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DRUG DELIVERY

EDITORIAL

Drug Delivery in the Twenty-First Century: A New Paradigm

B Smith[1] and K Uhl[2]

Once evidence exists that a particular chemical entity positively affects a disease state and possesses a reasonable safety profile, proper treatment comes from adjusting the dose to appropriately minimize the risks and maximize the benefits of the therapeutic entity. With knowledge of environmental and/or genetic factors, physicians and other health-care practitioners commonly make dosage adjustments tailored to best meet an individual patient's needs. Sometimes, however, dose adjustments alone are insufficient to produce desired therapeutic gain, and alternative modes of drug delivery are necessary.

In a study of Alzheimer's disease patients, Lefèvre *et al*. found that the pharmacokinetic (PK) profiles of two drug delivery systems of rivastigmine—the transdermal patch and twice-daily oral capsules—differ vastly, and that these PK differences are entirely dependent on the modes of drug delivery (**Figure 1**).[1] In addition, the authors demonstrated that the patch and capsules produce not only different PK profiles but also distinctly different pharmacodynamic (PD) responses.[1] The implications of different delivery systems of rivastigmine are much broader than PK and PD differences alone, because additional research comparing the two delivery systems demonstrated that the transdermal patch was more tolerable than oral capsules, with equivalent efficacy.[2] Nausea was reported by 7% of the subjects in the 10-cm^2 patch group; this was comparable to the placebo group, 5% of which reported nausea, but much lower than the 23% of subjects in the 12-mg/day capsule group who reported nausea. On the other hand, the mean change from baseline score on the Alzheimer's Disease Assessment Scale–Cognitive Subscale at week 24 was –0.6 for subjects in the 10-cm^2 patch group. This is identical to the mean reduction from baseline for the 12-mg/day capsule group but much different from the mean change for the placebo group, which was a 1-unit increase.[2]

One of the basic tenets of clinical pharmacology and personalized medicine is delivery of the right drug at the right dose to the right patient so as to optimize therapeutics. It is readily apparent, then, given the rivastigmine example above, that the method of drug delivery is an equally important factor. These tenets must therefore also include *how* the drug is delivered—i.e., the correct route of drug administration. This issue of *Clinical Pharmacology & Therapeutics* is devoted to the concept of drug delivery and how advances in the pharmaceutical sciences and other scientific fields, e.g., nanotechnology and stem cell research, have enabled substantial progress in drug delivery. More traditional ways of viewing drug

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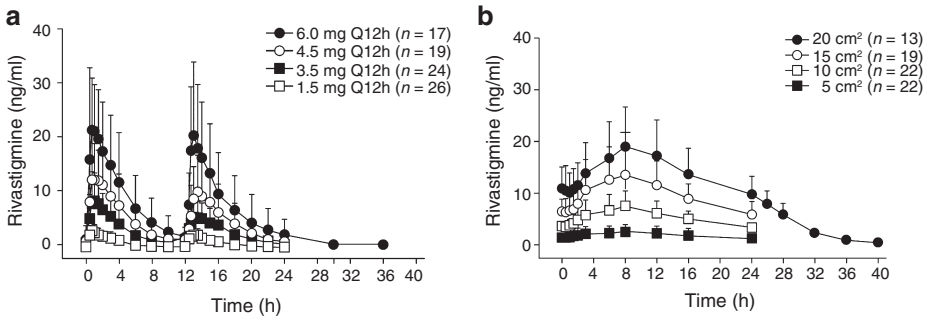

Figure 1 Rivastigmine mean (SD) plasma concentration following (**a**) capsule or (**b**) patch application. Reprinted from ref. 1.

delivery look at different routes of drug administration, e.g., oral, parenteral, transdermal, and inhalational. Here we explore the tip of the iceberg of emerging and evolving technologies that make possible more sophisticated applications toward drug delivery.

