even less accolade will be forthcoming elsewhere.

2.1. Oncology drug approvals and survival

The number of new oncology drugs and new indications for existing oncology drugs increased drastically over the last decade compared to previous decades (Fig. 1).

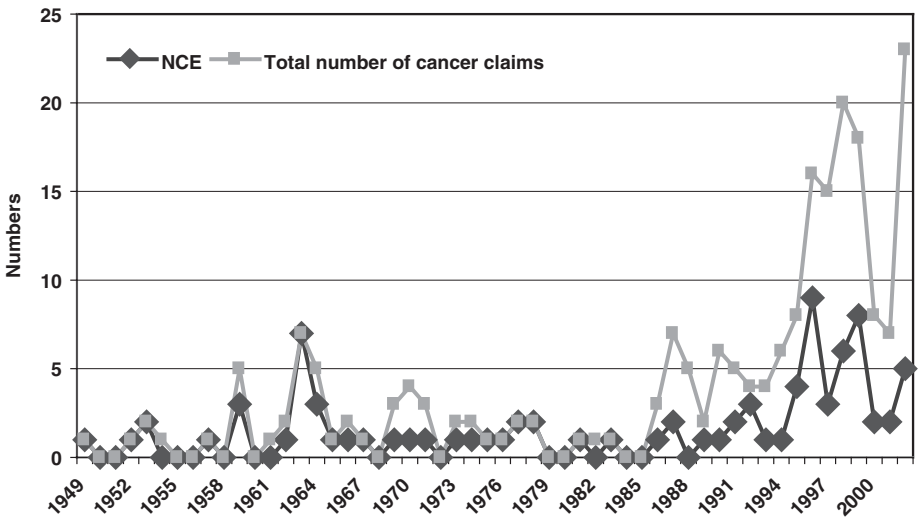

Fig. 1. Oncology drug approval trends [10]. New Chemical Entities approved by FDA are shown in blue, whilst the total number of cancer indications by new or existing NCEs claimed (and approved by FDA) are represented in red.

This trend reflects four key developments over the last decade:

1. the maturation of molecular biology to give an increase in understanding of the molecular mechanisms associated with different types of cancer. These have allowed the development of safer, targeted treatment (both small molecules and biologicals). This is reflected in the reduction in the risk of lethal toxicity with the newer, non-cytotoxic agents [11] for participating cancer patients.
2. rational selection based on target properties to decide whether to use a biological (protein) or small molecule approach;
3. Privileged libraries (arrays of compounds synthesised with some insight into the targets structure activity relationships, after gained from related proteins) and recombinant targets that have finally moved high throughput screening to a successful technology;
4. increased investment in oncology by pharmaceutical companies [12].

The increase in cancer medicines on the market is paralleled by the improved cancer survival statistics in both the UK and the US. Marketed oncology drugs have demonstrated clear year on year improvement in population survival benefits [13]. The rates of improvement differ for different cancer types, e.g. breast cancer (31 approved drugs) [14] show higher survival improvements than lung cancer, for which 14 drugs have been approved. The improvement in 5-year survival across the US in whites rose from 60% in 1950–54 to 88% in 1992–99 for breast cancer and 43% to 98% for prostate cancer respectively [15]. Childhood cancer survival improved from 20% to 79% and leukaemia from 10% to 48% (26 approved drugs). In contrast, lung cancer 5-year survival improved only from 6% to 15% during that time period. A similarly poor outlook also still exists for brain tumors.

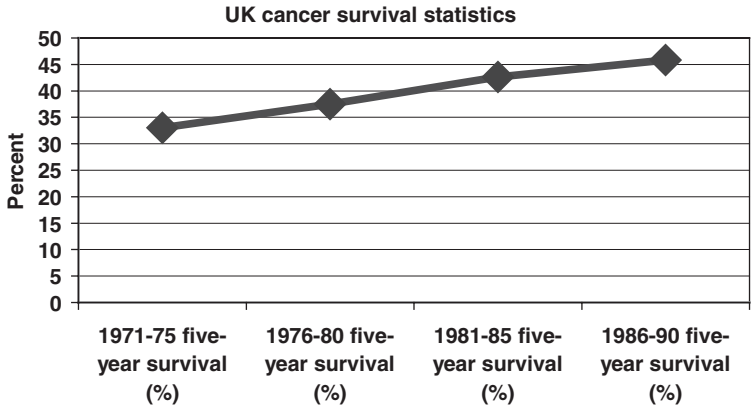

Fig. 2. UK 5-year cancer survival statistics [16].

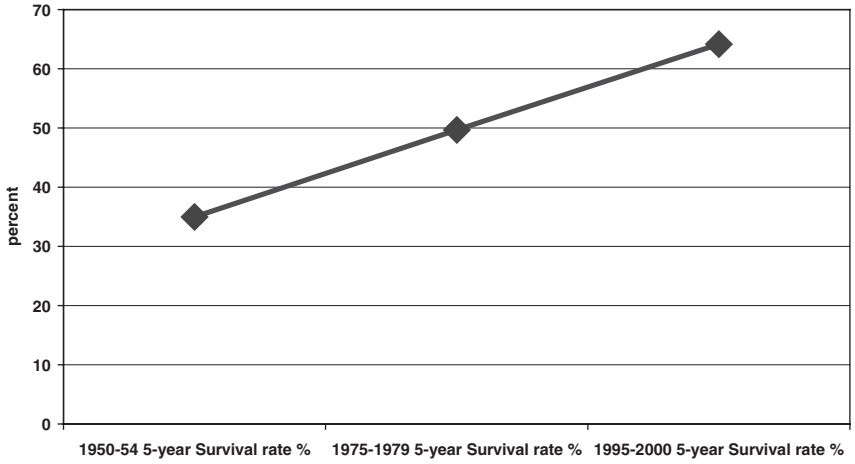

Fig. 3. US 5-year cancer survival statistics (data derived from [17], [15] and [18]).

2.1.1. Survival statistics for cancer patients

Despite other positive developments, e.g. improved surgical techniques, radiation therapy and earlier diagnosis, new drugs are thought to have accounted for about 50–60% of the improvement in survival rates and about 10.7% of the overall increase in US life expectancy [15] (Figs. 2,3).

2.1.2. Interplay of radical and incremental innovation in oncology

For decades the mainstay of cancer treatment were cytotoxic drugs, radiation therapy and surgery. Through incremental innovation, these treatment modalities were and still are being optimised, which is evident in the range of combinations explored and approved for various cancer indications. The interplay of technology advances in diagnosis, conventional treatment (e.g. radiation, bone marrow transplants, and surgery) and tailored drug treatment has resulted in childhood cancer 5-year survival improvement from 20% to 79% [15].

Over the recent decade, targeted, less toxic, treatments have taken on much greater significance. Amongst the most prominent small molecules is Gleevec (imatinib), which inhibits the abnormal, constitutively active Bcr-Abl tyrosine kinase that leads to cell proliferation in chronic myeloid leukaemia (CML) [19]. Recent statistics show 8-year survival of 78.5% for patients achieving a response to Gleevec, 22.6% for the control group and 6.2% for patients not responsive to Gleevec [20]. Gleevec demonstrates the impact of radical innovations on cancer treatment. This drug also demonstrates that the last decade of investment in recombinant technologies, biomarkers and compound subsets can indeed pay off.

It is very difficult to predict whether a new molecule, antibody, combination therapy or clinical treatment design will result in a radical breakthrough. Many

times more modest results are achieved, e.g. treatment modalities for brain cancers. Temodar (temozolomide), first launched in 1999 for refractory brain tumors (glioblastoma), has demonstrated survival benefits (from 12.1 to 14.6 months) if given concomitantly with radiation [21] and has been approved early 2005 for newly diagnosed glioblastoma. Gliadel Wafer, a new formulation of carmustin, a cytotoxic drug, given as adjunct to surgery and radiation, improved survival rates from 11.6 months to 13.9 months [22].

A similar evolution of disease management through incremental steps is evident for breast cancer. Recent studies comparing aromatase inhibitors, such as Femara (letrozole) with selective estrogen receptor modulators, such as Nolvadex (tamoxifen) indicate that the further "upstream" in the estrogen pathway the inhibition, the greater the benefits in terms of recurrence and side effects [23].

The example of Avastin, an antibody against the vascular endothelial growth factor to block angiogenesis and thus tumor growth, highlights the erratic nature of research into breakthrough discoveries and the need to value both incremental and more radical improvements in patient care. Although Avastin did not work in breast cancer patients, it is now an approved first line therapy for metastatic colon cancer. The value of incremental discoveries in highly complex and fragmented diseases only becomes evident when such pieces of the "jigsaw" come together to form a bigger picture. The advances in diagnostics have also contributed greatly to the understanding of the molecular mechanisms underlying different cancer types. Herceptin (trastuzumab) is an example of an antibody exploiting this understanding of the molecular basis of tumors. It targets the HER2/neu protein, which is overexpressed in 20–30% of breast cancers and responsible for tumor growth [24]. It remains to be seen whether medicinal chemistry will be able to provide the next wave of small molecule based, targeted cancer treatments, in the wake of these improved, mostly antibody-based therapies. Some small molecule drugs have already reached the market, such as Tarceva (erlotinib), an epidermal growth factor receptor inhibitor for non-small cell lung cancer, or Gleevec (imatinib). Following these compounds are molecules like SU-11248 [25], an orally active inhibitor of platelet-derived growth factor tyrosine kinase (TK) and other TK signalling pathways. This compound is showing high promise in gastro-intestinal and renal carcinoma. Biological approaches showing early promise include CP-675,206, an anti-CTLA-4 antibody which boosts the patients' own antibody response to malignant melanoma. Some patients are disease free after more than a year of treatment [26]. It is hoped that the pipeline of agents will herald in the beginning of a new era in cancer management.

2.2. HIV and cardiovascular drugs and survival

Death rates from AIDS in the US fell 70%, HIV transmission to newborns by their HIV infected mothers in the US have reduced from 25% to 2% through drug intervention [27]. In wealthy countries, HIV has been converted from a disease, which in the 1980s progressed quickly to its lethal state once symptoms occurred, to

a chronic disorder, which can be managed through a cocktail of successive drug interventions. Further innovations are occurring through greater understanding of the relationship between host and virus. Of exciting promise are CCR5 inhibitors, such as UK-427,857, which will add to the current HIV arsenal of nucleoside and non-nucleoside reverse transcriptase inhibitors and protease inhibitors. UK-427,857 is showing great promise and is now in Phase 3 clinical trials. The programme shows all the benefits of the advanced science investment the industry has made over the past decade. Genomic information on HIV resistant individuals [28] identified the Δ32 CCR5 receptor polymorphism as a key factor. From this finding the concept of a CCR5 antagonist as an innovative new HIV drug was born. Lead compounds were identified using high throughput screening of files, including privileged libraries. Screening cascades included *in vitro* assessment of key liabilities such as inhibition of the cardiac potassium channel IKr [29] and potential cardiotoxicity.

Large trial data emerging frequently over recent years show the huge value to society in tackling abnormal lipid and cholesterol balance. Statin therapy reduced hospital interventions by a third in the US [30] and deaths from heart disease between 2002–2003 fell by 3.6 percent [31]. The Framingham heart study showed that 10-year cumulative mortality from cardiovascular disease in the 1970 cohort was 43 percent less than that in the 1950 cohort and 37 percent less than that in the 1960 cohort [32]. This more recent cardiovascular drug class, statins, demonstrated survival benefits in patients with coronary artery disease and heart failure [33]. Lipitor (atorvastatin) showed a significant risk reduction in both fatal and non-fatal strokes in hypertensive patients with at least three other cardiovascular risk factors and baseline cholesterol [34]. Similarly, older drugs, such as diuretics and beta-blockers have contributed to survival of patients with cardiovascular risk factors [35]. Considerable advances will still be made in this area. Cholesteryl ester transfer protein (CETP) is now genetically linked, in that a polymorphic deficiency in the enzyme raises plasma high-density lipopoprotein cholesterol (HDL-c). These individuals appear to have an absence of cardiovascular disease and a longer natural lifespan [36]. CETP inhibitors might recreate this deficiency. Encouraging results from clinical studies with CETP inhibitors such as torcetrapib suggest that when used in combination with a statin, very favourable lipid profiles are produced in patients [37].

3. INNOVATION CAPABILITIES OF THE PHARMACEUTICAL INDUSTRY

The genomic and technology revolutions have given way to a new realism. Corporate compound files increased in size initially, but a new focus on quality has improved the contents of compound files as well as chemical synthesis plans. High throughput technology, such as plate based chemistry, and auto purification have taken on a prominent role in the process changes in Discovery to improve efficiency.

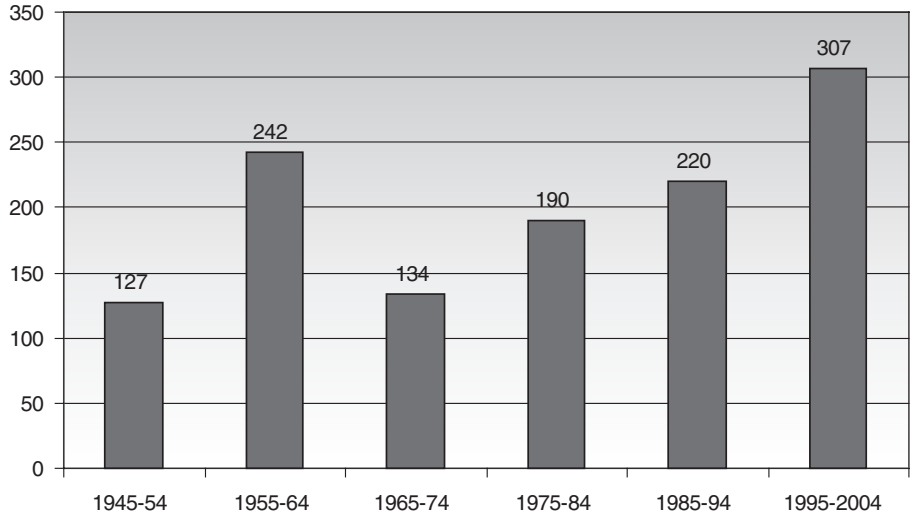

Fig. 4. Innovation rates over six decades. FDA approved new chemical entities by approval decade ([38] source data: Food and Drug Administration Drug Approvals, raw data sheet provided by F. Lichtenberg).

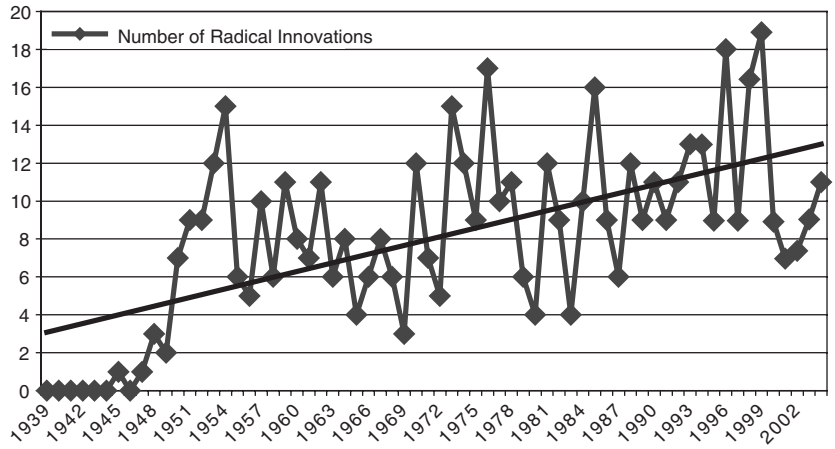

Fig. 5. Priority reviewed new chemical entities by approval year [38].

These new strategies are only just starting to pay off. Whilst the cost per medicine has spiralled out of control (it now stands at almost one billion dollars per new drug approval), the numbers of new innovations have steadily increased over the decades [38] (Figs. 4–6).

Almost half the US population and more than eighty percent of the elderly (65 and older) take at least one prescription medicine [40]. According to Frank

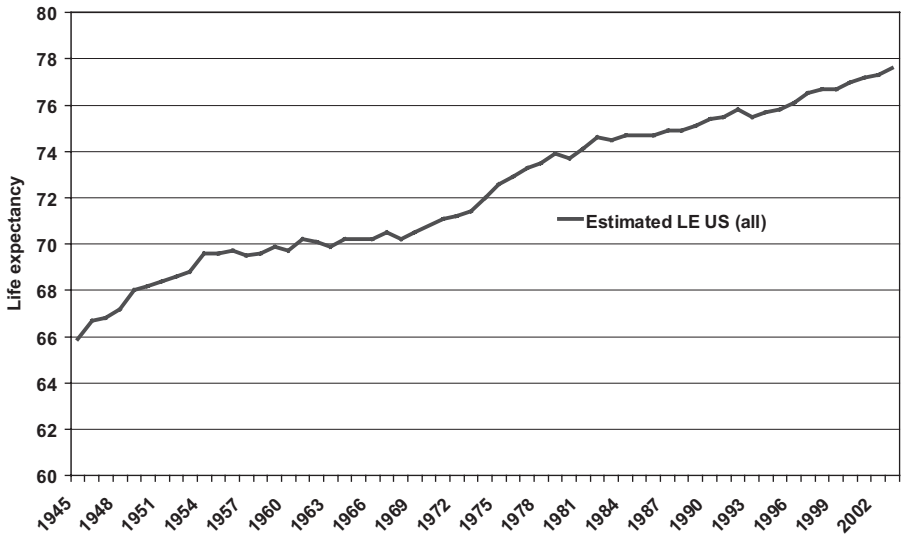

Fig. 6. Improvements in US life expectancy [39].

Lichtenberg [41] there is strong evidence that both new drug approvals and public expenditure on medical care contributed to longevity increase in the US during 1960–1997.

4. SUSTAINING INNOVATIONS IN THE MARKET PLACE – THE ISSUE OF RISK

The availability of new and better medicines year on year has increased expectations in their efficacy and safety ratios. Recent drug withdrawals, such as Baycol (cerivastatin) [42]), which was linked to about 100 deaths worldwide [43] and Vioxx (rofecoxib), which increased the risk of cardiovascular events in certain patient populations, highlighted the importance and the value associated with availability of several related drugs in a given class. Only over a period of years sometimes does the difference between a range of therapies become apparent, whether in side effect profile or enhanced therapeutic effect. The factors behind this may be obvious or very subtle. These include intrinsic potency, intrinsic selectivity (very broad ligand binding indicates differences in selectivity for closely related drugs), molecular structure, pharmacokinetics, administered dose and even formulation. The description ''me-too'' is used commonly to describe follow-on entities to a drug class. In fact, to optimise the benefit of radical innovations it is essential that follow-on drugs enter the market. Table 1 lists examples where the innovator drug was subsequently withdrawn due to side effects, but the drug class was hugely beneficial to patients and society. Follow-on compounds included structural modification, improved selectivity, increased potency, etc.

Table 1. Examples of valuable drug classes in which the original innovator drug was withdrawn or severely limited in use due to side effects. Note: only one follow-on is listed

Drug class	Therapeutic area	Modification	Innovator	Follow-on
β-agonists	Asthma	Improved selectivity	Isoprenaline	Salbutamol
β1-selective blockers	Cardiovascular	Structural change	Practolol	Atenolol
Sulfonyl hypoglycemics	Diabetes	Structural change	Carbutamide	Tolbutamide
Glitazones	Diabetes	Improved potency	Troglitazone	Rosiglitazone
Acetylcholinesterase inhibitors	Alzheimers	Structural change and improved potency	Tacrine	Donepezil
5-HT4 antagonists	Gastro-intestinal	No follow on	Cisapride	-

5. CONCLUSION

Pharmaceutical innovation has clear benefits for the public. Mortality rates are one of the clearest outcome measures, and whilst not all the improvements can be attributed to new medicines, a large proportion can. New scientific knowledge and subsequent investment in pharmaceutical innovation made major contributions to the positive trends seen in high medical need areas such as HIV and oncology. R&D has become more expensive, but the new research paradigms applied in Medicinal Chemistry, Drug Metabolism, and Biology, have already impacted positively on the number of new medicines reaching the market. Whether Medicinal Chemistry will be able to harness the scientific breakthroughs that have clearly already happened in antibody and protein based therapies, remains to be seen, but there is a high likelihood that the next wave of oncology drugs will be small molecule modulators of important pathways downstream from the cell surface receptors currently targeted by antibodies.

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To Market, To Market—2004

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Contents

1. INTRODUCTION

The number of new chemical entities and biologics entering the market in 2004 for therapeutic use was 19, which included five first-in-class therapies. While these numbers were lower than previous years, the emphasis in 2004 was clearly on line extensions comprised of new formulations, new indications and new combinations of existing drugs to provide enhanced value to the patient population [1–5]. For the first time in decades, line extensions accounted for more than half of the drug launches last year. However, it is worth noting that an unusually high number of new molecular entities received approval in the final weeks of the year. Although not in time for launch before year-end, many were projected to appear on the market in the early part of 2005. From the 17 NCEs and two NBEs introduced last year, the US was the most active market with nine new product launches, followed by Europe and Japan with five and four, respectively, and one new drug reached its first market in the Republic of Korea. Of the major pharmaceutical companies,

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Eli Lilly and Pfizer had a marketing or co-marketing role in two new launches each, followed by AstraZeneca and Novartis, each launching one new product. In addition to the new molecular entities, the year also saw the entry of several new diagnostic agents based on novel technologies.

The anticancer field was the most prolific therapeutic area in 2004, with the introduction of seven new entities, including three first-in-class therapies. Avastin™ (bevacizumab), a monoclonal antibody targeting vascular endothelial growth factor (VEGF), is the first angiogenesis inhibitor to reach the market, and it is indicated for the treatment of colorectal cancer. In addition, Plenaxis™ (abarelix), the first gonadotropin releasing-hormone (GnRH) receptor antagonist, and Vidaza™ (azacitidine), the first DNA methyltransferase inhibitor, were introduced last year for the treatment of prostate cancer and myelodysplastic syndrome (MDS), respectively. The portfolio of options for the second- and third-line treatment of non-small cell lung cancer (NSCLC) grew considerably with the addition of two NCEs. Tarceva™ (erlotinib), an epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, and Alimta® (pemetrexed), a multi-targeted antifolate, were launched for this indication. Pemetrexed is also labeled for the treatment of malignant pleural mesothelioma, a rare form of cancer associated with asbestos exposure. The other oncolytic drugs launched last year include Laserphyrin (talaporfin), a photodynamic therapy for early stage lung cancer, and Camtobell® (belotecan), a new camptothecin analog, for the treatment of ovarian and small cell lung cancer. Belotecan is the third camptothecin derivative to reach the market behind topotecan and irinotecan, which were introduced in previous years.

In the area of endocrine and metabolic diseases, three new drugs appeared on the market. Sensipar™ (cinacalcet), the first entry in a new class of therapeutics called the calcimimetics, was launched as an oral treatment for secondary hyperparathyroidism and hypercalcemia. Glufast® (mitiglinide), a non-sulfonylurea hypoglycemic agent and inhibitor of ATP-dependent potassium channels in beta cells, was introduced in Japan for the treatment of type 2 diabetes. In addition, Protelos® (strontium ranelate) was launched for the treatment of postmenopausal osteoporosis, specifically to reduce the risk of vertebral and hip fractures. Strontium ranelate is the first osteoporosis drug able to stimulate the formation of new bone as well as prevent loss of existing bone, thereby representing a major advancement in treating the disease.