Tobacco: illustrating modes of drug delivery

The early story of tobacco use illustrates the importance of the method of delivery. It appears that tobacco, found primarily in its natural state in South America, was first cultivated 3,000 to 5,000 years ago.[3] Tobacco had then, and continues to have, a significant role in shaping South American culture. Johannes Wilbert, the author of *Tobacco and Shamanism in South America*, explains tobacco's use as a medicine, a recreational drug, and a spiritual tool.[4] Tobacco was long recognized to have analgesic properties and was therefore used for the relief of toothaches. It was also recognized as an effective insecticide and was used to rid individuals of lice or other parasites. Perhaps the most interesting use of tobacco was in preparing the traditional healer, the shaman, to practice his religious trade. At high concentrations, tobacco has hallucinogenic effects and can cause paralysis. South American natives interpreted the hallucinogenic effects of tobacco as a communications channel to the gods and the paralytic effects as a near-death experience. Because they believed that an individual who had a near-death experience was most able to treat disease, the potential shaman's reaction to high concentrations of tobacco was necessary to identify him as a shaman, which in turn allowed him to serve as an intermediary between the human and spirit worlds.

Tobacco was smoked both as cigars and in pipes. As we know, smoking tobacco permits quick and efficient nicotine delivery to the brain.[5] Other methods of administration, however, were widespread among South American natives. There is evidence that tobacco was chewed, drunk, licked, used as snuff, used in an enema, applied to broken skin, applied ocularly, and blown onto others. When the goal was to induce paralysis, tobacco was administered by many of these methods simultaneously, as well as in combination with a variety of herbs. South American natives made use of just about all possible routes of administration available to them.[4] The pipe seen on the cover of this issue of *CPT* highlights one of the earliest uses of a medical device as a modality to deliver a drug (nicotine). A cigar, another option for smoking tobacco and delivering nicotine, is relatively fragile and easily damaged or destroyed, whereas a pipe, which is filled with loose

tobacco, is relatively easy to transport. This convenience is partly responsible for the widespread use of the pipe to smoke tobacco and its extension for illustrative purposes to a drug (nicotine)–device (pipe) combination.

Why different drug delivery systems?

Throughout the history of medicine, there has been a need for alternative methods of delivering a drug to a patient. The reasons include the obvious clinical scenarios of vomiting and unconsciousness that preclude oral administration. Other aims are to improve ease of administration, ensure or improve patient compliance, decrease toxicity, improve drug bioavailability, and achieve precise therapeutic targeting. Patients' convenience and preferences were probably the initial impetus behind developments in drug delivery. Technologies associated with drug delivery are now commonly used to create and extend markets, with the pharmaceutical industry turning to drug delivery to extend the revenue-earning lifetime of their biggest products. It has been projected that the US demand for drug delivery systems will expand more than 10% annually to $132 billion in 2012.[6] Growth opportunities for drug delivery systems extend into all therapeutic classes of pharmaceuticals and encompass a wide range of compounds and formulations and a wide variety of options in the world market. Safety and efficacy shortcomings seen in current conventional medicines and special formulation needs for large molecules (e.g., liposomes, monoclonal antibodies) may be among the driving forces behind emerging technologies in drug delivery. The discovery of better delivery systems, in conjunction with the discovery of new pharmacological compounds, will potentially advance disease diagnosis and treatment beyond our wildest imagination. The "medical tricorder," a handheld diagnostics and drug delivery device used by Dr. Beverly Crusher on "Star Trek: The Next Generation," may not be so far-fetched and is certainly an amazing advancement over the simple pipe.

Often, however, drug–device combination products provide therapeutic advantages over either the device or the drug alone. For this reason, some forecast that combination products will double in global value (to US$11.5 billion) by 2010 from their 2004 value.[7] The regulatory path for these products is not always straightforward and can be particularly challenging if one is not aware of the regulations that govern combination products and how they differ from single-entity products. In this issue, Lauritsen, of the US Food and Drug Administration's Office of Combination Products, discusses the challenging regulatory, policy, scientific, and review–management issues presented by combination products.[7]