The CNS area was represented by the entry of three new drugs: Cymbalta® (duloxetine) for the treatment of depression, Lyrica® (pregabalin) for the treatment of epilepsy and peripheral neuropathic pain, and Sensiron® (indisetron hydrochloride) for the treatment of chemotherapy-induced nausea and vomiting. Duloxetine, a selective serotonin and norepinephrine reuptake inhibitor (SSNRI), is additionally indicated for treating pain caused by diabetic peripheral neuropathy. Pregabalin is the second GABA analog marketed by Pfizer, and it has higher potency and improved pharmacokinetic properties as compared to its predecessor, gabapentin (Neurontin®). Indisetron is a dual serotonin 5-HT3/5-HT4 receptor antagonist and has proven efficacy in reducing the number and duration of emetic episodes up to 24 hours after the administration of anticancer agents.

The anti-infective domain had two new drugs entering the market in 2004. Factive® (gemifloxacin), a new member of the fluoroquinolone class of antibacterials, was launched for the treatment of community-acquired pneumonia (CAP) caused by multi-drug resistant *S. pneumoniae* and for the treatment of acute bacterial exacerbations of chronic bronchitis (AECB). Prodif® (fosfluconazole), a phosphate pro-drug of fluconazole, was also introduced last year in Japan as an intravenous injection for treating *Candida* and *Cryptococcus* infections. Fosfluconazole undergoes nearly quantitative hydrolysis *in vivo* to provide >97% bioavailability of fluconazole, but its improved water-solubility as compared with fluconazole allows a significant reduction in the infusion volume. Unlike in previous years, there were no new drug entities launched in 2004 for HIV and AIDS therapy. Instead, the focus was on combination drugs to simplify the dosing regimens while maintaining efficacy and tolerability, a considerable benefit to patients. Two drugs entering this market were Epzicom™ (abacavir sulfate/lamivudine) and Truvada™ (tenofovir disoproxil fumarate/emtricitabine), and both were based on combinations of previously marketed reverse transcriptase inhibitors.

The cardiovascular sector had one NCE and two important combination therapies entering the market in 2004, all of which offer substantial projected value to patients. Exanta® (ximelagatran) was introduced in Europe as an oral anticoagulant for the prevention of venous thromboembolic events in patients undergoing hip or knee replacement surgery. Vytorin™, a combination of ezetimbe and simvastatin, was launched for the treatment of hypercholesterolemia. Ezetimbe is an inhibitor of cholesterol absorption in the intestine, whereas simvastatin is an inhibitor of cholesterol biosynthesis. Caduet®, a combination of amlodipine besylate and atorvastatin calcium, also entered the market as a simultaneous treatment for hypertension and high cholesterol, two important risk factors directly associated with cardiovascular mortality.

Certican® (everolimus), a derivative of rapamycin, was launched as an oral immunosuppressant for the prevention of kidney and heart transplant rejection. Everolimus has immunosuppressive properties similar to rapamycin, but with significantly improved oral bioavailability and pharmacokinetic profile. It is indicated for use in combination with Neoral® (cyclosporine for microemulsion) and corticosteroids.

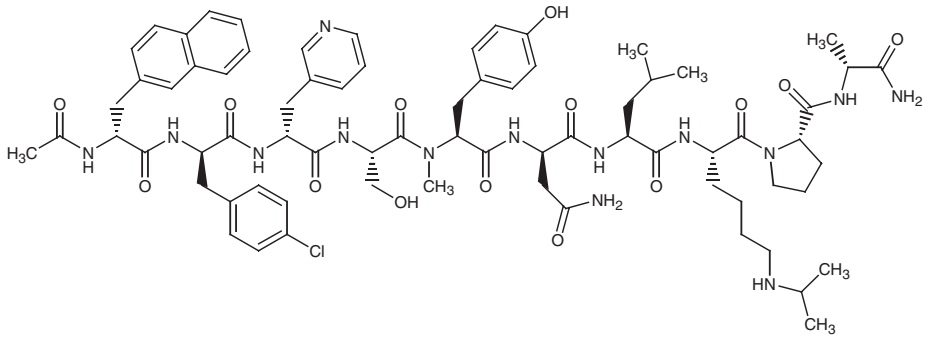
Two new urologic drugs entered their first markets in Europe last year. Vesicare® (solifenacin), an M3 muscarinic receptor antagonist, was introduced as once-daily oral treatment for treating overactive bladder with symptoms of urge incontinence and increased urinary frequency and urgency. Duloxetine, in addition to being marketed under the trade name Cymbalta® for treating depression and diabetic peripheral neuropathic pain as mentioned above, was also launched in the UK under the trade name Yentreve® as a twice-daily oral treatment of stress urinary incontinence in women.

Tysabri® (natalizumab), a humanized monoclonal antibody, was launched in the final days of last year as treatment for relapsing forms of multiple sclerosis. It acts by inhibiting adhesion molecules on the surface of immune cells and preventing the migration of immune cells into the CNS, where they can cause inflammation and potentially damage nerve fibers and their insulation.

Finally, several new diagnostic agents were introduced last year, including two heavy metal derivatives: Primovist® (gadoxate disodium) for the MRI detection and characterization of liver lesions including liver tumors and metastases, and NeutroSpec™ (technetium [Tc 99 m] fanolesomab), an anti CD15 monoclonal antibody, for the diagnosis of appendicitis. Human secretin, a synthetic peptide identical to the natural hormone and a stimulant of pancreatic secretions, was also introduced as an agent to aid in the diagnosis of pancreatic exocrine dysfunction and gastrinoma and in ERCP procedures. Although these diagnostic agents are not considered drug entities for therapeutic use and not covered in this review, they represent significant technological advancements in the field.

2. ABARELIX (ANTICANCER) [6–8]

Country of Origin:	US
Originator:	Praecis
First Introduction:	US
Introduced by:	Praecis
Trade Name:	Plenaxis
CAS Registry No:	183552-38-7
Molecular Weight:	1416.06

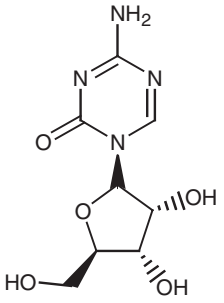


Abarelix is an antagonist of the gonadotropin releasing-hormone (GnRH) receptor, and it was launched last year as an intramuscular injection for the palliative treatment of advanced symptomatic prostate cancer. Hormonal therapy of prostate cancer is based on the modulation of testosterone to achieve medical castration levels. The inhibition of GnRH activity causes the suppression of luteinizing hormone (LH) and follicle stimulating hormone (FSH) secretion, thereby reducing the secretion of testosterone by the testes. Abarelix is the first GnRH antagonist to reach its market. Hormonal therapy with GnRH agonists such as leuprolide, buserelin, and goserelin has been in use for over two decades. Drugs of this type achieve the suppression of LH and FSH by a feedback inhibition mechanism, which involves an initial rise in the LH and FSH levels and, consequently, in the levels of testosterone. A testosterone surge may induce a clinical tumor flare that worsens

cancer-related symptoms. This phenomenon is observed in 4–33% of patients receiving a GnRH agonist. A GnRH antagonist such as abarelix acts by direct inhibition of LH and FSH secretion, which avoids the initial surge in serum testosterone concentrations. Abarelix is a decapeptide, and it is prepared by a typical coupling cycle for peptide synthesis using Boc-amino acids and a methylbenzhydrylamine (MBHA) resin. Abarelix has high binding affinity for GnRH receptor ($K_d = 0.1$ nM). Following intramuscular administration of a 100 mg dose, abarelix is absorbed slowly with a C_{max} of 43.4 ng/mL observed approximately 3 days after the injection and has a half-life of about 13 days. The apparent volume of distribution is over 4000 L, suggesting extensive distribution. Abarelix has high protein binding (96–99%), and it is primarily metabolized via hydrolysis of peptide bonds. Following a dose of 15 μg/kg in humans, approximately 13% of abarelix is recovered unchanged in the urine, with no detectable metabolites. The renal clearance of abarelix is 14.4 L/day following a 100 mg dose. Two randomized, open label, comparative clinical trials involving 348 patients demonstrated the efficacy of abarelix versus a GnRH agonist (leuprolide) as well as a combination of GnRH agonist and anti-androgen (leuprolide + bicalutamide). In these trials, both abarelix and the comparators reduced testosterone to medical castration levels (< 50 ng/dL) by day 29 of therapy in 94–98% of the patients. However, a significant difference was observed between the two groups for the occurrence of testosterone surge (0% in the abarelix group versus 82% in the GnRH agonist group) and for the rapidity of attaining castration levels (72% versus 0% on day 8 in the abarelix and GnRH agonist groups, respectively). Both groups maintained medical castration levels of testosterone with similar efficacy between days 29 and 85 of treatment. Abarelix was generally well tolerated in these trials. Approximately 3% of the patients experienced an immediate-onset allergic reaction. Other adverse events were similar to comparator controls and included hot flushes, sleep disturbance, pain, and breast enlargement. The recommended dosage of abarelix is 100 mg intramuscular injection on days 1, 15, and 29 of therapy, and every 4 weeks thereafter.

3. AZACITIDINE (ANTICANCER) [9–13]

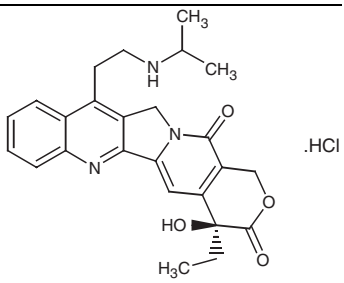
Country of Origin:	US
Originator:	Pharmion
First Introduction:	US
Introduced by:	Pharmion
Trade Name:	Vidaza
CAS Registry No:	320-67-2
Molecular Weight:	244.2



Azacitidine is an antineoplastic agent launched last year for the treatment of myelodysplastic syndrome (MDS). MDS is a group of closely related diseases caused by abnormal blood-forming stem cells of the bone marrow. They are characterized by a hyperproliferative bone marrow, the presence of clonal blood cells with impaired morphology and maturation, and peripheral blood cytopenias resulting from ineffective blood cell production. The initial stem cell injury can be from cytotoxic chemotherapy, radiation exposure, chemical exposure, or genetic predisposition. Subsequently a clonal mutation predominates over bone marrow thereby suppressing healthy stem cells. Azacitidine is indicated for the treatment of all five subtypes of MDS, which consist of refractory anemia, refractory anemia with ringed sideroblasts, refractory anemia with excess blasts, refractory anemia with excess blasts in transformation, and chronic myelomonocytic leukemia. Azacitidine is an analog of cytidine in which the carbon at position 5 of the pyrimidine ring has been replaced by nitrogen. It is prepared by the ribosylation of 5-azacytosine, which is derived from the condensation of amidinourea with either an alkyl orthoformate or *N*,*N*-dimethylformamide dialkyl acetal. The antineoplastic activity of azacitidine is derived from a combination of two different mechanisms. It inhibits DNA methyltransferase, which causes demethylation or hypomethylation of DNA. In addition, it exerts direct cytotoxicity on hyperproliferating abnormal stem cells in the bone marrow. After *in vivo* phosphorylation, azacitidine incorporates into DNA and forms covalent adducts with cellular DNA methyltransferase, thereby depleting the cells from enzyme activity and causing hypomethylation of genomic DNA. Hypomethylation restores normal function to tumor-suppressor genes, which are responsible for regulating cell differentiation and growth. The cytotoxic effects of azacitidine cause the death of rapidly dividing cells, including cancer cells that are no longer responsive to normal growth control mechanisms. Non-proliferating cells are relatively insensitive to azacitidine. The recommended dosage of azacitidine is $75\,mg/m^2$ subcutaneously once daily for 7 days, and the cycle is repeated every 4 weeks. It is rapidly absorbed with peak concentrations achieved within 30 minutes of the dose. It has a bioavailability of 89% and a mean volume of distribution of 76 ± 26 liters. Mean clearance of azacitidine is 167 ± 49 L/hour, and the mean half-life of the parent drug is 41 ± 8 minutes. The primary route of elimination for azacitidine and its metabolites is renal (85%), with a cumulative mean elimination half-life of 4 hours. The efficacy of azacitidine was demonstrated in a randomized, open-label, controlled clinical trial and two non-randomized trials involving 300 patients with any of the five subtypes of MDS. Approximately 14–19% of patients in these trials had an overall response rate (complete response + partial response) to azacitidine, which consisted of normalization of blood counts and decrease in bone marrow blasts percentage. The initial response was generally observed by the fifth cycle of treatment. The need for transfusions was also eliminated in responder patients. In all three studies, approximately 19% of patients met the criteria for improvement with a median duration of 195 days. The most common adverse events with azacitidine therapy include nausea, vomiting, myelosuppression, and infection. Dose-limiting toxicities include neutropenia and thrombocytopenia.

4. BELOTECAN (ANTICANCER) [14–17]

Country of Origin:	S. Korea
Originator:	CKD Pharmaceuticals
First Introduction:	S. Korea
Introduced by:	CKD Pharmaceuticals
Trade Name:	Camtobell
CAS Registry No:	213819-48-8
Molecular Weight:	469.97 (hydrochloride)



Belotecan, a DNA topoisomerase I inhibitor, is an analog of camptothecin. It was launched last year in the Republic of Korea as an injectable formulation for the treatment of ovarian and small cell lung cancer. Although camptothecin exhibits potent antineoplastic activity *in vitro*, its clinical application is hampered by severe toxicity and poor water solubility. Several synthetic and semi-synthetic analogs of camptothecin with improved solubility and lower toxicity have been developed over the past two decades. Two drugs from this class, topotecan and irinotecan, have been launched in previous years and belotecan is the newest member to reach the market. It is prepared by a two-step semi-synthesis starting from camptothecin, first by converting to 7-methylcamptothecin via a free-radical methylation reaction using a combination of acetic acid, *tert*-butylhydroperoxide, ferrous sulfate and sulfuric acid, and subsequently, in the second step, a Mannich reaction with isopropylamine hydrochloride and dimethylsulfoxide. A total synthesis of belotecan in seventeen steps starting from ethyl acetopyruvate is also reported. Belotecan inhibits topoisomerase I with approximately equal potency as camptothecin and about 3-fold higher potency than topotecan, with respective IC_{50} values of 0.119, 0.123 and 0.33 μg/mL. Its cytotoxic activity is comparable to that of camptothecin, with IC_{50} values ranging from 2 ng/mL to 2 μg/mL against 26 different human cancer cell lines. In studies using human tumor xenografts in nude mice, 80-100 mg/kg of belotecan dosed every four days for four doses produced 67 to 94% tumor regression rates against HT-29, WIDR and CX-1 colon, LX-1 lung, MX-1 breast and SKOV-3 ovarian carcinomas. Pharmacokinetic studies of belotecan in rats at intravenous doses of 2.6–8.9 mg/kg demonstrated that both C_{max} and AUC increased in a dose-dependent manner. Total clearances, volumes of distribution and mean residence times did not change significantly with increasing doses. The elimination half-life ranged between 9.2 to 11.2 hours. In a Phase I study of belotecan, the fraction of renal clearance was found to be 33.1 to 50.3%, and the protein-binding fraction was 53 to 87%. Approximately 9.5% of the administered dose was excreted via the hepatobiliary system. In clinical studies involving 20 patients with recurrent or refractory ovarian cancer, intravenous administration of 0.5 mg/m^2/day

of belotecan for 5 days every 3 weeks over a median of six dosing cycles resulted in an overall response rate of 45%. All patients had grade 3 or 4 neutropenia as the most significant adverse event.

5. BEVACIZUMAB (ANTICANCER) [18–22]

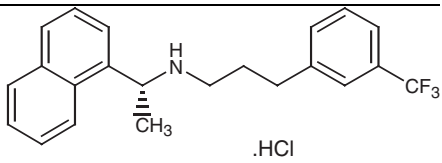
Country of Origin:	US
Originator:	Genentech
First Introduction:	US
Introduced by:	Genentech/Roche
Trade Name:	Avastin
CAS Registry No:	216974-75-3
Class:	Recombinant humanized antibody
Type:	IgG1 monoclonal anti-VEGF
Molecular Weight:	149 kDa
Expression system:	CHO cell line
Manufacturer:	Genentech

Bevacizumab, a humanized IgG1 monoclonal antibody against vascular endothelial growth factor (VEGF), inhibits tumor angiogenesis and delays disease progression. It was launched in the US last year as an intravenous infusion for the treatment of metastatic colorectal cancer in combination with fluorouracil-based chemotherapy. Bevacizumab was developed by engineering the VEGF binding residues of the murine neutralizing antibody A.4.6.1 into the framework of the consensus human IgG1. Its amino acid sequence is approximately 93% human IgG and 7% murine antibody and is produced in a CHO cell expression system. Bevacizumab binds VEGF with high affinity ($K_d = 0.5$ nM) and prevents its interaction with tyrosine kinase receptors VEGFR1 and VEGFR2 on the surface of endothelial cells, thereby inhibiting cell proliferation and microvascular growth. In mouse models, administration of bevacizumab blocked the growth of human tumor xenografts and reduced the size and number of metastases. The recommended dosage of bevacizumab is 5 mg/kg administered once every 2 weeks as an intravenous infusion until disease progression is detected. Based on a population pharmacokinetic analysis of patients who received 1–20 mg/kg of bevacizumab once every 1–3 weeks, the estimated half-life was approximately 20 days, and the predicted time to reach steady state was 100 days. The maximum and minimum steady-state serum concentrations at 2.5 mg/kg/week dose were 226 and 88 μg/mL, respectively. Clearance of bevacizumab is low, and varies with body weight, gender and tumor burden. In patients with colorectal cancer receiving bevacizumab 5–10 mg/kg in combination with fluorouracil and leucovorin, mean total clearance was 2.79 ml/kg/day. In clinical studies involving the administration of bevacizumab (5 mg/kg every 2 weeks) or placebo in addition to bolus-IFL (irinotecan 125 mg/m^2 i.v., 5-fluorouracil 500 mg/m^2 i.v., and leucovorin 20 mg/m^2 i.v. administered once weekly for four weeks every

six weeks), the median overall survival was significantly increased from 15.6 months in the bolus IFL + placebo arm to 20.3 months in the bolus IFL + bevacizumab arm. Similar increases were also seen in progression-free survival (6.4 versus 10.6 months), overall response rate (35% versus 45%), and duration of response (7.1 months versus 10.4 months). The most common adverse events in these trials were hypertension, diarrhea and leucopenia. Other clinically significant adverse events reported occasionally were gastrointestinal perforations, thromboembolic events, bleeding and proteinuria. Because wound healing may be impaired by inhibition of VEGF, bevacizumab therapy is not recommended until 28 days after primary surgery.

6. CINACALCET (HYPERPARATHYROIDISM) [23–29]

Country of Origin:	US
Originator:	NPS pharmaceuticals
First Introduction:	US
Introduced by:	Amgen
Trade Name:	Sensipar
CAS Registry No:	364782-34-3
Molecular Weight:	393.88 (Hydrochloride)

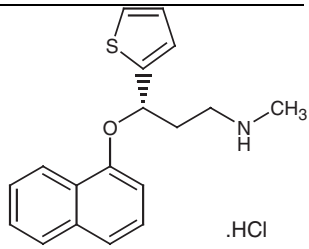


Cinacalcet is the first entry in a new class of therapeutic agents called the calcimimetics. It was launched last year as an oral treatment for secondary hyperparathyroidism (SHPT) in patients with chronic kidney disease on dialysis and for hypercalcemia in patients with parathyroid carcinoma. SHPT is associated with increased parathyroid hormone (PTH) secretion, which is triggered by low serum levels of calcium resulting from the failure of the kidney to clear phosphorous from the body and its inability to produce sufficient quantities of vitamin D. The consequences of increased PTH include stimulation of osteoclastic activity, cortical bone resorption and marrow fibrosis. PTH secretion is primarily regulated by the calcium-sensing receptor (CaR), which is located on the surface of the chief cell of the parathyroid gland. Calcimimetics bind to CaR and increase the sensitivity of CaR to extracellular calcium, thereby enabling its activation at subnormal levels of serum calcium. As a result, in the presence of these agents, the low levels of endogenous calcium in patients with renal failure are able to exert a suppressive effect on PTH secretion. Parathyroid carcinoma is also associated with elevated PTH levels, which are driven by autonomous parathyroid gland activity and subsequently lead to hypercalcemia. Although surgical resection is the primary therapy for treating hypercalcemia in parathyroid carcinoma patients, calcimimetics offer a nonsurgical alternative for patients with failed parathyroidectomy, metastatic parathyroid carcinoma, or high surgical risk. The recommended dosage of cinacalcet for the treatment of SHPT in chronic kidney disease is 30 mg once daily at start and subsequent titration to 60, 90, 120 or 180 mg once daily. The dosage

for the treatment of hypercalcemia in patients with parathyroid carcinoma is 30 mg twice daily at start and subsequent titration to 60 or 90 mg twice daily, or 90 mg three or four times daily as necessary to normalize serum calcium level. After oral administration of cinacalcet, maximum plasma concentration is achieved in approximately 2 to 6 hours. It has a terminal half-life of 30 to 40 hours and steady-state drug levels are reached within 7 days. Cinacalcet has a high volume of distribution (1000 L) and high protein binding (93%–97%). It is extensively metabolized in the liver, mainly by CYP3A4, CYP2D6 and CYP1A2. The primary routes of elimination are in the urine (80%) and in the feces (15%). In Phase III clinical trials involving 1136 patients with SHPT, administration of cinacalcet at 30–180 mg/day doses for 6 months produced 38–48% decrease in intact PTH. Overall, 64% of patients given cinacalcet achieved at least a 30% reduction in PTH, versus 11% of placebo patients. Calcium-phosphorous product was reduced 14% by the active treatment and did not change in the placebo group. In a much smaller clinical study involving 21 hypercalcemic patients with parathyroid carcinoma, administration of 60–360 mg/day doses of cinacalcet resulted in 71% of patients achieving a target reduction of $\geqslant$1 mg/dL in serum calcium. The most common adverse events in these trials were nausea and vomiting. *In vitro*, cinacalcet is a strong inhibitor of CYP2D6; therefore, dose adjustments may be required when coadministered with medications that are predominantly metabolized by CYP2D6 and have a narrow therapeutic index (e.g. flecainide, vinblastine, thioridazine and most tricyclic antidepressants). Cinacalcet is prepared in a two-step synthesis starting from 3-[3-(trifluoromethyl)phenyl]propionaldehyde, by first condensing with (*R*)-(1-naphthyl)ethylamine to form the corresponding imine and subsequent reduction of the imine with sodium cyanoborohydride.

7. DULOXETINE (ANTIDEPRESSANT) [30–38]

Country of Origin:	US
Originator:	Lilly
First Introduction:	US
Introduced by:	Lilly/Boehringer-Ingelheim
Trade Name:	Cymbalta
CAS Registry No:	136434-34-9
Molecular Weight:	333.9 (Hydrochloride)



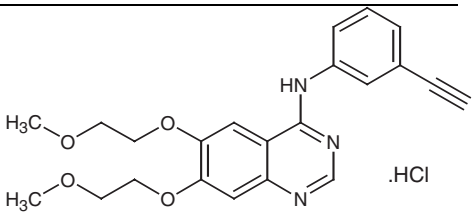
Duloxetine is a selective serotonin (5-HT) and norepinephrine reuptake inhibitor (SSNRI) for oral administration, and it is currently approved in the US and in Europe for the treatment of major depressive disorder (MDD) and diabetic peripheral neuropathic pain (DPN). Additionally, it is indicated for the treatment of stress urinary incontinence (SUI) in Europe. However, the US approval for SUI is

still pending, and Lilly recently withdrew the NDA for this indication. It is expected that additional clinical trials may be required to secure FDA approval for SUI treatment due to concern over the potential of duloxetine to prolong the QT interval in patients taking concomitant CYP2D6 or CYP1A2 inhibitors such as tricyclic antidepressants, type 1C antiarrhythmics and phenothiazines. Duloxetine is the second SSNRI to enter the market. Its predecessor, venlafaxine, has been available since 1994 for the treatment of depression. Duloxetine has a higher affinity for human norepinephrine ($K_i = 7.5$ nM) and 5-HT transporters ($K_i = 0.8$ nM) than venlafaxine ($K_i = 2480$ nM and 82 nM, respectively). Additionally, it shows a more balanced ratio of 5-HT to norepinephrine uptake inhibition than venlafaxine. Duloxetine is a moderate inhibitor of dopamine reuptake ($K_i = 300$ nM in rat brain preparations) and shows only weak affinity for muscarinic, α_1-and α_2 adrenergic and histamine H1 receptors ($K_i = > 2.3$ μM). It is devoid of any activity against monoamine oxidase. *In vivo*, duloxetine inhibits 5-HT and norepinephrine uptake in rats, with ED_{50} values of 12 mg/kg and 22 mg/kg p.o., respectively, and has no effect on dopamine uptake at doses up to 30 mg/kg. The antidepressant and central pain-inhibitory action of SSNRIs are derived from the inhibition of both 5-HT and norepinephrine uptake, which increases the availability of neurotransmitters and ultimately increases serotonergic and noradrenergic function within the CNS. In the treatment of SUI with duloxetine, the increased neurotransmitter concentration is believed to increase the tone and contractions of the urethral sphincter at the opening of the bladder, helping to prevent accidental urine leakage. The absolute stereochemistry of duloxetine is shown to be *S*(+). It is prepared in four steps starting from 2-acetylthiophene. A key intermediate in the synthesis of duloxetine is (*S*)-3-dimethylamino-1-(2-thienyl)-1-propanol, which is obtained from the corresponding ketone, either by reduction with sodium borohydride and subsequent chiral resolution of the alcohol product with *S*-mandelic acid, or by enantioselective reduction with a chiral amine complex of lithium aluminum hydride. Etherification of this intermediate with 1-fluoronaphthalene, followed by *N*-demethylation with trichloroethylchloroformate affords duloxetine. Orally administered duloxetine is well absorbed, with C_{max} achieved in 6 hours post dose. It exhibits dose-proportional pharmacokinetics at doses of 20–40 mg twice daily, with an average bioavailability of about 50% and an elimination half-life of about 12 hours. The volume of distribution is high (1943 L) and steady-state plasma levels are achieved after 3 days of dosing. Duloxetine is extensively metabolized, mainly by CYP2D6 and CYP1A2. The primary routes of elimination are in the urine (70%) and in the feces (20%). The recommended dosages of duloxetine are 20 mg to 30 mg twice daily for treating MDD, 60 mg once daily for treating DPN, and 40 mg twice daily for treating SUI. In clinical trials with adult MDD outpatients ($n = 1059$), duloxetine at doses of 40–120 mg daily for 8 to 9 weeks demonstrated superiority over placebo as measured by improvement in the 17-item Hamilton Depression Rating Scale total score. In trials involving 791 DPN patients, 60 and 120 mg once daily dose of duloxetine statistically significantly improved the endpoint mean pain scores from baseline, and increased the proportion of patients with at least 50% reduction in pain score from baseline. All doses of duloxetine resulted in superior results than placebo. In phase III studies of adult women with predominant

symptoms of SUI ($n = 1635$), duloxetine 40 mg twice daily reduced the median incontinence episode frequency from baseline to a significantly greater extent than placebo (50.0–53.6% versus 27.5–40.0%). In addition, duloxetine recipients experienced significantly greater increases in their average voiding interval than placebo recipients (15.0–20.4 versus 1.7–8.5 minutes). The most commonly observed adverse events associated with duloxetine therapy are nausea, dry mouth, constipation, decreased appetite, fatigue, somnolence, and increased sweating. As with other 5-HT reuptake inhibitors, duloxetine is contraindicated in patients taking MAO inhibitors. Additionally, it is contraindicated in patients with uncontrolled narrow-angle glaucoma.

8. ERLOTINIB (ANTICANCER) [39–42]

Country of Origin:	US
Originator:	Pfizer
First Introduction:	US
Introduced by:	OSI Pharmaceuticals/ Genentech/Roche
Trade Name:	Tarceva
CAS Registry No:	183319-69-9
Molecular Weight:	429.9 (Hydrochloride)

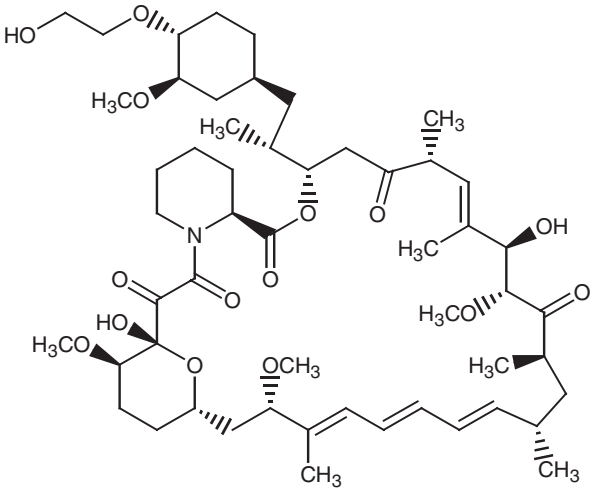


Erlotinib, launched last year as once daily oral treatment for patients with non-small-cell lung cancer (NSCLC), is an inhibitor of the epidermal growth factor receptor (EGFR) tyrosine kinase, and it is the second small-molecule drug to be marketed with this mechanism of action. Both erlotinib and its predecessor, gefitinib, are members of the anilinoquinazoline class of tyrosine kinase inhibitors. They compete with the binding of ATP to the intracellular tyrosine kinase domain of EGFR, thereby inhibiting receptor autophosphorylation and blocking downstream signal transduction. Erlotinib is prepared by the condensation of 3-ethynylaniline with 4-chloro-6,7-bis(2-methoxyethoxy)quinazoline, which is a key intermediate obtained in five synthetic steps starting from ethyl 3,4-dihydroxybenzoate. *In vitro*, Erlotinib inhibits purified human EGFR tyrosine kinase with an IC_{50} of 2 nM and blocks EGFR autophosphorylation in cellular assays with an IC_{50} of 20 nM. Treatment of human colon cancer cells with erlotinib was associated with growth inhibition, G1 cell cycle arrest, and apoptosis. Oral administration of erlotinib in athymic mice produced potent antitumor effects with an ED_{50} of 9.2 mg/kg/day for HN5 head and neck xenografts and 14 mg/kg/day for A431 epidermoid xenografts. The absorption of Erlotinib following oral dosing is approximately 60%. Food greatly enhances the absorption allowing for almost 100% bioavailability of the dose. The time to reach peak plasma levels of the

drug is about 4 hours, and the half-life is approximately 36 hours. Steady-state drug levels are reached in 7 to 8 days. Erlotinib has high protein binding (93%) and has an apparent volume of distribution of 232 L. It is metabolized primarily by CYP3A4 and to a lesser extent by CYP1A2 and CYP1A1. The drug is mainly excreted in the feces with less than 9% of the dose found in the urine. Erlotinib is labeled for the treatment of patients with locally advanced or metastatic NSCLC who have failed one or more previous chemotherapy regimens. The recommended dosage is 150 mg daily until disease progression is detected. In a randomized, double blind, placebo-controlled trial involving 731 patients, 150 mg/day oral dose of erlotinib resulted in a median overall survival of 6.7 months compared with 4.7 months in the placebo group ($p < 0.001$). Progression-free survival was 9.9 weeks and 7.9 weeks in the erlotinib and placebo groups, respectively ($p < 0.001$). Survival at one year was 31.2% in the erlotinib group versus 21.5% in the placebo group. The use of erlotinib showed greater benefit in patients with EGFR positive tumors and in those who never smoked. The most common adverse events reported in clinical trials were rash (9%) and diarrhea (6%). Elevations in liver function tests were also seen; however, these effects were mainly transient or associated with liver metastases. As previously noted for gefitinib, erlotinib is also shown to lack any clinical benefit in concurrent administration with platinum-based chemotherapy.

9. EVEROLIMUS (IMMUNOSUPPRESSANT) [43–46]

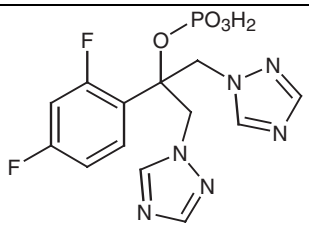
Country of Origin:	Switzerland
Originator:	Novartis
First Introduction:	Germany
Introduced by:	Novartis
Trade Name:	Certican
CAS Registry No:	159351-69-6
Molecular Weight:	958.22



Everolimus, an oral immunosuppressant for the treatment of kidney and heart transplant rejection, is the 40-*O*-(2-hydroxyethyl) derivative of rapamycin. It has immunosuppressive properties similar to those of rapamycin, but with improved pharmacokinetic profile. In addition, the 40-*O*-(2-hydroxyethyl) group alters the physico-chemical properties of the macrolide to allow galenic formulation. Everolimus is prepared in a two-step semisynthesis starting from rapamycin, by alkylation of the 40-hydroxyl group with *t*-butyldimethylsilyloxyethyl triflate and subsequent cleavage of the silyl protecting group. Everolimus, like rapamycin, is a proliferation signal inhibitor that exerts its immunosuppressive effect by inhibiting the activation of p70 S6 kinase, thereby blocking growth factor-driven proliferation of T cells, B cells and vascular smooth muscle cells, and arresting cell cycle at the G1 phase. Inhibition of p70 S6 kinase activation by everolimus and rapamycin is mediated by their binding to FKBP12 (FK506 binding-protein 12). Everolimus inhibits FK506 binding to FKBP12 with an IC_{50} of 1.8–2.6 nM, and it is about 3- to 5-fold less potent than rapamycin ($IC_{50} = 0.4$–0.9 nM). The *in vitro* immunosuppressive activity of everolimus is also slightly less than that of rapamycin as demonstrated in a mixed lymphocyte reaction (MLR) assay ($IC_{50} = 0.2$–1.6 nM versus 0.07–0.5 nM, respectively) and in antigen-specific human helper T-cell clones ($IC_{50} = 0.05$–0.17 nM versus 0.014–0.37 nM, respectively). However, the *in vivo* immunosuppressive activity of oral everolimus 1–5 mg/kg/day is similar to that of rapamycin at equivalent doses in rat models of renal or cardiac transplantation, localized graft-versus-host disease, and autoimmune glomerulonephritis. The recommended dosage of everolimus is 0.75 mg twice daily, and it is used in combination with cyclosporine microemulsion and corticosteroids. Following oral dosing, the peak concentration (C_{max}) of everolimus is estimated between 1.5 to 2 hours, and steady state is achieved within 4 days. The terminal elimination half-life is 21 to 35 hours. By comparison, rapamycin has a longer elimination half-life (60 hours) and longer time to reach steady state (7 to 14 days). Consequently, rapamycin treatment requires a large loading dose, followed by once daily maintenance dose, whereas everolimus is administered twice daily but without the need of a loading dose. Everolimus is extensively metabolized, primarily by CYP3A4. Approximately 80% of the dose is excreted in the feces and about 5% in the urine. In clinical trials with adult cardiac transplant recipients, oral everolimus 0.75 or 1.5 mg twice daily significantly reduced the incidence of efficacy failure as well as cardiac allograft vasculopathy (CAV) up to 2 years after transplantation as compared with azathioprene 1–3 mg/kg/day. However, graft and patient survival rates at 1 year were similar in patients receiving everolimus and azathioprene. In trials involving renal transplant recipients, the combined incidence of biopsy-confirmed acute rejection, graft loss, death, or loss to follow-up was similar in patients receiving everolimus 1.5 or 3 mg/day or mycophenolate mofetil 2 g/day up to 3 years after transplantation. Everolimus was well tolerated in transplant patients. The incidence of viral infection including cytomegalovirus (CMV) was reduced in comparison to azathioprene and mycophenolate mofetil, but bacterial infections were more frequent. Main adverse events associated with everolimus were thrombocytopenia, leucopenia, and elevated serum lipids and creatinine.

10. FOSFLUCONAZOLE (ANTIFUNGAL) [47–50]

Country of Origin:	Japan
Originator:	Pfizer
First Introduction:	Japan
Introduced by:	Pfizer
Trade Name:	Prodif
CAS Registry No:	194798-83-9
Molecular Weight:	386.25

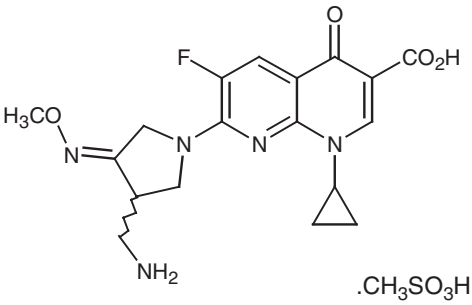


Fosfluconazole is a phosphate prodrug of fluconazole, and it was launched last year in Japan as an intravenous injection for the treatment of candidiasis and cryptococcosis infections. Fluconazole, a triazole antifungal agent, is a selective inhibitor of fungal cytochrome P450 sterol C-14 alpha-demethylation, and it is widely used for the treatment of patients with serious systemic fungal infections. Fluconazole is marketed in both oral and intravenous formulations, the latter being a dilute (2 mg/mL) infusion in saline due to the relatively poor water solubility of the drug. In patients needing high doses (>400 mg) of fluconazole, a drawback of the IV formulation is the requirement of a high-volume infusion, which is undesirable in critically ill patients in whom fluid overload must be avoided. Fosfluconazole is a prodrug with approximately 40-fold higher water solubility than fluconazole, thereby achieving a substantial reduction in infusion volume. It is prepared in three steps starting from fluconazole. In the first step, fluconazole is converted to its dibenzyl phosphite derivative by reaction with phosphorous trichloride and benzyl alcohol. Subsequent oxidation of the phosphite to the corresponding phosphate with hydrogen peroxide and cleavage of the benzyl protecting groups by hydrogenolysis affords fosfluconazole. *In vitro*, fosfluconazole is at least 25-fold less potent than fluconazole against single isolates of *Candida* species and *Cryptococcus neoformans*. *In vivo*, it is rapidly hydrolyzed to fluconazole by phosphatase enzymes and exhibits similar efficacy to fluconazole in experimental models of fungal disease. The hydrolysis potential of fosfluconazole was initially demonstrated in homogenates of kidney, lung and liver of rat, dog, and human. Subsequently, in clinical trials with healthy volunteers ($n = 24$), fosfluconazole was shown to hydrolyze rapidly and almost completely to provide a 97% mean bioavailability of fluconazole. Less than 1% of the administered dose of fosfluconazole was excreted unchanged in the urine, with the majority (85.6%) of the dose eliminated as fluconazole. The terminal half-life was about 2.3 hours, and the volume of distribution was 0.2 L/kg. The time to reach steady state drug levels with 500 mg daily dose was about 10 days, which could be shortened to 3 days by administering loading doses of 1000 mgs on days 1 and 2 followed by 500 mg daily. Further studies showed that hepatic or renal impairment did not significantly alter the pharmacokinetic profile of fosfluconazole. In phase III studies in patients with deep-seated mycosis due to *Candida* or *Cryptococcus* ($n = 160$), a 2-day loading dose regimen of fosfluconazole provided

efficacy range of 73.8% (in Japanese patients) to 91.7% (patients of non-Japanese origin). The adverse events seen in these trials were similar to those previously known with fluconazole therapy and included rash (3.1%), abnormal liver function values (2.5%), asthma (1.9%), and lightheadedness (1.9%).

11. GEMIFLOXACIN (ANTIBACTERIAL) [51–57]

Country of Origin:	S. Korea
Originator:	LG Life Sciences
First Introduction:	US
Introduced by:	Oscient
Trade Name:	Factive
CAS Registry No:	210353-53-0
Molecular Weight:	485.49 (Mesylate)

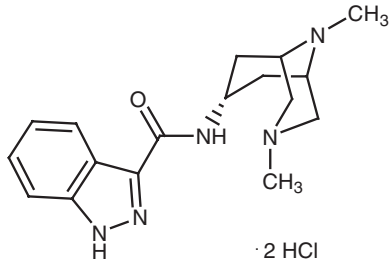


Gemifloxacin is a new fluoroquinolone derivative that is active against both Gram-positive and Gram-negative bacteria, and it was launched last year as an oral treatment for bacterial respiratory infections. Compared with other fluoroquinolones currently on the market, gemifloxacin possesses enhanced *in vitro* activity against *Streptococcus pneumoniae*, including isolates resistant to β-lactams, macrolides, and ciprofloxacin. Gemifloxacin is specifically indicated for treating community-acquired pneumonia (CAP) caused by multi-drug resistant *S. pneumoniae* and for treating acute bacterial exacerbations of chronic bronchitis (AECB). The recommended dose of gemifloxacin is 320 mg daily for 5 days for AECB and 320 mg daily for 7 days for CAP. Fluoroquinolones derive their antibacterial activity by inhibiting either DNA gyrase or DNA topoisomerase IV or both. In *S. pneumoniae*, gemifloxacin is about 10-fold more potent inhibitor of topoisomerase IV (IC_{50} = 1.4 μg/mL) than DNA gyrase (IC_{50} = 47.5 μg/mL), indicating that topoisomerase IV is the primary target in these bacteria. In the same species, gemifloxacin is about 5-fold more potent inhibitor of topoisomerase IV than ciprofloxacin (IC_{50} = 6.4 μg/mL). In measurements of *in vitro* antibacterial activity against 300 isolates of *S. pneumoniae*, gemifloxacin is >30-fold more active than ciprofloxacin, with average MIC_{90} values of 0.06 and 2 μg/mL, respectively. The activity of gemifloxacin against *S. pneumoniae* is relatively unaffected by penicillin or erythromycin resistance, with MIC_{90} values of 0.03 and 0.032 μg/mL, respectively, in the resistant species. Its activity against Gram-negative organisms is generally equivalent to or superior to that of ciprofloxacin, with MIC_{90} values ranging from 0.001 to 0.5 μg/mL depending on the species. The chemical synthesis of gemifloxacin utilizes a chloronaphthyridone intermediate, which is obtained in a multistep sequence and has been widely used in the preparation of fluoroquinolone antibiotics previously.

Gemifloxacin is obtained by the condensation of the chloronaphthyridone intermediate with 4-(aminomethyl)-3-(methoxyimino)pyrrolidine, which in turn is obtained from *N*-Boc-4-cyano-3-pyrrolidone in three steps by sequential condensation with methoxyamine hydrochloride, deprotection of the Boc group, and reduction of the cyano group by catalytic hydrogenation. Gemifloxacin is marketed as a racemic mixture. However, the individual enantiomers have been separated by chiral chromatography and shown to possess equivalent antibacterial activities. In addition, the two enantiomers are shown to have similar oral bioavailability, plasma profile, and pharmacokinetic parameters in rats and dogs. Orally administered gemifloxacin is rapidly absorbed, with the peak concentration being observed in 0.5 to 2 hours. The bioavailability is about 71%. Approximately 60–70% of the drug is protein bound, and the volume of distribution is 4.18 L/kg. Gemifloxacin is metabolized to a minimal extent by the liver. It does not undergo CYP 450 mediated metabolism to a clinically important extent. The half-life is approximately 7 hours, which is not altered by hepatic or renal impairment. Approximately 65% of the drug is excreted unchanged. Urine excretion is 36%, and the fecal excretion is 61%. In a phase III study in CAP patients, gemifloxacin once-daily for 7 days resulted in a clinical success rate of 88.7%, similar to that reported for high-dose amoxicillin + clavulanate given three times a day for 10 days (87.6%). Gemifloxacin also eradicated 95.7% of *S. pneumoniae* clinical isolates, including those resistant to penicillin and erythromycin. In another randomized, double blind study comparing gemifloxacin with levofloxacin in patients with AECB, gemifloxacin 320 mg once-daily for 5 days resulted in a higher clinical success rate (88.2%) as compared to levofloxacin 500 mg once-daily for 7 days (85.1%). The difference in clinical success rates at long-term follow-up was significant, with 80.8% for gemifloxacin and 70.8% for levofloxacin. Gemifloxacin is generally well tolerated. Side effects are minimal and include nausea, vomiting, diarrhea, and abdominal pain.

12. INDISETRON (ANTIEMETIC) [58–62]

Country of Origin:	Japan
Originator:	Nisshin
First Introduction:	Japan
Introduced by:	Nisshin Kyorin
Trade Name:	Sinseron
CAS Registry No:	160472-97-9
Molecular Weight:	386.32

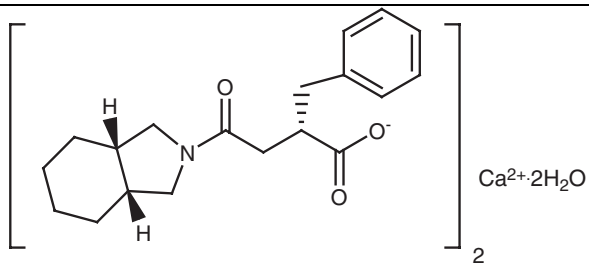


Concomitant with the emesis provoked by radiation and cytotoxic drugs is an increase in 5-hydroxytryptamine (5-HT) concentration in the brain stem and intestinal mucosa, which in turn stimulates the 5-HT_3 receptors on the adjacent vagal afferent nerves. The depolarization of the vagal afferents is responsible for inducing

the vomiting reflex. Indisetron, a 5-HT_3/5-HT_4 antagonist, has, therefore, been launched in Japan as an anti-emetic. Since 5-HT_4 is implicated in intestinal motility, dual antagonism should improve the anti-emetic effect, although this remains to be demonstrated. The diazabicycloamine portion of indisetron is prepared in four steps starting with methylamine and bromoacetaldehyde dimethylacetal. Coupling to the indazole core is accomplished via the acid chloride of 1*H*-indazole-3-carboxylic acid. *In vitro*, indisetron displaced [^{3}H]GR-65630 binding to the 5-HT_3 receptor in rat brain membranes in a concentration-dependent manner with a pK_i value of 8.77, which is comparable to the activities of other 5-HT_3 antagonists such as granisetron and ondansetron. In animal models (ferrets and dogs), indisetron reduced the number and duration of cisplatin-induced emetic episodes when administered orally at 0.1–1 mg/kg prior to cisplatin treatment. Compared to granisetron and ondansetron, there were no significant differences in the frequency of vomiting; however, the duration was significantly reduced with indisetron. After the administration of anticancer agents, including cisplatin, in phase II and phase III clinical trials, indisetron prevented vomiting in 62% of patients (67 of 108) for 24 h. It faired even better in a phase III clinical study where the anticancer agents did not include cisplatin; emesis was averted in 34 of 40 patients (85%). In a single oral dose study (4, 8, 16, and 32 mg under fasting conditions or a 16 mg dose post-prandial) in healthy males, indisetron showed high oral bioavailability (nearly 100%), and C_{max} and AUC increased in a dose-dependent manner. While four subjects experienced adverse effects, they were considered mild and not clinically significant. In a phase I study comprised of 16 mg of indisetron administered b.i.d. over 7 consecutive days, the adverse reactions were also deemed clinically insignificant although it was noted that two patients presented with constipation and an increase in serum amylase. Steady state plasma levels were achieved by day 3. Repeated administration did not change the pharmacokinetics and accumulation of indisetron. Indisetron is metabolized by a host of CYP enzymes (1A1, 2C9, 2D6, and 3A4) in the liver; however, it is unlikely to cause drug interactions at clinical doses because the plasma concentrations are lower than those necessary for CYP inhibition. Finally, as a 5-HT_3 antagonist, indisetron should avoid the adverse effects of the current dopamine-blocking anti-emetics, such as, sedation, ataxia, diarrhea, and tasikinesia, making it a viable alternative to existing therapy.