Therapeutic implications of manipulating drug delivery

Several therapeutic areas have reaped substantial benefit from technologies that modify, enhance, or target drug delivery. The development of combination drug–device products—including traditional small molecules, macromolecules, and larger cellular entities—will probably continue as our population ages and develops chronic diseases. Cardiovascular disease, for example, is the No. 1 killer of men and women in the developed world, with increasing incidence and prevalence with advancing age. Treatment of atherosclerotic heart disease, including myocardial infarction and occlusive coronary artery disease, was revolutionized with the introduction of the drug-eluting

stent (DES), an implantable drug–device combination product. DESs are currently used in the majority of coronary stent procedures in the United States. They have generally been shown to be superior to bare-metal stents; however, safety concerns have arisen with reports of increased risk of late stent thrombosis. In this issue, Maluenda *et al.*[8] provide a review of the safety and efficacy of different DESs, including new-generation DESs. Stem cell–based biologics have been examined for structural and functional repair of the myocardium for approximately a decade. Bartunek *et al.*[9] discuss limitations associated with current approaches to stem cell–based cardiovascular regenerative medicine, as well as potential improvements that may advance this area of technology. The recent approval of new therapies for the treatment of age-related macular degeneration is only one reason for renewed interest in ophthalmic drug delivery systems. As mentioned earlier, tobacco was sometimes administered ocularly, but one can only imagine the resultant burning that would certainly compromise the acceptability of this delivery method. Still, ocular drug delivery may be very effective for many eye disorders and can easily and quickly deliver effective drug concentrations to target tissues. Advances in ophthalmic drug delivery systems, including products in development and the unique regulatory aspects of ophthalmic product development, are reviewed by Novack.[10]

The consequences of poor patient compliance may include hospitalization, work absence, and increased morbidity and mortality. The major consequence of nonuse or incorrect use of contraception, however, is unintended pregnancy —of which there are annually more than 80 million worldwide—which may result in substantial lifetime financial, social, medical, and emotional consequences. Female contraception is an area in which pharmaceutics advances have resulted in many new drug delivery and dosing methods, advancing from the oral contraceptive "pill" to intramuscular, subdermal implantable, transdermal, intrauterine, and vaginal dosage forms in addition to low-dose and extended-dose oral contraception dosage forms. These impressive advances in contraceptive technology over the past 50 years, as well as future developments, are elucidated by Merkatz *et al.*[11]

Nanotechnology to modulate drug delivery

Nanotechnology is being used to manipulate not only the size of drug particles but the physical characteristics and thus the extent and location of drug delivery. Although the topic has been addressed in prior *CPT* issues, including nanoparticle applications[12] and transdermal vaccine delivery,[13] nanotechnologies are again examined as they relate specifically to drug delivery. Elman *et al.*[14] discuss the promise of advances in microelectromechanical systems and miniaturization technologies for passive and active drug delivery microdevices. The application of nanotechnology to the diagnosis and treatment of cancers is seen as potentially nanotechnology's largest public health contribution. Orringer *et al.*[15] describe targeting brain tumors with nanoparticles through antigen-dependent (specific) or antigen-independent (nonspecific) mechanisms. New technologies in science and medicine frequently present unique regulatory, moral, and ethical dilemmas, and nanotechnology is no exception. Ferrari *et al.*[16] discuss potential ethical issues associated with the wide range of materials used in nanotechnology and potential societal applications of nanomedicine while also recognizing nanotechnology's potential benefits.

Final thoughts

Drug delivery is a sizable topic, and many aspects are not touched on in this issue. What can be gleaned from these articles, however, is that many exciting new therapies are conceivable only with alternative means of delivering the drug to patients. This revelation raises enthusiasm about what the future holds, which may include surprises in which the method of drug delivery is found to be the critical link in clinical outcomes. The surprise may be unwarranted, however, if one remembers South American tobacco use:[3]

> *The astonishing diversity of tobacco habits reflects not only the multitudinous purposes it served, but also the different climatic conditions in which the weed was employed. For instance, it was hard to smoke in the thin, dry air of the Andes, so snuffing tended to prevail. Similarly, in the swamplands of the Amazon, where fires could not be kindled readily, tobacco was taken as a drink. Different methods of tobacco consumption often existed side by side—one form for everyday use, another for magic or ritual.*

CONFLICT OF INTEREST

B.S. is a full-time employee of Amgen. K.U. declared no conflict of interest.