13. MITIGLINIDE (ANTIDIABETIC) [63–67]

Country of Origin:	Japan
Originator:	Kissei
First Introduction:	Japan
Introduced by:	Kissei
Trade Name:	Glufast
CAS Registry No:	145525-41-3
Molecular Weight:	704.91



Mitiglinide is a non-sulfonylurea hypoglycemic agent that has been developed and launched in Japan for the treatment of type-2 diabetes. Similar to the sulfonylurea insulinotropic drugs, mitiglinide adopts a U-shaped configuration in which the base of the U contains an amide linkage, and each branch of the U incorporates a hydrophobic side chain. This similarity in conformation suggests that mitiglinide also binds to the sulfonylurea receptor to cause the direct closing of ATP-sensitive potassium channels in pancreatic β-cells; the result is stimulation of insulin secretion. In contrast to typical sulfonylurea agents that frequently cause hypoglycemia due to slowly reversed insulinotropic activity, mitiglinide's short duration of action should be advantageous in preventing this adverse effect. It also enjoys a rapid onset of insulin release. Mitiglinide can be prepared by several closely related methods, which involve either classical resolution of racemic intermediates, or enantioselective methods, such as, chiral enolate alkylation, and asymmetric hydrogenation with a rhodium or ruthenium-based chiral diphosphine complex. A highly efficient preparative method for mitiglinide involves the diasteroselective alkylation of a chiral acylsultam intermediate that is obtained by the reaction of 3-phenylpropionyl chloride with (-)-camphorsultam. The chiral enolate of the acylsultam is derived by using sodium hexamethyldisilazane as the base, and is subsequently alkylated with *tert*-butyl bromoacetate to achieve >93% diastereomeric purity of the product. Following cleavage of the *tert*-butyl ester with trifluoroacetic acid, the resultant acid is coupled with (3a*R*,7a*S*)octahydro-1*H*-isoindole, and the camphorsultam chiral auxiliary is removed by saponification to produce the parent acid of mitiglinide in high yield. *In vitro*, mitiglinide has about a 1000-fold greater affinity for the Kir6.2/SUR1 form of potassium-ATP channels expressed in β-cells ($IC_{50} = 4$ nM) than for the Kir6.2/SUR2A or Kir6.2/SUR2B channel types found in cardiac and smooth muscle. In fact, it is significantly less potent in blocking potassium-ATP channels than the prototype sulfonylurea glyburide ($IC_{50} = 42$ μM vs. 0.13 μM, respectively); thus, it possesses a more favorable cardiac safety profile. Phase III clinical data demonstrated that mitiglinide significantly improved indices of blood glucose control (postprandial glucose and fasting plasma glucose levels) in a double blind, comparative study. It was also confirmed that the incidence of hypoglycemia, a frequent adverse effect, remained as low as placebo. In another placebo-controlled study involving twenty-two patients with type-2 diabetes, mitiglinide 5 mg t.i.d. treatment significantly suppressed postprandial plasma glucose elevations (181 vs. 261 mg/dL with placebo), and the daily change in blood glucose level was reduced with no subjective symptoms. No episodes of hypoglycemia or abnormal clinical laboratory parameters were noted. Regarding the pharmacokinetics, a single oral dose (unspecified) of mitiglinide reached its peak plasma concentration of about 0.5 μg/mL at 30 minutes post dose and then steadily declined to about 0.04 μg/mL at 4 h.

14. NATALIZUMAB (MULTIPLE SCLEROSIS) [68–74]

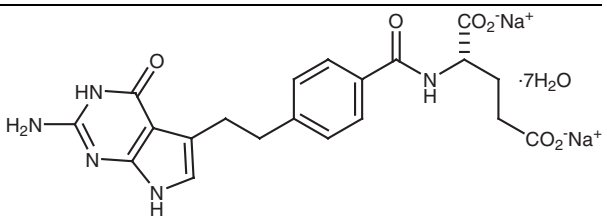
Country of Origin:	UK
Originator:	Elan
First Introduction:	US
Introduced by:	Elan/Biogen Idec
Trade Name:	Tysabri
CAS Registry No:	189261-10-7
Manufacturer:	Biogen Idec
Class:	Recombinant humanized, murine monoclonal antibody
Type:	Humanized $IgG4_{\kappa}$, anti-VLA4
Molecular Weight:	149 kDa
Expression system:	murine myeloma cells

The α4 family of integrins expressed on the surface of leukocytes are involved in cell adhesion processes. The α4 integrin can pair with either of two β subunits to generate a heterodimeric cell surface receptor known as α4β1 (VLA4) or α4β7. Ligands for VLA4 include vascular cell adhesion molecule-1 (VCAM-1), which is expressed on activated vascular endothelium, while α4β7 interacts predominantly with mucosal addressin cell adhesion molecule-1 (MadCAM-1) existing on vascular endothelial cells of the gastrointestinal tract. By virtue of this α4–mediated interaction between leukocytes and vascular endothelial cells that leads to trans-endothelial infiltration of various leukocytes (lymphocytes, monocytes, T-cells, etc.) at the site of inflammation, interference with the adhesion of the α4 integrin has been deemed a viable approach for disrupting the inflammatory cascade. As an antibody that binds to the α4 integrin subunit, natalizumab has been developed and launched for the treatment of multiple sclerosis, a chronic inflammatory disorder of the central nervous system. It is also being developed for other chronic inflammatory diseases, such as, Crohn's disease, rheumatoid arthritis, and irritable bowel syndrome (IBS). Natalizumab is a recombinant humanized monoclonal antibody produced in murine myeloma cells. It contains human framework regions and the complementarity-determining regions of an antibody that is targeted to the α4 integrin. For the treatment of irritable bowel diseases (Crohn's disease, ulcerative colitis, and IBS), the target is the α4β7 glycoprotein while efficacy in treating MS is attributed to binding to the α4 subunit of α4β1. For MS, the binding of natalizumab prevents docking of VCAM-1 to its receptor on leukocytes, thereby, effectively inhibiting leukocyte trafficking across the blood brain-barrier (BBB). A reduction in migration across the BBB translates into a reduction in lesions and relapses. In a two-year, placebo-controlled, double blind phase III study, a once-monthly, 300 mg i.v. infusion of natalizumab reduced relapses by 66% compared to placebo. All of the secondary endpoints, such as, the number of new or newly enlarging T2-hyperintense lesions, the number of gadolinium-enhancing lesions, and the proportion of relapse-free patients, were all met. Regarding side effects, headache, fatigue, and arthralgia were reported in 5% of natalizumab patients, 2%

more common than observed with placebo. Serious hypersensitivity-like reactions were experienced in 1% of the natalizumab group. In these cases, adverse effects usually developed within two hours of the onset of the infusion. The symptoms included urticaria, fever, rash, rigors, dizziness, pruritus, nausea, flushing, dyspnea, hypotension, and chest pain. Antibodies to natalizumab are believed to be responsible, and any patient experiencing hypersensitivity should discontinue further treatment. Since adequate studies have not been performed in the pregnant, pediatric, and elderly, natalizumab is currently contraindicated in these patient populations. In addition, this drug should not be taken concurrent with medications that suppress the immune system, such as, corticosteroids; the combination increases the risk for serious infections. With a dose of 300 mg to MS patients, the long half-life of 11 ± 4 days results in a once-a-month trip to the physician for the one-hour infusion. Natalizumab is cleared at a rate of 16 mL/h with a C_{max} of 98 μg/mL and a mean steady-state concentration of approximately 30 μg/mL.

15. PEMETREXED (ANTICANCER) [75–77]

Country of Origin:	US
Originator:	Eli Lilly
First Introduction:	US
Introduced by:	Eli Lilly
Trade Name:	Alimta
CAS Registry No:	150399-23-8
Molecular Weight:	597.49

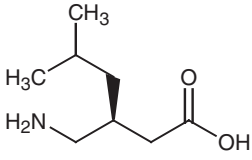


Pemetrexed, a pyrrolo[2,3-*d*]pyrimidine-based antifolate that disrupts cell replication by inhibiting multiple folate-dependent metabolic processes, was initially developed and launched in the US for the treatment of malignant pleural mesothelioma in conjunction with cisplatin. Patients who are not candidates for surgery may benefit from this combination therapy. Clinical data demonstrated that the median overall survival time increased to 12.1 months, compared with 9.3 months for patients receiving cisplatin alone. In August of 2004, the FDA also approved pemetrexed as a second-line treatment of non-small-cell lung cancer (NSCLC). While median survival is comparable to the standard second-line treatment docetaxel, the improved toxicity profile (significant reduction in neutropenia) accelerated the approval for NSCLC. Its effectiveness as an anticancer drug is derived from its ability to gain internal cell access via the reduced folate carrier and membrane folate binding protein transport systems. Once inside, pemetrexed undergoes polyglutamation, and the resultant polyglutamate forms (predominantly the pentaglutamate) inhibit the folate-dependent enzymes thymidylate synthase

(TS), dihydrofolate reductase (DHFR), and glycinamide ribonucleotide formyltransferase (GARFT). Against recombinant human TS, pemetrexed has a Ki of 109 nM while the triglutamate and pentaglutamate forms have Ki values of 1.6 nM and 1.3 nM, respectively. All forms of pemetrexed display similar potency against recombinant human DHFR (7 nM), but the pentaglutamate form is significantly more potent against recombinant murine GARFT than the parent (Ki = 65 nM versus 9.3 μM). The selectivity of pemetrexed may be explained by the fact that polyglutamation is more likely to occur in cancer cells compared to normal cells while its prolonged duration of action may be attributed to decreased cellular efflux of the polyglutamate forms. While several different routes have provided pemetrexed, one of the most efficient exploits the propensity of 2,6-diamino-3*H*-pyrimidin-4-one to undergo Michael additions at its unsubstituted C-5 position. Using ethyl 4-(4-nitrobut-3-enyl)benzoate as the Michael acceptor, the resulting adduct is then converted to the ultimate precursor for glutamyl coupling via a one-pot, three-step process (Nef reaction to transform the nitro to the aldehyde, intramolecular condensation to afford the pyrrole, and saponification of the ethyl ester). A typical treatment regimen involves intravenous administration of pemetrexed, infused over ten minutes, at a dose of 500 mg/m^2 followed by a thirty minute wash-out period and then cisplatin intravenously over two hours at a dose of 75 mg/m^2. Both drugs are given on Day 1 of a 21-day cycle. In order to reduce treatment-related hematological and GI toxicity, patients are instructed to take folic acid and vitamin B_{12} as a prophylactic measure. Pretreatment with a corticosteroid is also recommended to prevent possible skin rashes. Pemetrexed is primarily excreted intact in the urine, with 70–90% of the dose being recovered within 24 hours of administration. The half-life of pemetrexed is 3.5 hours in patients with normal renal function, and the total systemic clearance is 91.8 mL/min. As expected, clearance decreases as renal impairment increases. The drug's plasma protein binding is 81%, and it has a steady state volume of distribution of 16.1 L. The pharmacokinetics of pemetrexed is linear with dose and remains unchanged over multiple treatment cycles. While *in vitro* studies suggest that pemetrexed would not interfere with drugs metabolized by CYP3A4, CYP2D6, CYP2C9, and CYP1A2, ibuprofen (400 mg q.d.) does reduce pemetrexed clearance by 20%. Caution should, therefore, be taken when administering pemetrexed concurrently with ibuprofen to patients with renal insufficiency and should not be given at all to patients whose creatinine clearance is <45 mL/min.

16. PREGABALIN (ANTIEPILEPTIC) [78–81]

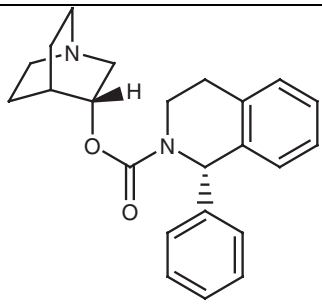
Country of Origin:	US
Originator:	Warner-Lambert
First Introduction:	UK
Introduced by:	Pfizer
Trade Name:	Lyrica
CAS Registry No.:	148553-50-8
Molecular Weight:	159.23



As a follow-up to its γ-aminobutyric acid (GABA) agonist gabapentin, Pfizer has developed and launched pregabalin for the treatment of epilepsy and neuropathic pain. Although pregabalin is a structural analog of GABA, it does not interact with GABA-A or GABA-B receptors or influence GABA uptake. The exact mechanism of action is unclear, but pregabalin may reduce excitatory neurotransmitter release by binding to the α_2-δ protein subunit of voltage-gated calcium channels. The resulting inhibition of excess neuronal activity is believed to be the basis for pregabalin's efficacy in epilepsy and neuropathic pain alleviation. Since the activity is attributed to the (*S*)-enantiomer alone, an efficient asymmetric synthesis is employed for commercial production. The key step is the asymmetric hydrogenation of 3-cyano-5-methyl-3-hexenoic acid using a chiral rhodium catalyst to afford an intermediate that is enriched in the (*S*)-enantiomer. The cyano group is ultimately reduced by routine hydrogenation with a nickel catalyst. Further enrichment of the final product is realized by selective recrystallization with (*S*)-mandelic acid or simply recrystallizing from water/isopropanol. Compared to gabapentin, pregabalin is 2- to 10-fold more potent in various animal models. For example, in preventing maximal electroshock seizures (MES) in mice, pregabalin has an ED_{50} of 20 mg/kg p.o. versus 87 mg/kg for gabapentin. A comparable increase in potency is also observed in the rat MES model ($ED_{50} = 1.8$ mg/kg p.o. for pregabalin versus 10.3 mg/kg for gabapentin). In addition, pregabalin's linear pharmacokinetics (C_{max} relates to dose) translates to better predictability of pharmacological effects. It has 90% oral bioavailability, with an elimination half-life of approximately 6 h. The primary route of excretion is via the renal system with negligible metabolism. Furthermore, its lack of activity at the cytochrome P450 enzymes was reflected in an absence of pharmacokinetic drug-drug interactions in relevant studies. In a placebo-controlled, fixed dose (up to 600 mg/day) trial with pregabalin as an adjunctive therapy for epilepsy, 14 to 51% of patients showed at least a 50% decrease in seizure frequency with a clear dose-response relationship. In a flexible dosing group, (150 mg/day to 600 mg/day), the seizure reduction rate was 35.4% compared to 40.3% for a fixed dose of 600 mg/day and 10.6% for placebo. The most common side effects were dizziness (29%) and somnolence (21%). In addition, weight gain (equal to or more than 7% increase from baseline) occurred in 40% of patients in the 12-week study; however, there was no affect on male fertility or efficacy of oral contraceptives in women. Regarding the use of pregabalin in treating painful diabetic peripheral neuropathy, oral administration of 300 and 600 mg/day t.i.d. was superior to placebo (39–48% compared to 15–18% with placebo) in relieving pain and improving pain-related sleep interference. While pregabalin was originally developed as an anticonvulsant for epilepsy, its success in treating neuropathic pain has led to its exploration in treating other CNS disorders, such as, anxiety, social phobia, and fibromyalgia.

17. SOLIFENACIN (POLLAKIURIA) [82–87]

Country of Origin:	Japan
Originator:	Yamanouchi
First Introduction:	UK
Introduced by:	Yamanouchi
Trade Name:	Vesicare
CAS Registry No:	242478-38-2
Molecular Weight:	362.46

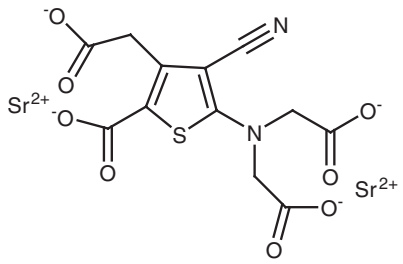


Solifenacin is an M3 muscarinic receptor antagonist that was developed and launched for the treatment of overactive bladder (pollakiuria) in Europe. M3 receptors have been implicated in neurally evoked smooth muscle contractions of the bladder, and M2 receptors have also been suspected of playing a role because of their dominance in the detrusor muscle. Solifenacin displays affinity for both M3 and M2 receptors with K_i values of 9.9 nM and 120 nM, respectively. Since muscarinic salivary glands are of the M3 persuasion, a common side effect of antimuscarinic therapy is dry mouth. At the cellular level, solifenacin possesses a selective preference for bladder over salivary gland that is 15-fold greater than that of atropine suggesting a lower probability of inducing dry mouth at pharmacologically relevant doses. The synthesis of solifenacin involves the preparation of racemic 1-phenyl-1,2,3,4-tetrahydroisoquinoline via cyclization of *N*-(2-phenylethyl)benzamide, and subsequent reaction with ethyl chloroformate and transesterification with (*R*)-3-quinuclidinol. Chiral chromatography affords the isolation of the desired diastereomer. Alternatively, 1-phenyl-1,2,3,4-tetrahydroisoquinoline may be subjected to optical resolution with (+)-tartaric acid prior to treatment with ethyl chloroformate and subsequent transesterification. The pooled results of four phase III trials concluded that 63% of women receiving 5 mg of solifenacin once daily and 68% of women receiving 10 mg once daily reported a 50% or more reduction in urgency episodes, compared to 44% of women taking placebo. This compares with a 53% reduction in patients receiving tolterodine twice daily. In another placebo-controlled trial, with the change in the number of micturitions in a 24-h period as the primary endpoint, once-daily solifenacin recorded an 18% decrease for a 5-mg dose and a 21% decrease for a 10-mg dose compared to 10% with placebo. Pharmacokinetic studies have demonstrated that solifenacin has an oral bioavailability of 90%, a long elimination half-life (50 h), low clearance (9.39 L/h), a mean V_{ss} of 599 L, a C_{max} of approximately 14 ng/mL, and a time to maximal plasma concentration of 4 h making it suitable for q.d. dosing. Furthermore, these PK parameters are not affected by food ingestion. Solifenacin is excreted predominantly in the feces with only 3–6% found in

urine. It is contraindicated in patients with hepatic impairment, gastric retention, urinary retention, or uncontrolled narrow angle glaucoma. Further precautions, such as dose adjustment, should be considered for patients with concurrent use of ketoconazole or other potent CYP3A4 inhibitors or for patients with a history of QT prolongation or currently on medications known to prolong the QT interval. Finally, while other muscarinic antagonists have been explored in the treatment of irritable bowel syndrome (IBS), it is too early to predict the therapeutic utility of solifenacin for IBS although animal studies are promising.

18. STRONTIUM RANELATE (OSTEOPOROSIS) [88–90]

Country of Origin:	Japan
Originator:	Fujisawa
First Introduction:	UK
Introduced by:	Servier
Trade Name:	Protelos
CAS Registry No:	135459-87-9
Molecular Weight:	521.55

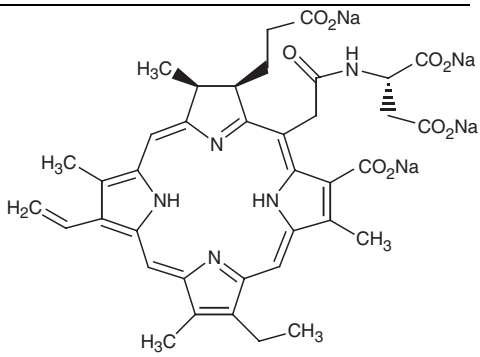


Strontium ranelate, a divalent strontium salt of ranelic acid, has been developed and launched for the treatment of osteoporosis. As early as 1910, investigations suggested that strontium stimulates the formation of osteoid tissues while simultaneously repressing the resorptive process in bones. Specifically, strontium enhances pre-osteoblastic cell replication, inhibits pre-osteoclast differentiation, and suppresses the bone-resorbng activity of osteoclasts. From the evaluation of 26 strontium salts, ranelic acid was selected as the ideal strontium carrier due to its physicochemical and pharmacokinetic properties. The thiophene core of ranelic acid is constructed by the condensation of dialkyl 3-oxoglutarate, malononitrile, and sulfur in a suitable alcohol in the presence of morpholine or diethylamine. The resultant diester of 5-amino-3-carboxymethyl-4-cyano-2-thiophenecarboxylic acid is subsequently dialkylated with an alkyl bromoacetate to provide the tetraester precursor to strontium ranelate. Strontium ranelate is supplied in a 2 g sachet, and the drug is evenly suspended in water prior to consumption. Since the simultaneous ingestion of either calcium or food has a negative influence on the bioavailability of strontium ranelate, it is recommended that strontium ranelate be administered once a day at bedtime. Following this regimen, the absolute bioavailability of strontium is 27% while that of ranelic acid is 2.5%. Because strontium ranelate dissociates after intake, and ranelic acid has negligible absorption, the effects of the drug on

bone metabolism are dependent on the pharmacokinetics of strontium. In postmenopausal women, the half-life of strontium is 6.3 ± 2.3 days, and renal clearance accounts for 57% of the total clearance of 12 mL/min. After a 25-day treatment, the maximum plasma concentration of strontium is 20 ± 2.3 mg/L. In addition, not only is perfect stability of strontium plasma concentration achieved within 3 to 24 months of chronic administration so is stabilization of strontium incorporation into bones. Strontium is incorporated into bone by two mechanisms. The predominant mode involves the rapid, saturable surface exchange with calcium. A slower mechanism embodies the incorporation of strontium into the crystal lattice of the bone mineral; however, only a small amount of calcium in the apatite is substituted by strontium at pharmacological doses. A phase II clinical trial assessed the effect of various strontium ranelate doses in postmenopausal women with established osteoporosis. The primary efficacy endpoint for this double-blind, randomized, placebo-controlled trial was the measure of mean lumbar bone mineral density (BMD) by dual-energy X-ray absorptiometry. A statistically significant dose-dependent increase in lumbar BMD was observed; increases of 1.3, 5.9, 8.3, and 13.6% were recorded for placebo, 500-, 1000-, and 2000-mg doses of strontium ranelate, respectively. In a phase III trial encompassing 1,649 osteoporotic postmenopausal women from 12 countries, the efficacy of a 2 g/day dose in preventing new vertebral fractures was evaluated. The mean lumbar BMD was 0.73 g/cm^2 while the mean age at baseline was 70 years. All of the enrolled patients had at least one prior vertebral fracture. The primary end point for this study was a reduction in the incidence of patients experiencing fractures. While 222 women in the placebo group experienced a new vertebral fracture, only 139 patients treated with strontium ranelate presented with new fractures. Furthermore, the risk of fracture was reduced by 51% in the third year alone, implicating the sustained efficacy of the drug. For both the phase II and phase III studies, strontium ranelate was well tolerated with most of the adverse events being mild-to-moderate in severity. The most commonly reported events in all treatment groups were musculoskeletal disorders (back pain, arthralgia, and lumbar pain). As for laboratory measurements, only creatine phosphokinase, the musculoskeletal isoenzyme, was significantly elevated in the 1000-mg and 2000-mg strontium ranelate groups; however, this did not translate into any particular clinical or biological abnormality. Without relevant data regarding bone safety in patients with renal impairment, strontium ranelate is currently contraindicated in patients with creatine clearance below 30 mL/min.

19. TALAPORFIN SODIUM (ANTICANCER) [91–95]

Country of Origin:	Japan
Originator:	Nippon Petrochem.
First Introduction:	Japan
Introduced by:	Nippon Petrochem./ Light Sciences Corp./Meiji Seika Kaisha
Trade Name:	Laserphyrin
CAS Registry No:	110230-98-3
Molecular Weight:	799.69

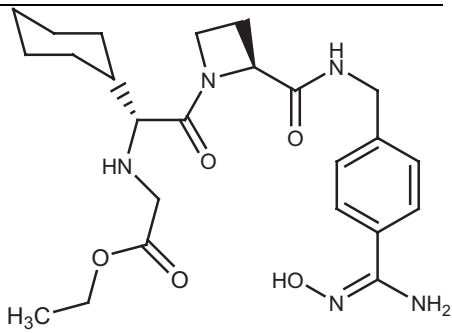


In collaboration with Light Sciences Corp. and Meiji Seika Kaisha, Nippon Petrochemicals has developed and launched the injectable photosensitizer talaporfin sodium in Japan for the photodynamic therapy (PDT) of cancer. The initial approval is for the treatment of early stage lung cancer, but Light Sciences Corp. and its subsidiaries are also developing talaporfin sodium for other hyperproliferative diseases, such as, liver metastases arising from colorectal cancer, wet age-related macular degeneration, and atherosclerosis. Talaporfin sodium is typically supplied as a lyophilized green powder, and it is synthesized via a carbodiimide-mediated coupling of chlorin e_6 (obtained from precursors that were extracted from natural sources) with L-aspartic acid. Since chlorin e_6 contains three carboxylic acid groups, the coupling reaction produces a mixture of aspartic acid conjugates. The desired site of conjugation is the acetic acid side chain of C-20, and the other regioisomers are removed by chromatographic purification. Compared to other photosensitizers, talaporfin sodium is associated with minimal cutaneous photosensitivity, is activated at long wavelengths permitting deeper tissue penetration, and requires a shorter interval between intravenous administration and photoactivation. It has a serum half-life of nine hours and is excreted unmetabolized, predominantly by the biliary system. In the clinical study of patients with early lung cancer, a complete response was obtained in approximately 86% of the lesions (administration at 40 mg/m^2 followed by laser irradiation at 100 J/cm^2, 4–6 hours later). In addition to laser activation, a clinical study involving a variety of refractory solid tumors demonstrated that intratumoral delivery of light-emitting diodes was effective; a 33% overall response rate was observed with no cutaneous phototoxicity. Regardless of the mode of activation, the outcome is the same; the activated photosensitizer reacts with endogenous oxygen to generate singlet oxygen that ultimately leads to apoptosis and vascular ischemia of the targeted tissue. The most significant

adverse effect associated with talaporfin sodium was generalized cutaneous photosensitivity, and erythema and oedema were common as well. Individual cases of nausea, vomiting, diarrhea, heartburn, headache, and pruritus were also reported.

20. XIMELAGATRAN (ANTICOAGULANT) [96–98]

Country of Origin:	Germany
Originator:	AstraZeneca
First Introduction:	Germany
Introduced by:	AstraZeneca
Trade Name:	Exanta
CAS Registry No.:	192939-46-1
Molecular Weight:	473.57



Ximelagatran, a prodrug of melagatran with improved oral bioavailability, is a direct thrombin inhibitor that was launched for the prevention of venous thromboembolic events (VTE) in elective hip or knee replacement surgery in Germany with several European countries following with approval for the same indication. A mutual recognition European filing was subsequently submitted for the prevention of stroke and other thromboembolic complications associated with atrial fibrillation (AF). While studies indicate that ximelagatran is as effective as traditional therapies for preventing strokes and recurring blood clots, the U.S. Food and Drug Administration has currently declined approval due to potential hepatotoxicity. Elevation of alanine aminotransferase (three times the upper limit of normal) has been observed in the first four months of therapy, but levels regress to normal upon discontinuation of the drug. Despite the questions surrounding the toxicological consequences of this elevated liver enzyme, ximelagatran remains an attractive alternative to the current antithrombotic therapies that utilize either the low molecular weight heparin (LMWH) or warfarin. Since LMWH is administered subcutaneously once or twice daily, the oral agent ximelagatran is preferable for patient compliance. In addition to the convenience of oral therapy, ximelagatran does not require the frequent laboratory monitoring and dosage adjustment that is necessary with warfarin treatment. A clinical study comparing the efficacy of a fixed dose (36 mg b.i.d.) of ximelagatran with adjusted dose warfarin for stroke prevention in patients with nonvalvular atrial fibrillation concluded that ximelagatran is not inferior to warfarin, and major bleeding occurred at rates similar to warfarin. The synthesis route to ximelagatran involves the coupling of the three major components, cyclohexylglycine, azetidine-2-carboxylic acid, and protected

p-amidinobenzylamine, using solution-phase peptide chemistry. Subsequent alkylation of the *N*-terminus with ethyl bromoacetate, followed by deprotection of the amidine group and conversion to hydroxyamidine affords the double prodrug of melagatran. Delivery as ximelagatran provides reproducible oral bioavailability (18–25%), as measured by concentrations of the active metabolite melagatran formed by hydrolysis of the ethyl ester and dehydroxylation of the amidine. Melagatran reversibly binds to the arginine side pocket of both free and clot-bound thrombin ($K_i = 2\,nM$). Inhibition of thrombin ultimately blocks the conversion of fibrinogen to fibrin, the final step of the coagulation process. A linear relationship between ximelagatran dose and melagatran concentration exists with peak concentrations observed two to three hours post dose. Renal excretion is the primary route of elimination of melagatran (80%) with a half-life of 3–5 hours. Furthermore, the pharmacokinetics of ximelagatran is not influenced by the type of thromboembolic disease, obesity, ethnicity, gender, or age. In addition to the typical contraindications of current antithrombotic therapies, the increase in the liver enzyme alanine aminotransferase suggests that ximelagatran should not be used in patients with creatine clearance $< 30\,mL/min.$ pending further study in this population. While ximelagatran does not appear to have any interactions with the cytochrome P-450 system, combination with aspirin has been shown to increase adverse bleeding.

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GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
abacavir sulfate	antiviral	1999	35, 333
abarelix	anticancer	2004	40, 446
acarbose	antidiabetic	1990	26, 297
aceclofenac	antiinflammatory	1992	28, 325
acemannan	wound healing agent	2001	37, 259
acetohydroxamic acid	hypoammonuric	1983	19, 313
acetorphan	antidiarrheal	1993	29, 332
acipimox	hypolipidemic	1985	21, 323
acitretin	antipsoriatic	1989	25, 309
acrivastine	antihistamine	1988	24, 295
actarit	antirheumatic	1994	30, 296
adalimumab	rheumatoid arthritis	2003	39, 267
adamantanium bromide	antiseptic	1984	20, 315
adefovir dipivoxil	antiviral	2002	38, 348
adrafinil	psychostimulant	1986	22, 315
AF-2259	antiinflammatory	1987	23, 325
afloqualone	muscle relaxant	1983	19, 313
agalsidase alfa	fabry's disease	2001	37, 259
alacepril	antihypertensive	1988	24, 296
alclometasone dipropionate	topical anti-inflammatory	1985	21, 323
alefacept	plaque psoriasis	2003	39, 267
alemtuzumab	anticancer	2001	37, 260
alendronate sodium	osteoporosis	1993	29, 332
alfentanil HCl	analgesic	1983	19, 314
alfuzosin HCl	antihypertensive	1988	24, 296
alglucerase	enzyme	1991	27, 321
alitretinoin	anticancer	1999	35, 333
alminoprofen	analgesic	1983	19, 314
almotriptan	antimigraine	2000	36, 295
anakinra	antiarthritic	2001	37, 261
alosetron hydrochloride	irritable bowel syndrome	2000	36, 295
alpha-1 antitrypsin	protease inhibitor	1988	24, 297
alpidem	anxiolytic	1991	27, 322
alpiropride	antimigraine	1988	24, 296
alteplase	thrombolytic	1987	23, 326
amfenac sodium	antiinflammatory	1986	22, 315
amifostine	cytoprotective	1995	31, 338
aminoprofen	topical antiinflammatory	1990	26, 298
amisulpride	antipsychotic	1986	22, 316
amlexanox	antiasthmatic	1987	23, 327
amlodipine besylate	antihypertensive	1990	26, 298
amorolfine HCl	topical antifungal	1991	27, 322
amosulalol	antihypertensive	1988	24, 297
ampiroxicam	antiinflammatory	1994	30, 296

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
amprenavir	antiviral	1999	35, 334
amrinone	cardiotonic	1983	19, 314
amrubicin HCl	antineoplastic	2002	38, 349
amsacrine	antineoplastic	1987	23, 327
amtolmetin guacil	antiinflammatory	1993	29, 332
anagrelide HCl	hematological	1997	33, 328
anastrozole	antineoplastic	1995	31, 338
angiotensin II	anticancer adjuvant	1994	30, 296
aniracetam	cognition enhancer	1993	29, 333
anti-digoxin polyclonal antibody	antidote	2002	38, 350
APD	calcium regulator	1987	23, 326
apraclonidine HCl	antiglaucoma	1988	24, 297
aprepitant	antiemetic	2003	39, 268
APSAC	thrombolytic	1987	23, 326
aranidipine	antihypertensive	1996	32, 306
arbekacin	antibiotic	1990	26, 298
argatroban	antithromobotic	1990	26, 299
arglabin	anticancer	1999	35, 335
aripiprazole	neuroleptic	2002	38, 350
arotinolol HCl	antihypertensive	1986	22, 316
arteether	antimalarial	2000	36, 296
artemisinin	antimalarial	1987	23, 327
aspoxicillin	antibiotic	1987	23, 328
astemizole	antihistamine	1983	19, 314
astromycin sulfate	antibiotic	1985	21, 324
atazanavir	antiviral	2003	39, 269
atomoxetine	attention deficit hyperactivity disorder	2003	39, 270
atorvastatin calcium	dyslipidemia	1997	33, 328
atosiban	preterm labor	2000	36, 297
atovaquone	antiparasitic	1992	28, 326
auranofin	chrysotherapeutic	1983	19, 314
azacitidine	anticancer	2004	40, 447
azelnidipine	antihypertensive	2003	39, 270
azelaic acid	antiacne	1989	25, 310
azelastine HCl	antihistamine	1986	22, 316
azithromycin	antibiotic	1988	24, 298
azosemide	diuretic	1986	22, 316
aztreonam	antibiotic	1984	20, 315
balofloxacin	antibacterial	2002	38, 351
balsalazide disodium	ulcerative colitis	1997	33, 329
bambuterol	bronchodilator	1990	26, 299
barnidipine HCl	antihypertensive	1992	28, 326
beclobrate	hypolipidemic	1986	22, 317
befunolol HCl	antiglaucoma	1983	19, 315
belotecan	anticancer	2004	40, 449

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
benazepril HCl	antihypertensive	1990	26, 299
benexate HCl	antiulcer	1987	23, 328
benidipine HCl	antihypertensive	1991	27, 322
beraprost sodium	platelet aggreg. inhibitor	1992	28, 326
betamethasone butyrate prospinate	topical antiinflammatory	1994	30, 297
betaxolol HCl	antihypertensive	1983	19, 315
betotastine besilate	antiallergic	2000	36, 297
bevacizumab	anticancer	2004	40, 450
bevantolol HCl	antihypertensive	1987	23, 328
bexarotene	anticancer	2000	36, 298
biapenem	antibacterial	2002	38, 351
bicalutamide	antineoplastic	1995	31, 338
bifemelane HCl	nootropic	1987	23, 329
bimatoprost	antiglaucoma	2001	37, 261
binfonazole	hypnotic	1983	19, 315
binifibrate	hypolipidemic	1986	22, 317
bisantrene HCl	antineoplastic	1990	26, 300
bisoprolol fumarate	antihypertensive	1986	22, 317
bivalirudin	antithrombotic	2000	36, 298
bopindolol	antihypertensive	1985	21, 324
bortezomib	anticancer	2003	39, 271
bosentan	antihypertensive	2001	37, 262
brimonidine	antiglaucoma	1996	32, 306
brinzolamide	antiglaucoma	1998	34, 318
brodimoprin	antibiotic	1993	29, 333
bromfenac sodium	NSAID	1997	33, 329
brotizolam	hypnotic	1983	19, 315
brovincamine fumarate	cerebral vasodilator	1986	22, 317
bucillamine	immunomodulator	1987	23, 329
bucladesine sodium	cardiostimulant	1984	20, 316
budipine	antiParkinsonian	1997	33, 330
budralazine	antihypertensive	1983	19, 315
bulaquine	antimalarial	2000	36, 299
bunazosin HCl	antihypertensive	1985	21, 324
bupropion HCl	antidepressant	1989	25, 310
buserelin acetate	hormone	1984	20, 316
buspirone HCl	anxiolytic	1985	21, 324
butenafine HCl	topical antifungal	1992	28, 327
butibufen	antiinflammatory	1992	28, 327
butoconazole	topical antifungal	1986	22, 318
butoctamide	hypnotic	1984	20, 316
butyl flufenamate	topical antiinflammatory	1983	19, 316
cabergoline	antiprolactin	1993	29, 334
cadexomer iodine	wound healing agent	1983	19, 316
cadralazine	hypertensive	1988	24, 298
calcipotriol	antipsoriatic	1991	27, 323

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
camostat mesylate	antineoplastic	1985	21, 325
candesartan cilexetil	antihypertension	1997	33, 330
capecitabine	antineoplastic	1998	34, 319
captopril	antihypertensive agent	1982	13, 086
carboplatin	antibiotic	1986	22, 318
carperitide	congestive heart failure	1995	31, 339
carumonam	antibiotic	1988	24, 298
carvedilol	antihypertensive	1991	27, 323
caspofungin acetate	antifungal	2001	37, 263
cefbuperazone sodium	antibiotic	1985	21, 325
cefcapene pivoxil	antibiotic	1997	33, 330
cefdinir	antibiotic	1991	27, 323
cefditoren pivoxil	oral cephalosporin	1994	30, 297
cefepime	antibiotic	1993	29, 334
cefetamet pivoxil HCl	antibiotic	1992	28, 327
cefixime	antibiotic	1987	23, 329
cefmenoxime HCl	antibiotic	1983	19, 316
cefminox sodium	antibiotic	1987	23, 330
cefodizime sodium	antibiotic	1990	26, 300
cefonicid sodium	antibiotic	1984	20, 316
ceforanide	antibiotic	1984	20, 317
cefoselis	antibiotic	1998	34, 319
cefotetan disodium	antibiotic	1984	20, 317
cefotiam hexetil HCl	antibiotic	1991	27, 324
cefozopran HCl	injectable cephalosporin	1995	31, 339
cefpimizole	antibiotic	1987	23, 330
cefpiramide sodium	antibiotic	1985	21, 325
cefpirome sulfate	antibiotic	1992	28, 328
cefpodoxime proxetil	antibiotic	1989	25, 310
cefprozil	antibiotic	1992	28, 328
ceftazidime	antibiotic	1983	19, 316
cefteram pivoxil	antibiotic	1987	23, 330
ceftibuten	antibiotic	1992	28, 329
cefuroxime axetil	antibiotic	1987	23, 331
cefuzonam sodium	antibiotic	1987	23, 331
celecoxib	antiarthritic	1999	35, 335
celiprolol HCl	antihypertensive	1983	19, 317
centchroman	antiestrogen	1991	27, 324
centoxin	immunomodulator	1991	27, 325
cerivastatin	dyslipidemia	1997	33, 331
cetirizine HCl	antihistamine	1987	23, 331
cetrorelix	female infertility	1999	35, 336
cetuximab	anticancer	2003	39, 272
cevimeline hydrochloride	anti-xerostomia	2000	36, 299
chenodiol	anticholelithogenic	1983	19, 317
CHF-1301	antiparkinsonian	1999	35, 336
choline alfoscerate	nootropic	1990	26, 300
cibenzoline	antiarrhythmic	1985	21, 325

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cicletanine	antihypertensive	1988	24, 299
cidofovir	antiviral	1996	32, 306
cilazapril	antihypertensive	1990	26, 301
cilostazol	antithrombotic	1988	24, 299
cimetropium bromide	antispasmodic	1985	21, 326
cinacalcet	hyperparathyroidism	2004	40, 451
cinildipine	antihypertensive	1995	31, 339
cinitapride	gastroprokinetic	1990	26, 301
cinolazepam	hypnotic	1993	29, 334
ciprofibrate	hypolipidemic	1985	21, 326
ciprofloxacin	antibacterial	1986	22, 318
cisapride	gastroprokinetic	1988	24, 299
cisatracurium besilate	muscle relaxant	1995	31, 340
citalopram	antidepressant	1989	25, 311
cladribine	antineoplastic	1993	29, 335
clarithromycin	antibiotic	1990	26, 302
clobenoside	vasoprotective	1988	24, 300
cloconazole HCl	topical antifungal	1986	22, 318
clodronate disodium	calcium regulator	1986	22, 319
clopidogrel hydrogensulfate	antithrombotic	1998	34, 320
cloricromen	antithrombotic	1991	27, 325
clospipramine HCl	neuroleptic	1991	27, 325
colesevelam hydrochloride	hypolipidemic	2000	36, 300
colestimide	hypolipidaemic	1999	35, 337
colforsin daropate HCl	cardiotonic	1999	35, 337
crotelidae polyvalent immune fab	antidote	2001	37, 263
cyclosporine	immunosuppressant	1983	19, 317
cytarabine ocfosfate	antineoplastic	1993	29, 335
dalfopristin	antibiotic	1999	35, 338
dapiprazole HCl	antiglaucoma	1987	23, 332
daptomycin	antibiotic	2003	39, 272
defeiprone	iron chelator	1995	31, 340
defibrotide	antithrombotic	1986	22, 319
deflazacort	antiinflammatory	1986	22, 319
delapril	antihypertensive	1989	25, 311
delavirdine mesylate	antiviral	1997	33, 331
denileukin diftitox	anticancer	1999	35, 338
denopamine	cardiostimulant	1988	24, 300
deprodone propionate	topical antiinflammatory	1992	28, 329
desflurane	anesthetic	1992	28, 329
desloratadine	antihistamine	2001	37, 264
dexfenfluramine	antiobesity	1997	33, 332
dexibuprofen	antiinflammatory	1994	30, 298
dexmedetomidine hydrochloride	sedative	2000	36, 301
dexmethylphenidate HCl	psychostimulant	2002	38, 352

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dexrazoxane	cardioprotective	1992	28, 330
dezocine	analgesic	1991	27, 326
diacerein	antirheumatic	1985	21, 326
didanosine	antiviral	1991	27, 326
dilevalol	antihypertensive	1989	25, 311
dirithromycin	antibiotic	1993	29, 336
disodium pamidronate	calcium regulator	1989	25, 312
divistyramine	hypocholesterolemic	1984	20, 317
docarpamine	cardiostimulant	1994	30, 298
docetaxel	antineoplastic	1995	31, 341
dofetilide	antiarrhythmic	2000	36, 301
dolasetron mesylate	antiemetic	1998	34, 321
donepezil HCl	anti-Alzheimer	1997	33, 332
dopexamine	cardiostimulant	1989	25, 312
dornase alfa	cystic fibrosis	1994	30, 298
dorzolamide HCL	antiglaucoma	1995	31, 341
dosmalfate	antiulcer	2000	36, 302
doxacurium chloride	muscle relaxant	1991	27, 326
doxazosin mesylate	antihypertensive	1988	24, 300
doxefazepam	hypnotic	1985	21, 326
doxercalciferol	vitamin D prohormone	1999	35, 339
doxifluridine	antineoplastic	1987	23, 332
doxofylline	bronchodilator	1985	21, 327
dronabinol	antinauseant	1986	22, 319
drospirenone	contraceptive	2000	36, 302
drotrecogin alfa	antisepsis	2001	37, 265
droxicam	antiinflammatory	1990	26, 302
droxidopa	antiparkinsonian	1989	25, 312
duloxetine	antidepressant	2004	40, 452
dutasteride	5α reductase inhibitor	2002	38, 353
duteplase	anticougulant	1995	31, 342
ebastine	antihistamine	1990	26, 302
ebrotidine	antiulcer	1997	33, 333
ecabet sodium	antiulcerative	1993	29, 336
edaravone	neuroprotective	2001	37, 265
efalizumab	psoriasis	2003	39, 274
efavirenz	antiviral	1998	34, 321
efonidipine	antihypertensive	1994	30, 299
egualen sodium	antiulcer	2000	36, 303
eletriptan	antimigraine	2001	37, 266
emedastine difumarate	antiallergic/antiasthmatic	1993	29, 336
emorfazone	analgesic	1984	20, 317
emtricitabine	antiviral	2003	39, 274
enalapril maleate	antihypertensive	1984	20, 317
enalaprilat	antihypertensive	1987	23, 332
encainide HCl	antiarrhythmic	1987	23, 333
enfuvirtide	antiviral	2003	39, 275