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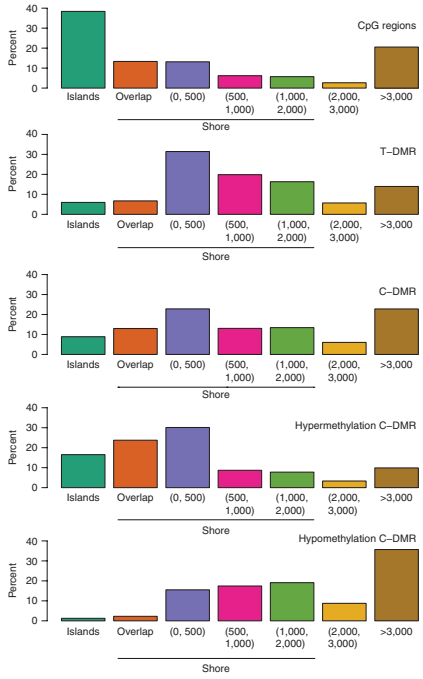
HIGHLIGHTS

Evaluating the human colon cancer methylome



Most studies of cancer methylation have failed to challenge the long-held assumption that functionally important DNA methylation (DNAm) will occur in promoters and that most DNAm changes in cancer occur in CpG islands. A new study published in *Nature Genetics* disputes this assumption by asserting that most methylation alterations in colon cancer actually occur in sequences up to 2 kb distant, termed "CpG island shores" by the researchers. The study aimed to answer three questions: where are DNAm changes that distinguish tissue type, where are DNAm alterations in cancer, and what is the functional role of these changes? A genome-wide analysis of DNAm revealed that most tissue-specific DNAm occurs at CpG island shores, that tissue-differential methylation regions (T-DMRs) are highly conserved between human and mouse, and that most cancer-related changes in DNAm correspond to T-DMRs. These findings indicate that epigenetic alterations affecting tissue-specific differentiation are potentially the predominant mechanism by which epigenetic changes cause cancer. (*Nat. Genet.* **41**, 178–186, 2009)

Highlights written by Elise Laffman-Johnson.

Determining the benefit of warfarin treatment in cancer patients

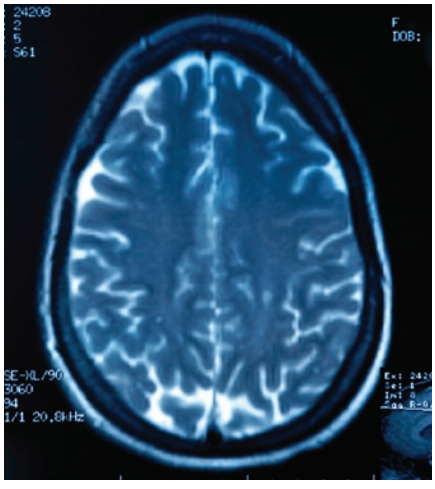
Warfarin remains a controversial treatment in cancer patients with central venous catheters (CVCs), prompting investigators to assess whether warfarin reduces catheter-related thrombosis compared with treatment without warfarin and whether dosage influences the thromboprophylactic effect. Venous thromboembolism, a known complication related to the use of CVCs, is evidenced in approximately 50% of postmortem examinations of individuals with cancer. In a study that sought to determine the benefit of warfarin treatment, 1,590 cancer patients receiving chemotherapy through CVCs were divided into three groups receiving either no warfarin, fixed-dose warfarin, or dose-adjusted warfarin per day. The findings indicate that treatment with warfarin does not reduce symptomatic catheter-related or other thromboses in cancer patients when compared with patients not receiving warfarin. Having determined that warfarin has little benefit to cancer patients, the researchers conclude that investigation into newer treatment options should be conducted. (*Lancet* **373**, 567–574, 2009)

Google: a better alternative to traditional influenza surveillance systems?