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enocitabine	antineoplastic	1983	19, 318
enoxacin	antibacterial	1986	22, 320
enoxaparin	antithrombotic	1987	23, 333
enoximone	cardiostimulant	1988	24, 301
enprostil	antiulcer	1985	21, 327
entacapone	antiparkinsonian	1998	34, 322
epalrestat	antidiabetic	1992	28, 330
eperisone HCl	muscle relaxant	1983	19, 318
epidermal growth factor	wound healing agent	1987	23, 333
epinastine	antiallergic	1994	30, 299
epirubicin HCl	antineoplastic	1984	20, 318
eplerenone	antihypertensive	2003	39, 276
epoprostenol sodium	platelet aggreg. inhib.	1983	19, 318
eprosartan	antihypertensive	1997	33, 333
eptazocine HBr	analgesic	1987	23, 334
eptilfibatide	antithrombotic	1999	35, 340
erdosteine	expectorant	1995	31, 342
erlotinib	anticancer	2004	40, 454
ertapenem sodium	antibacterial	2002	38, 353
erythromycin acistrate	antibiotic	1988	24, 301
erythropoietin	hematopoetic	1988	24, 301
escitalopram oxolate	antidepressant	2002	38, 354
esmolol HCl	antiarrhythmic	1987	23, 334
esomeprazole magnesium	gastric antisecretory	2000	36, 303
ethyl icosapentate	antithrombotic	1990	26, 303
etizolam	anxiolytic	1984	20, 318
etodolac	antiinflammatory	1985	21, 327
etoricoxibe	antiarthritic/analgesic	2002	38, 355
everolimus	immunosuppressant	2004	40, 455
exemestane	anticancer	2000	36, 304
exifone	nootropic	1988	24, 302
ezetimibe	hypolipidemic	2002	38, 355
factor VIIa	haemophilia	1996	32, 307
factor VIII	hemostatic	1992	28, 330
fadrozole HCl	antineoplastic	1995	31, 342
falecalcitriol	vitamin D	2001	37, 266
famciclovir	antiviral	1994	30, 300
famotidine	antiulcer	1985	21, 327
fasudil HCl	neuroprotective	1995	31, 343
felbamate	antiepileptic	1993	29, 337
felbinac	topical antiinflammatory	1986	22, 320
felodipine	antihypertensive	1988	24, 302
fenbuprol	choleretic	1983	19, 318
fenoldopam mesylate	antihypertensive	1998	34, 322
fenticonazole nitrate	antifungal	1987	23, 334
fexofenadine	antiallergic	1996	32, 307
filgrastim	immunostimulant	1991	27, 327

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
finasteride	5α-reductase inhibitor	1992	28, 331
fisalamine	intestinal antiinflammatory	1984	20, 318
fleroxacin	antibacterial	1992	28, 331
flomoxef sodium	antibiotic	1988	24, 302
flosequinan	cardiostimulant	1992	28, 331
fluconazole	antifungal	1988	24, 303
fludarabine phosphate	antineoplastic	1991	27, 327
flumazenil	benzodiazepine antag.	1987	23, 335
flunoxaprofen	antiinflammatory	1987	23, 335
fluoxetine HCl	antidepressant	1986	22, 320
flupirtine maleate	analgesic	1985	21, 328
flurithromycin ethylsuccinate	antibiotic	1997	33, 333
flutamide	antineoplastic	1983	19, 318
flutazolam	anxiolytic	1984	20, 318
fluticasone propionate	antiinflammatory	1990	26, 303
flutoprazepam	anxiolytic	1986	22, 320
flutrimazole	topical antifungal	1995	31, 343
flutropium bromide	antitussive	1988	24, 303
fluvastatin	hypolipaemic	1994	30, 300
fluvoxamine maleate	antidepressant	1983	19, 319
follitropin alfa	fertility enhancer	1996	32, 307
follitropin beta	fertility enhancer	1996	32, 308
fomepizole	antidote	1998	34, 323
fomivirsen sodium	antiviral	1998	34, 323
fondaparinux sodium	antithrombotic	2002	38, 356
formestane	antineoplastic	1993	29, 337
formoterol fumarate	bronchodilator	1986	22, 321
fosamprenavir	antiviral	2003	39, 277
foscarnet sodium	antiviral	1989	25, 313
fosfosal	analgesic	1984	20, 319
fosfluconazole	antifungal	2004	40, 457
fosinopril sodium	antihypertensive	1991	27, 328
fosphenytoin sodium	antiepileptic	1996	32, 308
fotemustine	antineoplastic	1989	25, 313
fropenam	antibiotic	1997	33, 334
frovatriptan	antimigraine	2002	38, 357
fudosteine	expectorant	2001	37, 267
fulveristrant	anticancer	2002	38, 357
gabapentin	antiepileptic	1993	29, 338
gadoversetamide	MRI contrast agent	2000	36, 304
gallium nitrate	calcium regulator	1991	27, 328
gallopamil HCl	antianginal	1983	19, 319
ganciclovir	antiviral	1988	24, 303
ganirelix acetate	female infertility	2000	36, 305
gatilfloxacin	antibiotic	1999	35, 340
gefitinib	antineoplastic	2002	38, 358
gemcitabine HCl	antineoplastic	1995	31, 344

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
gemeprost	abortifacient	1983	19, 319
gemifloxacin	antibacterial	2004	40, 458
gemtuzumab ozogamicin	anticancer	2000	36, 306
gestodene	progestogen	1987	23, 335
gestrinone	antiprogestogen	1986	22, 321
glatiramer acetate	Multiple Sclerosis	1997	33, 334
glimepiride	antidiabetic	1995	31, 344
glucagon, rDNA	hypoglycemia	1993	29, 338
GMDP	immunostimulant	1996	32, 308
goserelin	hormone	1987	23, 336
granisetron HCl	antiemetic	1991	27, 329
guanadrel sulfate	antihypertensive	1983	19, 319
gusperimus	immunosuppressant	1994	30, 300
halobetasol propionate	topical antiinflammatory	1991	27, 329
halofantrine	antimalarial	1988	24, 304
halometasone	topical antiinflammatory	1983	19, 320
histrelin	precocious puberty	1993	29, 338
hydrocortisone aceponate	topical antiinflammatory	1988	24, 304
hydrocortisone butyrate	topical antiinflammatory	1983	19, 320
ibandronic acid	osteoporosis	1996	32, 309
ibopamine HCl	cardiostimulant	1984	20, 319
ibudilast	antiasthmatic	1989	25, 313
ibutilide fumarate	antiarrhythmic	1996	32, 309
ibritunomab tiuxetan	anticancer	2002	38, 359
idarubicin HCl	antineoplastic	1990	26, 303
idebenone	nootropic	1986	22, 321
iloprost	platelet aggreg. inhibitor	1992	28, 332
imatinib mesylate	antineoplastic	2001	37, 267
imidapril HCl	antihypertensive	1993	29, 339
imiglucerase	Gaucher's disease	1994	30, 301
imipenem/cilastatin	antibiotic	1985	21, 328
imiquimod	antiviral	1997	33, 335
incadronic acid	osteoporosis	1997	33, 335
indalpine	antidepressant	1983	19, 320
indeloxazine HCl	nootropic	1988	24, 304
indinavir sulfate	antiviral	1996	32, 310
indisetron	antiemetic	2004	40, 459
indobufen	antithrombotic	1984	20, 319
influenza virus (live)	antiviral vaccine	2003	39, 277
insulin lispro	antidiabetic	1996	32, 310
interferon alfacon-1	antiviral	1997	33, 336
interferon gamma-1b	immunostimulant	1991	27, 329
interferon, gamma	antiinflammatory	1989	25, 314
interferon, gamma-1α	antineoplastic	1992	28, 332
interferon, β-1a	multiple sclerosis	1996	32, 311
interferon, β-1b	multiple sclerosis	1993	29, 339
interleukin-2	antineoplastic	1989	25, 314

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
ioflupane	diagnosis CNS	2000	36, 306
ipriflavone	calcium regulator	1989	25, 314
irbesartan	antihypertensive	1997	33, 336
irinotecan	antineoplastic	1994	30, 301
irsogladine	antiulcer	1989	25, 315
isepamicin	antibiotic	1988	24, 305
isofezolac	antiinflammatory	1984	20, 319
isoxicam	antiinflammatory	1983	19, 320
isradipine	antihypertensive	1989	25, 315
itopride HCl	gastroprokinetic	1995	31, 344
itraconazole	antifungal	1988	24, 305
ivermectin	antiparasitic	1987	23, 336
ketanserin	antihypertensive	1985	21, 328
ketorolac tromethamine	analgesic	1990	26, 304
kinetin	skin photodamage/ dermatologic	1999	35, 341
lacidipine	antihypertensive	1991	27, 330
lafutidine	gastric antisecretory	2000	36, 307
lamivudine	antiviral	1995	31, 345
lamotrigine	anticonvulsant	1990	26, 304
landiolol	antiarrhythmic	2002	38, 360
lanoconazole	antifungal	1994	30, 302
lanreotide acetate	acromegaly	1995	31, 345
lansoprazole	antiulcer	1992	28, 332
laronidase	mucopolysaccaridosis I	2003	39, 278
latanoprost	antiglaucoma	1996	32, 311
lefunomide	antiarthritic	1998	34, 324
lenampicillin HCl	antibiotic	1987	23, 336
lentinan	immunostimulant	1986	22, 322
lepirudin	anticoagulant	1997	33, 336
lercanidipine	antihyperintensive	1997	33, 337
letrazole	anticancer	1996	32, 311
leuprolide acetate	hormone	1984	20, 319
levacecarnine HCl	nootropic	1986	22, 322
levalbuterol HCl	antiasthmatic	1999	35, 341
levetiracetam	antiepileptic	2000	36, 307
levobunolol HCl	antiglaucoma	1985	21, 328
levobupivacaine hydrochloride	local anesthetic	2000	36, 308
levocabastine HCl	antihistamine	1991	27, 330
levocetirizine	antihistamine	2001	37, 268
levodropropizine	antitussive	1988	24, 305
levofloxacin	antibiotic	1993	29, 340
levosimendan	heart failure	2000	36, 308
lidamidine HCl	antiperistaltic	1984	20, 320
limaprost	antithrombotic	1988	24, 306
linezolid	antibiotic	2000	36, 309
liranaftate	topical antifungal	2000	36, 309

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
lisinopril	antihypertensive	1987	23, 337
lobenzarit sodium	antiinflammatory	1986	22, 322
lodoxamide tromethamine	antiallergic ophthalmic	1992	28, 333
lomefloxacin	antibiotic	1989	25, 315
lomerizine HCl	antimigraine	1999	35, 342
lonidamine	antineoplastic	1987	23, 337
lopinavir	antiviral	2000	36, 310
loprazolam mesylate	hypnotic	1983	19, 321
loprinone HCl	cardiostimulant	1996	32, 312
loracarbef	antibiotic	1992	28, 333
loratadine	antihistamine	1988	24, 306
lornoxicam	NSAID	1997	33, 337
losartan	antihypertensive	1994	30, 302
loteprednol etabonate	antiallergic ophthalmic	1998	34, 324
lovastatin	hypocholesterolemic	1987	23, 337
loxoprofen sodium	antiinflammatory	1986	22, 322
Lyme disease	vaccine	1999	35, 342
mabuterol HCl	bronchodilator	1986	22, 323
malotilate	hepatoprotective	1985	21, 329
manidipine HCl	antihypertensive	1990	26, 304
masoprocol	topical antineoplastic	1992	28, 333
maxacalcitol	vitamin D	2000	36, 310
mebefradil HCl	antihypertensive	1997	33, 338
medifoxamine fumarate	antidepressant	1986	22, 323
mefloquine HCl	antimalarial	1985	21, 329
meglutol	hypolipidemic	1983	19, 321
melinamide	hypocholesterolemic	1984	20, 320
meloxicam	antiarthritic	1996	32, 312
mepixanox	analeptic	1984	20, 320
meptazinol HCl	analgesic	1983	19, 321
meropenem	carbapenem antibiotic	1994	30, 303
metaclazepam	anxiolytic	1987	23, 338
metapramine	antidepressant	1984	20, 320
mexazolam	anxiolytic	1984	20, 321
micafungin	antifungal	2002	38, 360
mifepristone	abortifacient	1988	24, 306
miglitol	antidiabetic	1998	34, 325
miglustat	gaucher's disease	2003	39, 279
milnacipran	antidepressant	1997	33, 338
milrinone	cardiostimulant	1989	25, 316
miltefosine	topical antineoplastic	1993	29, 340
miokamycin	antibiotic	1985	21, 329
mirtazapine	antidepressant	1994	30, 303
misoprostol	antiulcer	1985	21, 329
mitiglinide	antidiabetic	2004	40, 460
mitoxantrone HCl	antineoplastic	1984	20, 321
mivacurium chloride	muscle relaxant	1992	28, 334

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
mivotilate	hepatoprotectant	1999	35, 343
mizolastine	antihistamine	1998	34, 325
mizoribine	immunosuppressant	1984	20, 321
moclobemide	antidepressant	1990	26, 305
modafinil	idiopathic hypersomnia	1994	30, 303
moexipril HCl	antihypertensive	1995	31, 346
mofezolac	analgesic	1994	30, 304
mometasone furoate	topical antiinflammatory	1987	23, 338
montelukast sodium	antiasthma	1998	34, 326
moricizine HCl	antiarrhythmic	1990	26, 305
mosapride citrate	gastroprokinetic	1998	34, 326
moxifloxacin HCL	antibiotic	1999	35, 343
moxonidine	antihypertensive	1991	27, 330
mupirocin	topical antibiotic	1985	21, 330
muromonab-CD3	immunosuppressant	1986	22, 323
muzolimine	diuretic	1983	19, 321
mycophenolate mofetil	immunosuppressant	1995	31, 346
mycophenolate sodium	immunosuppressant	2003	39, 279
nabumetone	antiinflammatory	1985	21, 330
nadifloxacin	topical antibiotic	1993	29, 340
nafamostat mesylate	protease inhibitor	1986	22, 323
nafarelin acetate	hormone	1990	26, 306
naftifine HCl	antifungal	1984	20, 321
naftopidil	dysuria	1999	35, 344
nalmefene HCl	dependence treatment	1995	31, 347
naltrexone HCl	narcotic antagonist	1984	20, 322
naratriptan HCl	antimigraine	1997	33, 339
nartograstim	leukopenia	1994	30, 304
natalizumab	multiple sclerosis	2004	40, 462
nateglinide	antidiabetic	1999	35, 344
nazasetron	antiemetic	1994	30, 305
nebivolol	antihypertensive	1997	33, 339
nedaplatin	antineoplastic	1995	31, 347
nedocromil sodium	antiallergic	1986	22, 324
nefazodone	antidepressant	1994	30, 305
nelfinavir mesylate	antiviral	1997	33, 340
neltenexine	cystic fibrosis	1993	29, 341
nemonapride	neuroleptic	1991	27, 331
neridronic acide	calcium regulator	2002	38, 361
nesiritide	congestive heart failure	2001	37, 269
neticonazole HCl	topical antifungal	1993	29, 341
nevirapine	antiviral	1996	32, 313
nicorandil	coronary vasodilator	1984	20, 322
nifekalant HCl	antiarrythmic	1999	35, 344
nilutamide	antineoplastic	1987	23, 338
nilvadipine	antihypertensive	1989	25, 316
nimesulide	antiinflammatory	1985	21, 330

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
nimodipine	cerebral vasodilator	1985	21, 330
nipradilol	antihypertensive	1988	24, 307
nisoldipine	antihypertensive	1990	26, 306
nitisinone	antityrosinaemia	2002	38, 361
nitrefazole	alcohol deterrent	1983	19, 322
nitrendipine	hypertensive	1985	21, 331
nizatidine	antiulcer	1987	23, 339
nizofenzone fumarate	nootropic	1988	24, 307
nomegestrol acetate	progestogen	1986	22, 324
norelgestromin	contraceptive	2002	38, 362
norfloxacin	antibacterial	1983	19, 322
norgestimate	progestogen	1986	22, 324
OCT-43	anticancer	1999	35, 345
octreotide	antisecretory	1988	24, 307
ofloxacin	antibacterial	1985	21, 331
olanzapine	neuroleptic	1996	32, 313
olimesartan Medoxomil	antihypertensive	2002	38, 363
olopatadine HCl	antiallergic	1997	33, 340
omalizumab	allergic asthma	2003	39, 280
omeprazole	antiulcer	1988	24, 308
ondansetron HCl	antiemetic	1990	26, 306
OP-1	osteoinductor	2001	37, 269
orlistat	antiobesity	1998	34, 327
ornoprostil	antiulcer	1987	23, 339
osalazine sodium	intestinal antinflamm.	1986	22, 324
oseltamivir phosphate	antiviral	1999	35, 346
oxaliplatin	anticancer	1996	32, 313
oxaprozin	antiinflammatory	1983	19, 322
oxcarbazepine	anticonvulsant	1990	26, 307
oxiconazole nitrate	antifungal	1983	19, 322
oxiracetam	nootropic	1987	23, 339
oxitropium bromide	bronchodilator	1983	19, 323
ozagrel sodium	antithrombotic	1988	24, 308
paclitaxal	antineoplastic	1993	29, 342
palonosetron	antiemetic	2003	39, 281
panipenem/betamipron	carbapenem antibiotic	1994	30, 305
pantoprazole sodium	antiulcer	1995	30, 306
parecoxib sodium	analgesic	2002	38, 364
paricalcitol	vitamin D	1998	34, 327
parnaparin sodium	anticoagulant	1993	29, 342
paroxetine	antidepressant	1991	27, 331
pazufloxacin	antibacterial	2002	38, 364
pefloxacin mesylate	antibacterial	1985	21, 331
pegademase bovine	immunostimulant	1990	26, 307
pegaspargase	antineoplastic	1994	30, 306
pegvisomant	acromegaly	2003	39, 281
pemetrexed	anticancer	2004	40, 463

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
pemirolast potassium	antiasthmatic	1991	27, 331
penciclovir	antiviral	1996	32, 314
pentostatin	antineoplastic	1992	28, 334
pergolide mesylate	antiparkinsonian	1988	24, 308
perindopril	antihypertensive	1988	24, 309
perospirone HCL	neuroleptic	2001	37, 270
picotamide	antithrombotic	1987	23, 340
pidotimod	immunostimulant	1993	29, 343
piketoprofen	topical antiinflammatory	1984	20, 322
pilsicainide HCl	antiarrhythmic	1991	27, 332
pimaprofen	topical antiinflammatory	1984	20, 322
pimecrolimus	immunosuppressant	2002	38, 365
pimobendan	heart failure	1994	30, 307
pinacidil	antihypertensive	1987	23, 340
pioglitazone HCL	antidiabetic	1999	35, 346
pirarubicin	antineoplastic	1988	24, 309
pirmenol	antiarrhythmic	1994	30, 307
piroxicam cinnamate	antiinflammatory	1988	24, 309
pitavastatin	hypocholesterolemic	2003	39, 282
pivagabine	antidepressant	1997	33, 341
plaunotol	antiulcer	1987	23, 340
polaprezinc	antiulcer	1994	30, 307
porfimer sodium	antineoplastic adjuvant	1993	29, 343
pramipexole HCl	antiParkinsonian	1997	33, 341
pramiracetam H_2SO_4	cognition enhancer	1993	29, 343
pranlukast	antiasthmatic	1995	31, 347
pravastatin	antilipidemic	1989	25, 316
prednicarbate	topical antiinflammatory	1986	22, 325
pregabalin	antiepileptic	2004	40, 464
prezatide copper acetate	vulnery	1996	32, 314
progabide	anticonvulsant	1985	21, 331
promegestrone	progestogen	1983	19, 323
propacetamol HCl	analgesic	1986	22, 325
propagermanium	antiviral	1994	30, 308
propentofylline propionate	cerebral vasodilator	1988	24, 310
propiverine HCl	urologic	1992	28, 335
propofol	anesthetic	1986	22, 325
prulifloxacin	antibacterial	2002	38, 366
pumactant	lung surfactant	1994	30, 308
quazepam	hypnotic	1985	21, 332
quetiapine fumarate	neuroleptic	1997	33, 341
quinagolide	hyperprolactinemia	1994	30, 309
quinapril	antihypertensive	1989	25, 317
quinfamide	amebicide	1984	20, 322
quinupristin	antibiotic	1999	35, 338
rabeprazole sodium	gastric antisecretory	1998	34, 328
raloxifene HCl	osteoporosis	1998	34, 328

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
raltitrexed	anticancer	1996	32, 315
ramatroban	antiallergic	2000	36, 311
ramipril	antihypertensive	1989	25, 317
ramosetron	antiemetic	1996	32, 315
ranimustine	antineoplastic	1987	23, 341
ranitidine bismuth citrate	antiulcer	1995	31, 348
rapacuronium bromide	muscle relaxant	1999	35, 347
rebamipide	antiulcer	1990	26, 308
reboxetine	antidepressant	1997	33, 342
remifentanil HCl	analgesic	1996	32, 316
remoxipride HCl	antipsychotic	1990	26, 308
repaglinide	antidiabetic	1998	34, 329
repirinast	antiallergic	1987	23, 341
reteplase	fibrinolytic	1996	32, 316
reviparin sodium	anticoagulant	1993	29, 344
rifabutin	antibacterial	1992	28, 335
rifapentine	antibacterial	1988	24, 310
rifaximin	antibiotic	1985	21, 332
rifaximin	antibiotic	1987	23, 341
rilmazafone	hypnotic	1989	25, 317
rilmenidine	antihypertensive	1988	24, 310
riluzole	neuroprotective	1996	32, 316
rimantadine HCl	antiviral	1987	23, 342
rimexolone	antiinflammatory	1995	31, 348
risedronate sodium	osteoporosis	1998	34, 330
risperidone	neuroleptic	1993	29, 344
ritonavir	antiviral	1996	32, 317
rivastigmin	anti-Alzheimer	1997	33, 342
rizatriptan benzoate	antimigraine	1998	34, 330
rocuronium bromide	neuromuscular blocker	1994	30, 309
rofecoxib	antiarthritic	1999	35, 347
rokitamycin	antibiotic	1986	22, 332
ronafibrate	hypolipidemic	1986	22, 326
ropinirole HCl	antiParkinsonian	1996	32, 317
ropivacaine	anesthetic	1996	32, 318
rosaprostol	antiulcer	1985	21, 332
rosiglitazone maleate	antidiabetic	1999	35, 348
rosuvastatin	hypocholesterolemic	2003	39, 283
roxatidine acetate HCl	antiulcer	1986	22, 326
roxithromycin	antiulcer	1987	23, 342
rufloxacin HCl	antibacterial	1992	28, 335
rupatadine fumarate	antiallergic	2003	39, 284
RV-11	antibiotic	1989	25, 318
salmeterol hydroxynaphthoate	bronchodilator	1990	26, 308
sapropterin HCl	hyperphenylalaninemia	1992	28, 336
saquinavir mesvlate	antiviral	1995	31, 349