Worldwide, 250,000 to 500,000 deaths and millions of respiratory illnesses each year are attributed to seasonal influenza epidemics. Staggering as these statistics are, a new strain of influenza virus against which no immunity exists could increase the number of fatalities into the millions. Early detection is the best means of reducing the impact of both seasonal and epidemic influenza, yet current surveillance systems lack the ability to report the needed data without a 1- to 2-week lag. With millions of Americans using online search tools to research diseases and medical problems each year, researchers now believe that Web search queries are uniquely valuable as a source of information

about health trends, as well as a potential means of faster detection of an influenza outbreak. Google's search logs provide access to queries from millions of users, thereby offering accelerated assessment of influenza data to determine potential outbreaks. (*Nature* **457**, 1012–1014, 2009)

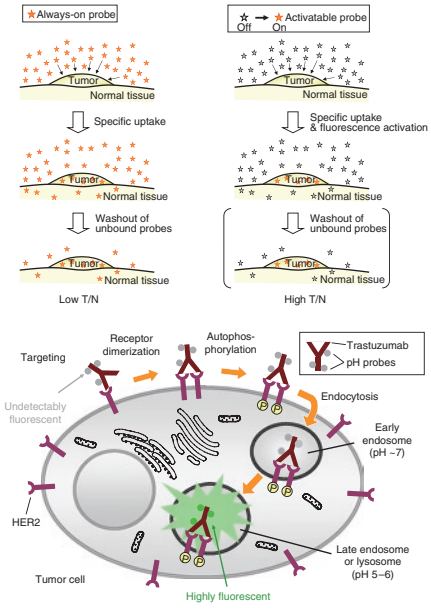
Safety and efficacy of autologous non-myeloablative hemopoietic stem cell transplantation



Over time, most individuals with relapsing-remitting multiple sclerosis (MS) develop secondary-progressive MS, the form of MS complicated by irreversible and gradual neurological impairments that progress without acute relapse. Patients with secondary progressive MS who underwent hemopoietic stem cell transplantation reported no improvement in neurological disability, but the disability stabilized for a period of time. A recent study evaluates the application of autologous non-myeloablative hemopoietic stem cell transplantation in patients with relapsing-remitting MS to determine safety and clinical outcome. Twenty-one patients with this form of the disease who had not responded to treatment with interferon-β were included in the study; after a mean of 37 months, all 21 subjects were free of progression, and 16 were free of relapses. The study's findings suggest that non-myeloablative autologous hemopoietic stem cell transplantation reverses neurological deficits in patients with relapsing-remitting MS, but the authors acknowledge that further studies are needed to confirm these results. (*Lancet* **8**, 244–253, 2009)

Probe conjugates as a clinical tool for cancer detection

The development of tumor-imaging techniques with sufficient specificity and sensitivity is integral to the future of cancer diagnosis. The injectable molecular imaging probes currently available often fail to detect small volumes of viable cancer because of low target-to-background ratios, highlighting the need to minimize the background signal originating from nontarget tissues. A recently published study demonstrates that macromolecule conjugates allow for the development of small-molecule, pH-activated fluorescence probes capable of targeting viable cancer cells. Testing on type 2–positive lung cancer cells in mice showed these probes to be effective in detecting tumors with minimal background signal. These results represent an important step toward adapting probe conjugates as a clinical tool for cancer detection. (*Nat. Med.* **15**, 104–109, 2009)



ASCPT NEWS

Call for 2010 symposia and workshop proposals

The Scientific Program Committee is calling for proposals for symposia and workshops to be presented at the 2010 Annual Meeting, which will be held in Atlanta, Georgia. Members of ASCPT are invited to submit proposals for consideration.

All proposals will be competitively peer-reviewed and ranked by members of ASCPT's Scientific Program Committee.

Additional information and guidelines about symposia and workshops can be found at http://www.ascpt.org. The deadline for online submission of proposals is Friday, 26 June 2009, at 3 PM Eastern time.

ASCPT receives accreditation from ACPE

Susan Abdel-Rahman, PharmD

ASCPT has received accreditation from the American Council on Pharmacy Education (ACPE) for a period of 6 years.

The Society would like to acknowledge and thank Susan Abdel-Rahman, PharmD, ASCPT's Vice Chair for CPE, for her leadership in the self-study process and for ensuring that ASCPT's CPE program continues to meet the criteria for accreditation.

Membership recruiters recognized

Saskia N. de Wildt, MD, PhD

Saskia N. de Wildt, MD, PhD, Erasmus MC Sophia Children's Hospital in the Netherlands, is the 2008–2009 top membership recruiter for ASCPT. Dr. de Wildt was recognized by the Society at the 2009 Annual Meeting for her outstanding efforts in recruitment of new members.

Other ASCPT members recognized for their efforts include:

Recruited five new members
Patricia W. Slattum, PharmD, PhD, CGP

Recruited two new members
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We thank these members for enhancing the voice of clinical pharmacology and ASCPT.

Call for 2010 Scientific Award nominations

The American Society for Clinical Pharmacology and Therapeutics (ASCPT) Scientific Awards program seeks to recognize excellence in clinical pharmacology, highlighting the continuum of drug discovery, development, regulation, and utilization of medicines.

The Scientific Awards Committee is now accepting nominations for the following awards:

- Leon I. Goldberg Young Investigator Award
- Henry W. Elliott Distinguished Service Award
- Gary Neil Prize for Innovation in Drug Development
- Oscar B. Hunter Memorial Award in Therapeutics
- Rawls-Palmer Progress in Medicine Award
- William B. Abrams Award in Geriatric Clinical Pharmacology

Nominations can be easily submitted online at http://www.ascpt.org. The deadline for receipt of nominations is Friday, 25 September 2009.

Member of the Month

Barry Mangum, PharmD, Associate Clinical Professor, Clinical Pharmacology, Duke University Medical Center, Durham, NC Dr. Mangum is the Chair of the ASCPT Government Affairs Committee and a member of the *Clinical Pharmacology & Therapeutics* Editorial Board. He also served on the Scientific Program Committee for the 2009 Annual Meeting. Since graduating from the Medical University of South Carolina in 1981, Dr. Mangum has designed and implemented global clinical research programs in more than 35 countries for 30 multinational pharmaceutical companies. He has over 16 years of experience with phase I–IV clinical trials in more than 40 clinical programs. In addition to his service to ASCPT, Dr. Mangum is an Executive Committee Member of CAPTN (Child and Adolescent Psychiatry Trials Network), whose goal is to develop and share knowledge that improves the care of pediatric patients through innovative clinical research. Dr. Mangum co-founded NeoFax "to help reduce drug dosing and nutritional errors in the difficult-to-treat pediatric population."

Dedicated Member

Alexander A. Vinks, PharmD, PhD, FCP, Director, Professor of Pediatrics and Pharmacology, Division of Clinical Pharmacology, Cincinnati Children's Hospital Medical Center, Cincinnati, OH Dr. Vinks has been a member of ASCPT since 2001. He credits the Society, its many leaders in clinical pharmacology, and the Annual Meetings as important sources of inspiration: "As a native of the Netherlands moving to Cincinnati in 2000, the Annual Meetings have been a wonderful resource for networking and personally meeting with many inspiring clinical pharmacologists from a wide variety of backgrounds that are part of this Society. As Chair of the Therapeutic Drug and Toxicology Monitoring Scientific Section and member of Coordinating Committee on Scientific Sections, I experienced the efforts to restructure scientific endeavors within

Welcome new ASCPT members!

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Associate in Training
Mindy N. Cohen, MD
University of Colorado at Denver

the drug discovery, development, regulatory, and utilization framework as a wonderful opportunity to experience the breadth of the Society."

Dr. Vinks currently mentors nine junior faculty members at his institution. He recalls one of many important landmarks that he has seen during his career: "the introduction of the programmable handheld calculator that allowed us to do simple pharmacokinetic calculations at the bedside. Now we have so much more insight and research tools to appreciate the many therapeutic complexities that have been discovered during my career. This does allow us to develop more intelligent methods for individualized drug dosing using computer technology and modeling and simulation to integrate both novel pharmacokinetic–pharmacodynamic approaches and pharmacogenetics." Since 1982, Dr. Vinks has given more than 150 invited lectures and presentations and published over 70 peer-reviewed research articles.