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sargramostim	immunostimulant	1991	27, 332
sarpogrelate HCl	platelet antiaggregant	1993	29, 344
schizophyllan	immunostimulant	1985	22, 326
seratrodast	antiasthmatic	1995	31, 349
sertaconazole nitrate	topical antifungal	1992	28, 336
sertindole	neuroleptic	1996	32, 318
setastine HCl	antihistamine	1987	23, 342
setiptiline	antidepressant	1989	25, 318
setraline HCl	antidepressant	1990	26, 309
sevoflurane	anesthetic	1990	26, 309
sibutramine	antiobesity	1998	34, 331
sildenafil citrate	male sexual dysfunction	1998	34, 331
simvastatin	hypocholesterolemic	1988	24, 311
sivelestat	anti-inflammatory	2002	38, 366
SKI-2053R	anticancer	1999	35, 348
sobuzoxane	antineoplastic	1994	30, 310
sodium cellulose PO4	hypocalciuric	1983	19, 323
sofalcone	antiulcer	1984	20, 323
solifenacin	pollakiuria	2004	40, 466
somatomedin-1	growth hormone insensitivity	1994	30, 310
somatotropin	growth hormone	1994	30, 310
somatropin	hormone	1987	23, 343
sorivudine	antiviral	1993	29, 345
sparfloxacin	antibiotic	1993	29, 345
spirapril HCl	antihypertensive	1995	31, 349
spizofurone	antiulcer	1987	23, 343
stavudine	antiviral	1994	30, 311
strontium ranelate	osteoporosis	2004	40, 467
succimer	chelator	1991	27, 333
sufentanil	analgesic	1983	19, 323
sulbactam sodium	β-lactamase inhibitor	1986	22, 326
sulconizole nitrate	topical antifungal	1985	21, 332
sultamycillin tosylate	antibiotic	1987	23, 343
sumatriptan succinate	antimigraine	1991	27, 333
suplatast tosilate	antiallergic	1995	31, 350
suprofen	analgesic	1983	19, 324
surfactant TA	respiratory surfactant	1987	23, 344
tacalcitol	topical antipsoriatic	1993	29, 346
tacrine HCl	Alzheimer's disease	1993	29, 346
tacrolimus	immunosuppressant	1993	29, 347
tadalafil	male sexual dysfunction	2003	39, 284
talaporfin sodium	anticancer	2004	40, 469
talipexole	antiParkinsonian	1996	32, 318
taltirelin	CNS stimulant	2000	36, 311
tamsulosin HCl	antiprostatic hypertrophy	1993	29, 347
tandospirone	anxiolytic	1996	32, 319

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
tasonermin	anticancer	1999	35, 349
tazanolast	antiallergic	1990	26, 309
tazarotene	antipsoriasis	1997	33, 343
tazobactam sodium	β-lactamase inhibitor	1992	28, 336
tegaserod maleate	irritable bowel syndrome	2001	37, 270
teicoplanin	antibacterial	1988	24, 311
telithromycin	antibiotic	2001	37, 271
telmesteine	mucolytic	1992	28, 337
telmisartan	antihypertensive	1999	35, 349
temafloxacin HCl	antibacterial	1991	27, 334
temocapril	antihypertensive	1994	30, 311
temocillin disodium	antibiotic	1984	20, 323
temoporphin	antineoplastic/ photosensitizer	2002	38, 367
temozolomide	anticancer	1999	35, 349
tenofovir disoproxil fumarate	antiviral	2001	37, 271
tenoxicam	antiinflammatory	1987	23, 344
teprenone	antiulcer	1984	20, 323
terazosin HCl	antihypertensive	1984	20, 323
terbinafine HCl	antifungal	1991	27, 334
terconazole	antifungal	1983	19, 324
tertatolol HCl	antihypertensive	1987	23, 344
thymopentin	immunomodulator	1985	21, 333
tiagabine	antiepileptic	1996	32, 319
tiamenidine HCl	antihypertensive	1988	24, 311
tianeptine sodium	antidepressant	1983	19, 324
tibolone	anabolic	1988	24, 312
tilisolol HCl	antihypertensive	1992	28, 337
tiludronate disodium	Paget's disease	1995	31, 350
timiperone	neuroleptic	1984	20, 323
tinazoline	nasal decongestant	1988	24, 312
tioconazole	antifungal	1983	19, 324
tiopronin	urolithiasis	1989	25, 318
tiotropium bromide	bronchodilator	2002	38, 368
tiquizium bromide	antispasmodic	1984	20, 324
tiracizine HCl	antiarrhythmic	1990	26, 310
tirilazad mesylate	subarachnoid hemorrhage	1995	31, 351
tirofiban HCl	antithrombotic	1998	34, 332
tiropramide HCl	antispasmodic	1983	19, 324
tizanidine	muscle relaxant	1984	20, 324
tolcapone	antiParkinsonian	1997	33, 343
toloxatone	antidepressant	1984	20, 324
tolrestat	antidiabetic	1989	25, 319
topiramate	antiepileptic	1995	31, 351
topotecan HCl	anticancer	1996	32, 320
torasemide	diuretic	1993	29, 348
toremifene	antineoplastic	1989	25, 319

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
tositumomab	anticancer	2003	39, 285
tosufloxacin tosylate	antibacterial	1990	26, 310
trandolapril	antihypertensive	1993	29, 348
travoprost	antiglaucoma	2001	37, 272
treprostinil sodium	antihypertensive	2002	38, 368
tretinoin tocoferil	antiulcer	1993	29, 348
trientine HCl	chelator	1986	22, 327
trimazosin HCl	antihypertensive	1985	21, 333
trimegestone	progestogen	2001	37, 273
trimetrexate glucuronate	*Pneumocystis carinii* pneumonia	1994	30, 312
troglitazone	antidiabetic	1997	33, 344
tropisetron	antiemetic	1992	28, 337
trovafloxacin mesylate	antibiotic	1998	34, 332
troxipide	antiulcer	1986	22, 327
ubenimex	immunostimulant	1987	23, 345
unoprostone isopropyl ester	antiglaucoma	1994	30, 312
valaciclovir HCl	antiviral	1995	31, 352
vadecoxib	antiarthritic	2002	38, 369
vaglancirclovir HCL	antiviral	2001	37, 273
valrubicin	anticancer	1999	35, 350
valsartan	antihypertensive	1996	32, 320
vardenafil	male sexual dysfunction	2003	39, 286
venlafaxine	antidepressant	1994	30, 312
verteporfin	photosensitizer	2000	36, 312
vesnarinone	cardiostimulant	1990	26, 310
vigabatrin	anticonvulsant	1989	25, 319
vinorelbine	antineoplastic	1989	25, 320
voglibose	antidiabetic	1994	30, 313
voriconazole	antifungal	2002	38, 370
xamoterol fumarate	cardiotonic	1988	24, 312
ximelagatran	anticoagulant	2004	40, 470
zafirlukast	antiasthma	1996	32, 321
zalcitabine	antiviral	1992	28, 338
zaleplon	hypnotic	1999	35, 351
zaltoprofen	antiinflammatory	1993	29, 349
zanamivir	antiviral	1999	35, 352
zidovudine	antiviral	1987	23, 345
zileuton	antiasthma	1997	33, 344
zinostatin stimalamer	antineoplastic	1994	30, 313
ziprasidone hydrochloride	neuroleptic	2000	36, 312
zofenopril calcium	antihypertensive	2000	36, 313
zoledronate disodium	hypercalcemia	2000	36, 314
zolpidem hemitartrate	hypnotic	1988	24, 313
zomitriptan	antimigraine	1997	33, 345
zonisamide	anticonvulsant	1989	25, 320
zopiclone	hypnotic	1986	22, 327
zuclopenthixol acetate	antipsychotic	1987	23, 345

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
gemeprost	ABORTIFACIENT	1983	19 (319)
mifepristone		1988	24 (306)
lanreotide acetate	ACROMEGALY	1995	31 (345)
pegvisomant		2003	39 (281)
nitrefazole	ALCOHOL DETERRENT	1983	19 (322)
omalizumab	ALLERGIC ASTHMA	2003	39 (280)
tacrine HCl	ALZHEIMER'S DISEASE	1993	29 (346)
quinfamide	AMEBICIDE	1984	20 (322)
tibolone	ANABOLIC	1988	24 (312)
mepixanox	ANALEPTIC	1984	20 (320)
alfentanil HCl	ANALGESIC	1983	19 (314)
alminoprofen		1983	19 (314)
dezocine		1991	27 (326)
emorfazone		1984	20 (317)
eptazocine HBr		1987	23 (334)
etoricoxib		2002	38 (355)
flupirtine maleate		1985	21 (328)
fosfosal		1984	20 (319)
ketorolac tromethamine		1990	26 (304)
meptazinol HCl		1983	19 (321)
mofezolac		1994	30 (304)
parecoxib sodium		2002	38 (364)
propacetamol HCl		1986	22 (325)
remifentanil HCl		1996	32 (316)
sufentanil		1983	19 (323)
suprofen		1983	19 (324)
desflurane	ANESTHETIC	1992	28 (329)
propofol		1986	22 (325)
ropivacaine		1996	32 (318)
sevoflurane		1990	26 (309)
levobupivacaine hydrochloride	ANESTHETIC, LOCAL	2000	36 (308)
azelaic acid	ANTIACNE	1989	25 (310)
betotastine besilate	ANTIALLERGIC	2000	36 (297)
emedastine difumarate		1993	29 (336)
epinastine		1994	30 (299)
fexofenadine		1996	32 (307)

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
nedocromil sodium		1986	22 (324)
olopatadine hydrochloride		1997	33 (340)
ramatroban		2000	36 (311)
repirinast		1987	23 (341)
suplatast tosilate		1995	31 (350)
tazanolast		1990	26 (309)
lodoxamide tromethamine	ANTIALLERGIC	1992	28 (333)
rupatadine fumarate		2003	39 (284)
loteprednol etabonate	OPHTHALMIC	1998	34 (324)
donepezil hydrochloride	ANTI-ALZHEIMERS	1997	33 (332)
rivastigmin		1997	33 (342)
gallopamil HCl	ANTIANGINAL	1983	19 (319)
cibenzoline	ANTIARRHYTHMIC	1985	21 (325)
dofetilide		2000	36 (301)
encainide HCl		1987	23 (333)
esmolol HCl		1987	23 (334)
ibutilide fumarate		1996	32 (309)
landiolol		2002	38 (360)
moricizine hydrochloride		1990	26 (305)
nifekalant HCl		1999	35 (344)
pilsicainide hydrochloride		1991	27 (332)
pirmenol		1994	30 (307)
tiracizine hydrochloride		1990	26 (310)
anakinra	ANTIARTHRITIC	2001	37 (261)
celecoxib		1999	35 (335)
etoricoxib		2002	38 (355)
meloxicam		1996	32 (312)
leflunomide		1998	34 (324)
rofecoxib		1999	35 (347)
valdecoxib		2002	38 (369)
amlexanox	ANTIASTHMATIC	1987	23 (327)
emedastine difumarate		1993	29 (336)
ibudilast		1989	25 (313)
levalbuterol HCl		1999	35 (341)
montelukast sodium		1998	34 (326)
pemirolast potassium		1991	27 (331)
seratrodast		1995	31 (349)
zafirlukast		1996	32 (321)
zileuton		1997	33 (344)

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
balofloxacin	ANTIBACTERIAL	2002	38 (351)
biapenem		2002	38 (351)
ciprofloxacin		1986	22 (318)
enoxacin		1986	22 (320)
ertapenem sodium		2002	38 (353)
fleroxacin		1992	28 (331)
gemifloxacin		2004	40 (458)
norfloxacin		1983	19 (322)
ofloxacin		1985	21 (331)
pazufloxacin		200	38 (364)
pefloxacin mesylate		1985	21 (331)
pranlukast		1995	31 (347)
prulifloxacin		2002	38 (366)
rifabutin		1992	28 (335)
rifapentine		1988	24 (310)
rufloxacin hydrochloride		1992	28 (335)
teicoplanin		1988	24 (311)
temafloxacin hydrochloride		1991	27 (334)
tosufloxacin tosylate		1990	26 (310)
arbekacin	ANTIBIOTIC	1990	26 (298)
aspoxicillin		1987	23 (328)
astromycin sulfate		1985	21 (324)
azithromycin		1988	24 (298)
aztreonam		1984	20 (315)
brodimoprin		1993	29 (333)
carboplatin		1986	22 (318)
carumonam		1988	24 (298)
cefbuperazone sodium		1985	21 (325)
cefcapene pivoxil		1997	33 (330)
cefdinir		1991	27 (323)
cefepime		1993	29 (334)
cefetamet pivoxil hydrochloride		1992	28 (327)
cefixime		1987	23 (329)
cefmenoxime HCl		1983	19 (316)
cefminox sodium		1987	23 (330)
cefodizime sodium		1990	26 (300)
cefonicid sodium		1984	20 (316)
ceforanide		1984	20 (317)
cefoselis		1998	34 (319)
cefotetan disodium		1984	20 (317)
cefotiam hexetil hydrochloride		1991	27 (324)
cefpimizole		1987	23 (330)

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
cefpiramide sodium		1985	21 (325)
cefpirome sulfate		1992	28 (328)
cefpodoxime proxetil		1989	25 (310)
cefprozil		1992	28 (328)
ceftazidime		1983	19 (316)
cefteram pivoxil		1987	23 (330)
ceftibuten		1992	28 (329)
cefuroxime axetil		1987	23 (331)
cefuzonam sodium		1987	23 (331)
clarithromycin		1990	26 (302)
dalfopristin		1999	35 (338)
dirithromycin		1993	29 (336)
erythromycin acistrate		1988	24 (301)
flomoxef sodium		1988	24 (302)
flurithromycin ethylsuccinate		1997	33 (333)
fropenam		1997	33 (334)
gatifloxacin		1999	35 (340)
imipenem/cilastatin		1985	21 (328)
isepamicin		1988	24 (305)
lenampicillin HCl		1987	23 (336)
levofloxacin		1993	29 (340)
linezolid		2000	36 (309)
lomefloxacin		1989	25 (315)
loracarbef		1992	28 (333)
miokamycin		1985	21 (329)
moxifloxacin HCl		1999	35 (343)
quinupristin		1999	35 (338)
rifaximin		1985	21 (332)
rifaximin		1987	23 (341)
rokitamycin		1986	22 (325)
RV-11		1989	25 (318)
sparfloxacin		1993	29 (345)
sultamycillin tosylate		1987	23 (343)
telithromycin		2001	37 (271)
temocillin disodium		1984	20 (323)
trovafloxacin mesylate		1998	34 (332)
meropenem	ANTIBIOTIC, CARBAPENEM	1994	30 (303)
panipenem/betamipron		1994	30 (305)
mupirocin	ANTIBIOTIC, TOPICAL	1985	21 (330)
nadifloxacin		1993	29 (340)
abarelix	ANTICANCER	2004	40 (446)
alemtuzumab		2001	37 (260)
alitretinoin		1999	35 (333)
arglabin		199	35 (335)

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
azacitidine		2004	40 (447)
belotecan		2004	40 (449)
bevacizumab		2004	40 (450)
bexarotene		2000	36 (298)
bortezomib		2003	39 (271)
cetuximab		2003	39 (272)
denileukin diftitox		1999	35 (338)
erlotinib		2004	40 (454)
exemestane		2000	36 (304)
fulvestrant		2002	38 (357)
gemtuzumab ozogamicin		2000	36 (306)
ibritumomab tiuxetan		2002	38 (359)
letrazole		1996	32 (311)
OCT-43		1999	35 (345)
oxaliplatin		1996	32 (313)
pemetrexed		2004	40 (463)
raltitrexed		1996	32 (315)
SKI-2053R		1999	35 (348)
talaporfin sodium		2004	40 (469)
tasonermin		1999	35 (349)
temozolomide		1999	35 (350)
topotecan HCl		1996	32 (320)
tositumomab		2003	39 (285)
valrubicin		1999	35 (350)
angiotensin II	ANTICANCER ADJUVANT	1994	30 (296)
chenodiol	ANTICHOLELITHOGENIC	1983	19 (317)
duteplase	ANTICOAGULANT	1995	31 (342)
lepirudin		1997	33 (336)
parnaparin sodium		1993	29 (342)
reviparin sodium		1993	29 (344)
ximelagatran		2004	40 (470)
lamotrigine	ANTICONVULSANT	1990	26 (304)
oxcarbazepine		1990	26 (307)
progabide		1985	21 (331)
vigabatrin		1989	25 (319)
zonisamide		1989	25 (320)
bupropion HCl	ANTIDEPRESSANT	1989	25 (310)
citalopram		1989	25 (311)
duloxetine		2004	40 (452)
escitalopram oxalate		2002	38 (354)
fluoxetine HCl		1986	22 (320)
fluvoxamine maleate		1983	19 (319)
indalpine		1983	19 (320)
medifoxamine fumarate		1986	22 (323)

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
metapramine		1984	20 (320)
milnacipran		1997	33 (338)
mirtazapine		1994	30 (303)
moclobemide		1990	26 (305)
nefazodone		1994	30 (305)
paroxetine		1991	27 (331)
pivagabine		1997	33 (341)
reboxetine		1997	33 (342)
setiptiline		1989	25 (318)
sertraline hydrochloride		1990	26 (309)
tianeptine sodium		1983	19 (324)
toloxatone		1984	20 (324)
venlafaxine		1994	30 (312)
acarbose	ANTIDIABETIC	1990	26 (297)
epalrestat		1992	28 (330)
glimepiride		1995	31 (344)
insulin lispro		1996	32 (310)
miglitol		1998	34 (325)
mitiglinide		2004	40 (460)
nateglinide		1999	35 (344)
pioglitazone HCl		1999	35 (346)
repaglinide		1998	34 (329)
rosiglitazone maleate		1999	35 (347)
tolrestat		1989	25 (319)
troglitazone		1997	33 (344)
voglibose		1994	30 (313)
acetorphan	ANTIDIARRHEAL	1993	29 (332)
anti-digoxin polyclonal antibody	ANTIDOTE	2002	38 (350)
crotelidae polyvalent immune fab		2001	37 (263)
fomepizole		1998	34 (323)
aprepitant	ANTIEMETIC	2003	39 (268)
dolasetron mesylate		1998	34 (321)
granisetron hydrochloride		1991	27 (329)
indisetron		2004	40 (459)
ondansetron hydrochloride		1990	26 (306)
nazasetron		1994	30 (305)
palonosetron		2003	39 (281)
ramosetron		1996	32 (315)
tropisetron		1992	28 (337)
felbamate	ANTIEPILEPTIC	1993	29 (337)

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
fosphenytoin sodium		1996	32 (308)
gabapentin		1993	29 (338)
levetiracetam		2000	36 (307)
pregabalin		2004	40 (464)
tiagabine		1996	32 (320)
topiramate		1995	31 (351)
centchroman	ANTIESTROGEN	1991	27 (324)
caspofungin acetate	ANTIFUNGAL	2001	37 (263)
fenticonazole nitrate		1987	23 (334)
fluconazole		1988	24 (303)
fosfluconazole		2004	40 (457)
itraconazole		1988	24 (305)
lanoconazole		1994	30 (302)
micafungin		2002	38 (360)
naftifine HCl		1984	20 (321)
oxiconazole nitrate		1983	19 (322)
terbinafine hydrochloride		1991	27 (334)
terconazole		1983	19 (324)
tioconazole		1983	19 (324)
voriconazole		2002	38 (370)
amorolfine hydrochloride	ANTIFUNGAL, TOPICAL	1991	27 (322)
butenafine hydrochloride		1992	28 (327)
butoconazole		1986	22 (318)
cloconazole HCl		1986	22 (318)
liranaftate		2000	36 (309)
flutrimazole		1995	31 (343)
neticonazole HCl		1993	29 (341)
sertaconazole nitrate		1992	28 (336)
sulconizole nitrate		1985	21 (332)
apraclonidine HCl	ANTIGLAUCOMA	1988	24 (297)
befunolol HCl		1983	19 (315)
bimatroprost		2001	37 (261)
brimonidine		1996	32 (306)
brinzolamide		1998	34 (318)
dapiprazole HCl		1987	23 (332)
dorzolamide HCl		1995	31 (341)
latanoprost		1996	32 (311)
levobunolol HCl		1985	21 (328)
travoprost		2001	37 (272)
unoprostone isopropyl ester		1994	30 (312)
acrivastine	ANTIHISTAMINE	1988	24 (295)
astemizole		1983	19 (314)
azelastine HCl		1986	22 (316)

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
cetirizine HCl		1987	23 (331)
desloratadine		2001	37 (264)
ebastine		1990	26 (302)
levocabastine hydrochloride		1991	27 (330)
levocetirizine		2001	37 (268)
loratadine		1988	24 (306)
mizolastine		1998	34 (325)
setastine HCl		1987	23 (342)
alacepril	ANTIHYPERTENSIVE	1988	24 (296)
alfuzosin HCl		1988	24 (296)
amlodipine besylate		1990	26 (298)
amosulalol		1988	24 (297)
aranidipine		1996	32 (306)
arotinolol HCl		1986	22 (316)
azelnidipine		2003	39 (270)
barnidipine hydrochloride		1992	28 (326)
benazepril hydrochloride		1990	26 (299)
benidipine hydrochloride		1991	27 (322)
betaxolol HCl		1983	19 (315)
bevantolol HCl		1987	23 (328)
bisoprolol fumarate		1986	22 (317)
bopindolol		1985	21 (324)
bosentan		2001	37 (262)
budralazine		1983	19 (315)
bunazosin HCl		1985	21 (324)
candesartan cilexetil		1997	33 (330)
carvedilol		1991	27 (323)
celiprolol HCl		1983	19 (317)
cicletanine		1988	24 (299)
cilazapril		1990	26 (301)
cinildipine		1995	31 (339)
delapril		1989	25 (311)
dilevalol		1989	25 (311)
doxazosin mesylate		1988	24 (300)
efonidipine		1994	30 (299)
enalapril maleate		1984	20 (317)
enalaprilat		1987	23 (332)
eplerenone		2003	39 (276)
eprosartan		1997	33 (333)
felodipine		1988	24 (302)
fenoldopam mesylate		1998	34 (322)
fosinopril sodium		1991	27 (328)
guanadrel sulfate		1983	19 (319)
imidapril HCl		1993	29 (339)
irbesartan		1997	33 (336)

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
isradipine		1989	25 (315)
ketanserin		1985	21 (328)
lacidipine		1991	27 (330)
lercanidipine		1997	33 (337)
lisinopril		1987	23 (337)
losartan		1994	30 (302)
manidipine hydrochloride		1990	26 (304)
mebefradil hydrochloride		1997	33 (338)
moexipril HCl		1995	31 (346)
moxonidine		1991	27 (330)
nebivolol		1997	33 (339)
nilvadipine		1989	25 (316)
nipradilol		1988	24 (307)
nisoldipine		1990	26 (306)
olmesartan medoxomil		2002	38 (363)
perindopril		1988	24 (309)
pinacidil		1987	23 (340)
quinapril		1989	25 (317)
ramipril		1989	25 (317)
rilmenidine		1988	24 (310)
spirapril HCl		1995	31 (349)
telmisartan		1999	35 (349)
temocapril		1994	30 (311)
terazosin HCl		1984	20 (323)
tertatolol HCl		1987	23 (344)
tiamenidine HCl		1988	24 (311)
tilisolol hydrochloride		1992	28 (337)
trandolapril		1993	29 (348)
treprostinil sodium		2002	38 (368)
trimazosin HCl		1985	21 (333)
valsartan		1996	32 (320)
zofenopril calcium		2000	36 (313)
captopril	ANTIHYPERTENSIVE AGENT	1982	13 (086)
daptomycin	ANTI INFECTIVE	2003	39 (272)
aceclofenac	ANTIINFLAMMATORY	1992	28 (325)
AF-2259		1987	23 (325)
amfenac sodium		1986	22 (315)
ampiroxicam		1994	30 (296)
amtolmetin guacil		1993	29 (332)
butibufen		1992	28 (327)
deflazacort		1986	22 (319)
dexibuprofen		1994	30 (298)
droxicam		1990	26 (302)
etodolac		1985	21 (327)