New Member

Eric J. Laïlle, MS, Manager, Clinical Pharmacology, Celgene Corporation, Overland Park, KS Mr. Laïlle is certain that being a member of ASCPT "will allow me to meet and interact with leaders in drug discovery, development, regulation, and use of safe and effective medication and be at the forefront of new developments and strategies in clinical pharmacology." In his current position, he is responsible for study design, protocol writing, data analysis, and report writing. He also manages drug metabolism, bioanalytical, and nonclinical studies. In addition, he prepares and reviews various publications, posters, and presentations for scientific meetings. He believes "the biggest challenges to clinical pharmacology are the individualization of pharmacological treatments and regimens, the identification of the influence of genetics on drug activity (both pharmacokinetics and pharmacodynamics), and the design of protocols to facilitate this research."

POINT/COUNTERPOINT

Studies Should Be Controlled, Randomized, and Blinded

RA Parker[1]

Rigorous assessment of the efficacy and safety of a new therapy requires a concurrent control group, randomization, and blinding. The control group allows comparison with a contemporaneous patient group. Randomization, properly done, avoids systematic bias between treatment groups and should balance other factors, reducing the likelihood of group differences due to patient characteristics. Blinding minimizes the risk of biases stemming from patients' and assessors' beliefs, actions, and hopes about the treatment received. This is critical given the substantial effect that such beliefs can have on outcome, often termed the "placebo effect." New and exciting technologies would probably be particularly susceptible to the placebo effect, making blinding even more critical when studying such treatments.

While this opinion piece on the importance of blinding in clinical trials was being prepared, a survey on physician use of placebos was published.[1] The news media widely reported that approximately half the physicians gave sham therapies to some patients. Strictly speaking, very few of the medications were true placebos, but they were being used as placebo treatments: "a treatment whose benefits derive from positive patient expectations and not from the physiological mechanism of the treatment itself."[1] Such treatments act through the placebo effect. The article focused on technical and ethical issues, not on whether such treatments work. Other studies have reported similar results from multiple countries. Practicing physicians believe that patients' beliefs can have clinical benefit. Thus, one needs to minimize this potential source of bias in a clinical trial. Effective blinding of treatment does this.

Many studies have shown that the beliefs and behaviors of patients and assessors can affect outcome; I describe only four such studies. In the 1980s, Guyatt and colleagues showed that encouragement during the 6-minute walk test significantly increased speed among individuals with chronic obstructive pulmonary disease.[2] In Parkinson's disease patients, differences between groups receiving actual and sham surgery were larger both in quality-of-life measurements and in ratings by blinded medical staff when compared by study subjects' perceived treatment group than when compared by actual treatment received.[3] In other words, what patients thought they had received had a greater effect than what they had actually received. Benedetti and colleagues showed that sham procedures produce objective changes in movement in Parkinson's disease patients.[4] The placebo effect can even be manipulated—supportive behavior by a health-care provider while administering a sham treatment can increase symptom relief and improvement in quality of life.[5]

For regulatory approval of a treatment, one or more adequate and well-controlled studies are required, normally a randomized, double-blinded, controlled trial. Logically, the decision to use a contemporaneous control group (whether active or placebo) comes first. Without such a group, it would only be possible to interpret results against historical experience, and randomization and blinding would be impossible. Once there is more than one group being studied, randomization with treatment allocation unknown before patient enrollment is possible. This avoids systematic bias in the way treatment is allocated to patients and should balance other patient characteristics, reducing the chance that differences in outcome between groups are due to differences in patient characteristics. Blinding of treatment allocation is also possible. Blinding investigators to assigned treatment minimizes systematic differences between study groups in treatment (e.g., concomitant medications, differential follow-up) and assessment. Blinding patients to assigned treatment minimizes systematic differences in such

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areas as self-reported adverse events, overall assessment of general health, effort expended in testing and assessment procedures, and even willingness to remain in the study. In ongoing studies, sponsors go to great lengths to remain blinded to interim results to avoid questions about how their knowledge might have compromised study integrity. Blinding not only helps to minimize the impact of potential biases but, more important, gives reason to believe that these biases are unlikely to be present. Therefore, blinding is routinely considered a basic feature of a well-controlled study.