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
flunoxaprofen		1987	23 (335)
fluticasone propionate		1990	26 (303)
interferon, gamma		1989	25 (314)
isofezolac		1984	20 (319)
isoxicam		1983	19 (320)
lobenzarit sodium		1986	22 (322)
loxoprofen sodium		1986	22 (322)
nabumetone		1985	21 (330)
nimesulide		1985	21 (330)
oxaprozin		1983	19 (322)
piroxicam cinnamate		1988	24 (309)
rimexolone		1995	31 (348)
sivelestat		2002	38 (366)
tenoxicam		1987	23 (344)
zaltoprofen		1993	29 (349)
fisalamine	ANTIINFLAMMATORY, INTESTINAL	1984	20 (318)
osalazine sodium		1986	22 (324)
alclometasone dipropionate	ANTIINFLAMMATORY, TOPICAL	1985	21 (323)
aminoprofen		1990	26 (298)
betamethasone butyrate propionate		1994	30 (297)
butyl flufenamate		1983	19 (316)
deprodone propionate		1992	28 (329)
felbinac		1986	22 (320)
halobetasol propionate		1991	27 (329)
halometasone		1983	19 (320)
hydrocortisone aceponate		1988	24 (304)
hydrocortisone butyrate propionate		1983	19 (320)
mometasone furoate		1987	23 (338)
piketoprofen		1984	20 (322)
pimaprofen		1984	20 (322)
prednicarbate		1986	22 (325)
pravastatin	ANTILIPIDEMIC	1989	25 (316)
arteether	ANTIMALARIAL	2000	36 (296)
artemisinin		1987	23 (327)
bulaquine		2000	36 (299)
halofantrine		1988	24 (304)
mefloquine HCl		1985	21 (329)
almotriptan	ANTIMIGRAINE	2000	36 (295)
alpiropride		1988	24 (296)
eletriptan		2001	37 (266)

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
frovatriptan		2002	38 (357)
lomerizine HCl		1999	35 (342)
naratriptan hydrochloride		1997	33 (339)
rizatriptan benzoate		1998	34 (330)
sumatriptan succinate		1991	27 (333)
zolmitriptan		1997	33 (345)
dronabinol	ANTINAUSEANT	1986	22 (319)
amrubicin HCl	ANTINEOPLASTIC	2002	38 (349)
amsacrine		1987	23 (327)
anastrozole		1995	31 (338)
bicalutamide		1995	31 (338)
bisantrene hydrochloride		1990	26 (300)
camostat mesylate		1985	21 (325)
capecitabine		1998	34 (319)
cladribine		1993	29 (335)
cytarabine ocfosfate		1993	29 (335)
docetaxel		1995	31 (341)
doxifluridine		1987	23 (332)
enocitabine		1983	19 (318)
epirubicin HCl		1984	20 (318)
fadrozole HCl		1995	31 (342)
fludarabine phosphate		1991	27 (327)
flutamide		1983	19 (318)
formestane		1993	29 (337)
fotemustine		1989	25 (313)
geftimib		2002	38 (358)
gemcitabine HCl		1995	31 (344)
idarubicin hydrochloride		1990	26 (303)
imatinib mesylate		2001	37 (267)
interferon gamma-1α		1992	28 (332)
interleukin-2		1989	25 (314)
irinotecan		1994	30 (301)
lonidamine		1987	23 (337)
mitoxantrone HCl		1984	20 (321)
nedaplatin		1995	31 (347)
nilutamide		1987	23 (338)
paclitaxal		1993	29 (342)
pegaspargase		1994	30 (306)
pentostatin		1992	28 (334)
pirarubicin		1988	24 (309)
ranimustine		1987	23 (341)
sobuzoxane		1994	30 (310)
temoporphin		2002	38 (367)
toremifene		1989	25 (319)

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
vinorelbine		1989	25 (320)
zinostatin stimalamer		1994	30 (313)
porfimer sodium	ANTINEOPLASTIC ADJUVANT	1993	29 (343)
masoprocol	ANTINEOPLASTIC, TOPICAL	1992	28 (333)
miltefosine		1993	29 (340)
dexfenfluramine	ANTIOBESITY	1997	33 (332)
orlistat		1998	34 (327)
sibutramine		1998	34 (331)
atovaquone	ANTIPARASITIC	1992	28 (326)
ivermectin		1987	23 (336)
budipine	ANTIPARKINSONIAN	1997	33 (330)
CHF-1301		1999	35 (336)
droxidopa		1989	25 (312)
entacapone		1998	34 (322)
pergolide mesylate		1988	24 (308)
pramipexole hydrochloride		1997	33 (341)
ropinirole HCl		1996	32 (317)
talipexole		1996	32 (318)
tolcapone		1997	33 (343)
lidamidine HCl	ANTIPERISTALTIC	1984	20 (320)
gestrinone	ANTIPROGESTOGEN	1986	22 (321)
cabergoline	ANTIPROLACTIN	1993	29 (334)
tamsulosin HCl	ANTIPROSTATIC HYPERTROPHY	1993	29 (347)
acitretin	ANTIPSORIATIC	1989	25 (309)
calcipotriol		1991	27 (323)
tazarotene		1997	33 (343)
tacalcitol	ANTIPSORIATIC, TOPICAL	1993	29 (346)
amisulpride	ANTIPSYCHOTIC	1986	22 (316)
remoxipride hydrochloride		1990	26 (308)
zuclopenthixol acetate		1987	23 (345)
actarit	ANTIRHEUMATIC	1994	30 (296)
diacerein		1985	21 (326)
octreotide	ANTISECRETORY	1988	24 (307)
adamantanium bromide	ANTISEPTIC	1984	20 (315)

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
drotecogin alfa	ANTISEPSIS	2001	37 (265)
cimetropium bromide	ANTISPASMODIC	1985	21 (326)
tiquizium bromide		1984	20 (324)
tiropramide HCl		1983	19 (324)
argatroban	ANTITHROMBOTIC	1990	26 (299)
bivalirudin		2000	36 (298)
defibrotide		1986	22 (319)
cilostazol		1988	24 (299)
clopidogrel hydrogensulfate		1998	34 (320)
cloricromen		1991	27 (325)
enoxaparin		1987	23 (333)
eptifibatide		1999	35 (340)
ethyl icosapentate		1990	26 (303)
fondaparinux sodium		2002	38 (356)
indobufen		1984	20 (319)
limaprost		1988	24 (306)
ozagrel sodium		1988	24 (308)
picotamide		1987	23 (340)
tirofiban hydrochloride		1998	34 (332)
flutropium bromide	ANTITUSSIVE	1988	24 (303)
levodropropizine		1988	24 (305)
nitisinone	ANTITYROSINAEMIA	2002	38 (361)
benexate HCl	ANTIULCER	1987	23 (328)
dosmalfate		2000	36 (302)
ebrotidine		1997	33 (333)
ecabet sodium		1993	29 (336)
egualen sodium		2000	36 (303)
enprostil		1985	21 (327)
famotidine		1985	21 (327)
irsogladine		1989	25 (315)
lansoprazole		1992	28 (332)
misoprostol		1985	21 (329)
nizatidine		1987	23 (339)
omeprazole		1988	24 (308)
ornoprostil		1987	23 (339)
pantoprazole sodium		1994	30 (306)
plaunotol		1987	23 (340)
polaprezinc		1994	30 (307)
ranitidine bismuth citrate		1995	31 (348)
rebamipide		1990	26 (308)
rosaprostol		1985	21 (332)
roxatidine acetate HCl		1986	22 (326)
roxithromycin		1987	23 (342)

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
sofalcone		1984	20 (323)
spizofurone		1987	23 (343)
teprenone		1984	20 (323)
tretinoin tocoferil		1993	29 (348)
troxipide		1986	22 (327)
abacavir sulfate	ANTIVIRAL	1999	35 (333)
adefovir dipivoxil		2002	38 (348)
amprenavir		1999	35 (334)
atazanavir		2003	39 (269)
cidofovir		1996	32 (306)
delavirdine mesylate		1997	33 (331)
didanosine		1991	27 (326)
efavirenz		1998	34 (321)
emtricitabine		2003	39 (274)
enfuvirtide		2003	39 (275)
famciclovir		1994	30 (300)
fomivirsen sodium		1998	34 (323)
fosamprenavir		2003	39 (277)
foscarnet sodium		1989	25 (313)
ganciclovir		1988	24 (303)
imiquimod		1997	33 (335)
indinavir sulfate		1996	32 (310)
interferon alfacon-1		1997	33 (336)
lamivudine		1995	31 (345)
lopinavir		2000	36 (310)
neflinavir mesylate		1997	33 (340)
nevirapine		1996	32 (313)
oseltamivir phosphate		1999	35 (346)
penciclovir		1996	32 (314)
propagermanium		1994	30 (308)
rimantadine HCl		1987	23 (342)
ritonavir		1996	32 (317)
saquinavir mesylate		1995	31 (349)
sorivudine		1993	29 (345)
stavudine		1994	30 (311)
tenofovir disoproxil fumarate		2001	37 (271)
valaciclovir HCl		1995	31 (352)
zalcitabine		1992	28 (338)
zanamivir		1999	35 (352)
zidovudine		1987	23 (345)
influenza virus live	ANTIVIRAL VACCINE	2003	39 (277)
cevimeline hydrochloride	ANTI-XEROSTOMIA	2000	36 (299)
alpidem	ANXIOLYTIC	1991	27 (322)

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
buspirone HCl		1985	21 (324)
etizolam		1984	20 (318)
flutazolam		1984	20 (318)
flutoprazepam		1986	22 (320)
metaclazepam		1987	23 (338)
mexazolam		1984	20 (321)
tandospirone		1996	32 (319)
atomoxetine	ATTENTION DEFICIT HYPERACTIVITY DISORDER	2003	39 (270)
flumazenil	BENZODIAZEPINE ANTAG.	1987	23 (335)
bambuterol	BRONCHODILATOR	1990	26 (299)
doxofylline		1985	21 (327)
formoterol fumarate		1986	22 (321)
mabuterol HCl		1986	22 (323)
oxitropium bromide		1983	19 (323)
salmeterol hydroxynaphthoate		1990	26 (308)
tiotropium bromide		2002	38 (368)
APD	CALCIUM REGULATOR	1987	23 (326)
clodronate disodium		1986	22 (319)
disodium pamidronate		1989	25 (312)
gallium nitrate		1991	27 (328)
ipriflavone		1989	25 (314)
neridronic acid		2002	38 (361)
dexrazoxane	CARDIOPROTECTIVE	1992	28 (330)
bucladesine sodium	CARDIOSTIMULANT	1984	20 (316)
denopamine		1988	24 (300)
docarpamine		1994	30 (298)
dopexamine		1989	25 (312)
enoximone		1988	24 (301)
flosequinan		1992	28 (331)
ibopamine HCl		1984	20 (319)
loprinone hydrochloride		1996	32 (312)
milrinone		1989	25 (316)
vesnarinone		1990	26 (310)
amrinone	CARDIOTONIC	1983	19 (314)
colforsin daropate HCL		1999	35 (337)
xamoterol fumarate		1988	24 (312)
cefozopran HCL	CEPHALOSPORIN, INJECTABLE	1995	31 (339)

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
cefditoren pivoxil	CEPHALOSPORIN, ORAL	1994	30 (297)
brovincamine fumarate	CEREBRAL VASODILATOR	1986	22 (317)
nimodipine		1985	21 (330)
propentofylline		1988	24 (310)
succimer	CHELATOR	1991	27 (333)
trientine HCl		1986	22 (327)
fenbuprol	CHOLERETIC	1983	19 (318)
auranofin	CHRYSOTHERAPEUTIC	1983	19 (314)
taltirelin	CNS STIMULANT	2000	36 (311)
aniracetam	COGNITION ENHANCER	1993	29 (333)
pramiracetam H2SO4		1993	29 (343)
carperitide	CONGESTIVE HEART	1995	31 (339)
nesiritide	FAILURE	2001	37 (269)
drospirenone	CONTRACEPTIVE	2000	36 (302)
norelgestromin		2002	38 (362)
nicorandil	CORONARY VASODILATOR	1984	20 (322)
dornase alfa	CYSTIC FIBROSIS	1994	30 (298)
neltenexine		1993	29 (341)
amifostine	CYTOPROTECTIVE	1995	31 (338)
nalmefene HCL	DEPENDENCE TREATMENT	1995	31 (347)
ioflupane	DIAGNOSIS CNS	2000	36 (306)
azosemide	DIURETIC	1986	22 (316)
muzolimine		1983	19 (321)
torasemide		1993	29 (348)
atorvastatin calcium	DYSLIPIDEMIA	1997	33 (328)
cerivastatin		1997	33 (331)
naftopidil	DYSURIA	1999	35 (343)
alglucerase	ENZYME	1991	27 (321)
erdosteine	EXPECTORANT	1995	31 (342)
fudosteine		2001	37 (267)
agalsidase alfa	FABRY'S DISEASE	2001	37 (259)
cetrorelix	FEMALE INFERTILITY	1999	35 (336)
ganirelix acetate		2000	36 (305)

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
follitropin alfa	FERTILITY ENHANCER	1996	32 (307)
follitropin beta		1996	32 (308)
reteplase	FIBRINOLYTIC	1996	32 (316)
esomeprazole magnesium	GASTRIC ANTISECRETORY	2000	36 (303)
lafutidine		2000	36 (307)
rabeprazole sodium		1998	34 (328)
cinitapride	GASTROPROKINETIC	1990	26 (301)
cisapride		1988	24 (299)
itopride HCL		1995	31 (344)
mosapride citrate		1998	34 (326)
imiglucerase	GAUCHER'S DISEASE	1994	30 (301)
miglustat		2003	39 (279)
somatotropin	GROWTH HORMONE	1994	30 (310)
somatomedin-1	GROWTH HORMONE INSENSITIVITY	1994	30 (310)
factor VIIa	HAEMOPHILIA	1996	32 (307)
levosimendan	HEART FAILURE	2000	36 (308)
pimobendan		1994	30 (307)
anagrelide hydrochloride	HEMATOLOGIC	1997	33 (328)
erythropoietin	HEMATOPOETIC	1988	24 (301)
factor VIII	HEMOSTATIC	1992	28 (330)
malotilate	HEPATOPROTECTIVE	1985	21 (329)
mivotilate		1999	35 (343)
buserelin acetate	HORMONE	1984	20 (316)
goserelin		1987	23 (336)
leuprolide acetate		1984	20 (319)
nafarelin acetate		1990	26 (306)
somatropin		1987	23 (343)
zoledronate disodium	HYPERCALCEMIA	2000	36 (314)
cinacalcet	HYPERPARATHYROIDISM	2004	40 (451)
sapropterin hydrochloride	HYPERPHENYL-ALANINEMIA	1992	28 (336)
quinagolide	HYPERPROLACTINEMIA	1994	30 (309)
cadralazine	HYPERTENSIVE	1988	24 (298)
nitrendipine		1985	21 (331)

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
binfonazole	HYPNOTIC	1983	19 (315)
brotizolam		1983	19 (315)
butoctamide		1984	20 (316)
cinolazepam		1993	29 (334)
doxefazepam		1985	21 (326)
loprazolam mesylate		1983	19 (321)
quazepam		1985	21 (332)
rilmazafone		1989	25 (317)
zaleplon		1999	35 (351)
zolpidem hemitartrate		1988	24 (313)
zopiclone		1986	22 (327)
acetohydroxamic acid	HYPOAMMONURIC	1983	19 (313)
sodium cellulose PO4	HYPOCALCIURIC	1983	19 (323)
divistyramine	HYPOCHOLESTEROLEMIC	1984	20 (317)
lovastatin		1987	23 (337)
melinamide		1984	20 (320)
pitavastatin		2003	39 (282)
rosuvastatin		2003	39 (283)
simvastatin		1988	24 (311)
glucagon, rDNA	HYPOGLYCEMIA	1993	29 (338)
acipimox	HYPOLIPIDEMIC	1985	21 (323)
beclobrate		1986	22 (317)
binifibrate		1986	22 (317)
ciprofibrate		1985	21 (326)
colesevelam hydrochloride		2000	36 (300)
colestimide		1999	35 (337)
ezetimibe		2002	38 (355)
fluvastatin		1994	30 (300)
meglutol		1983	19 (321)
ronafibrate		1986	22 (326)
modafinil	IDIOPATHIC HYPERSOMNIA	1994	30 (303)
bucillamine	IMMUNOMODULATOR	1987	23 (329)
centoxin		1991	27 (325)
thymopentin		1985	21 (333)
filgrastim	IMMUNOSTIMULANT	1991	27 (327)
GMDP		1996	32 (308)
interferon gamma-1b		1991	27 (329)
lentinan		1986	22 (322)
pegademase bovine		1990	26 (307)
pidotimod		1993	29 (343)

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
romurtide		1991	27 (332)
sargramostim		1991	27 (332)
schizophyllan		1985	22 (326)
ubenimex		1987	23 (345)
cyclosporine	IMMUNOSUPPRESSANT	1983	19 (317)
everolimus		2004	40 (455)
gusperimus		1994	30 (300)
mizoribine		1984	20 (321)
muromonab-CD3		1986	22 (323)
mycophenolate sodium		2003	39 (279)
mycophenolate mofetil		1995	31 (346)
pimecrolimus		2002	38 (365)
tacrolimus		1993	29 (347)
defeiprone	IRON CHELATOR	1995	31 (340)
alosetron hydrochloride	IRRITABLE BOWEL	2000	36 (295)
tegasedor maleate	SYNDROME	2001	37 (270)
sulbactam sodium	β-LACTAMASE INHIBITOR	1986	22 (326)
tazobactam sodium		1992	28 (336)
nartograstim	LEUKOPENIA	1994	30 (304)
pumactant	LUNG SURFACTANT	1994	30 (308)
sildenafil citrate	MALE SEXUAL DYSFUNCTION	1998	34 (331)
gadoversetamide	MRI CONTRAST AGENT	2000	36 (304)
telmesteine	MUCOLYTIC	1992	28 (337)
laronidase	MUCOPOLYSACCARIDOSIS	2003	39 (278)
interferon ß-1a	MULTIPLE SCLEROSIS	1996	32 (311)
interferon ß-1b		1993	29 (339)
glatiramer acetate		1997	33 (334)
natalizumab		2004	40 (462)
afloqualone	MUSCLE RELAXANT	1983	19 (313)
cisatracurium besilate		1995	31 (340)
doxacurium chloride		1991	27 (326)
eperisone HCl		1983	19 (318)
mivacurium chloride		1992	28 (334)
rapacuronium bromide		1999	35 (347)
tizanidine		1984	20 (324)
naltrexone HCl	NARCOTIC ANTAGONIST	1984	20 (322)
tinazoline	NASAL DECONGESTANT	1988	24 (312)

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
aripiprazole	NEUROLEPTIC	2002	38 (350)
clospipramine hydrochloride		1991	27 (325)
nemonapride		1991	27 (331)
olanzapine		1996	32 (313)
perospirone hydrochloride		2001	37 (270)
quetiapine fumarate		1997	33 (341)
risperidone		1993	29 (344)
sertindole		1996	32 (318)
timiperone		1984	20 (323)
ziprasidone hydrochloride		2000	36 (312)
rocuronium bromide	NEUROMUSCULAR BLOCKER	1994	30 (309)
edaravone	NEUROPROTECTIVE	1995	37 (265)
fasudil HCL		1995	31 (343)
riluzole		1996	32 (317)
bifemelane HCl	NOOTROPIC	1987	23 (329)
choline alfoscerate		1990	26 (300)
exifone		1988	24 (302)
idebenone		1986	22 (321)
indeloxazine HCl		1988	24 (304)
levacecarnine HCl		1986	22 (322)
nizofenzone fumarate		1988	24 (307)
oxiracetam		1987	23 (339)
bromfenac sodium	NSAID	1997	33 (329)
lornoxicam		1997	33 (337)
OP-1	OSTEOINDUCTOR	2001	37 (269)
alendronate sodium	OSTEOPOROSIS	1993	29 (332)
ibandronic acid		1996	32 (309)
incadronic acid		1997	33 (335)
raloxifene hydrochloride		1998	34 (328)
risedronate sodium		1998	34 (330)
strontium ranelate		2004	40 (467)
tiludronate disodium	PAGET'S DISEASE	1995	31 (350)
tadalafil	PDE5 INHIBITOR	2003	39 (284)
vardenafil		2003	39 (286)
temoporphin	PHOTOSENSITIZER	2002	38 (367)
verteporfin		2000	36 (312)
alefacept	PLAQUE PSORIASIS	2003	39 (267)

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
beraprost sodium	PLATELET AGGREG. INHIBITOR	1992	28 (326)
epoprostenol sodium		1983	19 (318)
iloprost		1992	28 (332)
sarpogrelate HCl	PLATELET ANTIAGGREGANT	1993	29 (344)
trimetrexate glucuronate	*PNEUMOCYSTIS CARINII* PNEUMONIA	1994	30 (312)
solifenacin	POLLAKIURIA	2004	40 (466)
histrelin	PRECOCIOUS PUBERTY	1993	29 (338)
atosiban	PRETERM LABOR	2000	36 (297)
gestodene	PROGESTOGEN	1987	23 (335)
nomegestrol acetate		1986	22 (324)
norgestimate		1986	22 (324)
promegestrone		1983	19 (323)
trimegestone		2001	37 (273)
alpha-1 antitrypsin	PROTEASE INHIBITOR	1988	24 (297)
nafamostat mesylate		1986	22 (323)
adrafinil	PSYCHOSTIMULANT	1986	22 (315)
dexmethylphenidate HCl		2002	38 (352)
dutasteride		2002	38 (353)
efalizumab	PSORIASIS	2003	39 (274)
finasteride	5α-REDUCTASE INHIBITOR	1992	28 (331)
surfactant TA	RESPIRATORY SURFACTANT	1987	23 (344)
Adalimumab	RHEUMATOID ARTHRITIS	2003	39 (267)
dexmedetomidine hydrochloride	SEDATIVE	2000	36 (301)
kinetin	SKIN PHOTODAMAGE/ DERMATOLOGIC	1999	35 (341)
tirilazad mesylate	SUBARACHNOID HEMORRHAGE	1995	31 (351)
APSAC	THROMBOLYTIC	1987	23 (326)
alteplase		1987	23 (326)
balsalazide disodium	ULCERATIVE COLITIS	1997	33 (329)
tiopronin	UROLITHIASIS	1989	25 (318)

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
propiverine hydrochloride	UROLOGIC	1992	28 (335)
Lyme disease	VACCINE	1999	35 (342)
clobenoside	VASOPROTECTIVE	1988	24 (300)
falecalcitriol	VITAMIN D	2001	37 (266)
maxacalcitol		2000	36 (310)
paricalcitol		1998	34 (327)
doxercalciferol	VITAMIN D PROHORMONE	1999	35 (339)
prezatide copper acetate	VULNERARY	1996	32 (314)
acemannan	WOUND HEALING AGENT	2001	37 (257)
cadexomer iodine		1983	19 (316)
epidermal growth factor		1987	23 (333)