This is not to say that blinding is always effective. There are occasions when effective blinding is impossible. Sometimes a study involves several active treatments associated with frequent but different side effects. The presence of the side effect identifies the treatment that many patients received. Even rarer, a treatment may be a breakthrough, and the patients' response reliably identifies those receiving the experimental treatment. In both these situations, a blinded assessor can and should be used for all other assessments. For example, in the first situation, an assessor blinded to the specific side effects related to treatment should assess other side effects not known to be related to a specific treatment and the outcome of the study. Admittedly, blinding is likely to be less than perfect, because the patient knows the treatment and may reveal information even if instructed not to, but certainly imperfect blinding is better than not attempting to assess outcome in a blinded manner.

Arguments have been made that placebo controls are unethical in general. Like any treatment, placebo treatment can cause harm. Because a placebo is assumed to have no potential for benefit, exposing patients to the unnecessary risks of an add-on placebo is unethical. A similar argument is raised when a randomized clinical trial is proposed for a therapy that "everyone knows" is better. These are arguments against having a contemporaneous control group, not arguments against randomization and blinding.

But without randomized controlled studies, how can we be confident that treatments actually work? In the 1960s, everyone knew that the Halsted radical mastectomy was better than less invasive surgery for the treatment of breast cancer. It was obvious that more surgery was better, and it had been the standard of care for more than 50 years. At that time, many considered it unethical to randomize patients to less invasive procedures.[6] But the procedure was *not* better, and much less invasive surgery is now the standard of care. In the 1980s, everyone knew that arrhythmia suppression reduced the risk of sudden deaths. The Cardiac Arrhythmia Suppression Trial (CAST) was even designed with a one-sided *P* value. The CAST results, however, showed that what everyone "knew" was not true, and the results "astounded most observers."[7]

A blinded study comparing surgical intervention with best medical care apparently raises the highest ethical hurdle. Blinding patients requires sham surgery on the control group, exposing them to the risks of the procedure with no expectation of benefit. It is difficult to argue that sham surgery could provide any benefit, so such studies appear to be unethical. Nonetheless, they are done. In Parkinson's disease, 20 uncontrolled studies reported benefits from transplantation of dopamine neurons before a randomized blinded study involving sham surgery found a much smaller benefit, only in younger patients and only when they were off medication.[8] Significantly, the benefits depended more on the treatment the individuals believed they had received than on the treatment that they actually received.[3] As another example, at least 15 studies had reported improvement after arthroscopic surgery in individuals with osteoarthritis of the knee, but a randomized controlled study using sham surgery failed to show any benefit in either subjective or objective measurements.[9] Randomized controlled studies are essential to accurately assess the benefit of therapy.

To make matters worse, few outcomes can be unambiguously assessed. Death is unambiguous, but the cause of death requires adjudication, and this may be biased. Although laboratory measurements are generally considered objective, they too are subject to potential biases. In a placebo-controlled blinded study of levodopa in Parkinson's disease patients, de la Fuente-Fernández and colleagues demonstrated increased dopamine release in patients exposed to placebo.[10] There was even a trend toward higher dopamine release among those who perceived an effect from the placebo. Such results in an objective measurement suggest that subjective components from a patient and/or assessor can affect most outcome measurements. Knowing that they have received an experimental therapy, patients may try harder on a physical test, feeling that they should be able to function better. Similarly, an investigator's assessments might be biased by knowing that a patient received the experimental treatment. The investigator may even consciously avoid assessing marginal changes favorably to restrain any tendency to favor the treatment, biasing the results in the opposite direction. Thus, treatment knowledge may affect the patient's reporting of outcome, the assessor's interpretation of the patient's performance, or both. Finally, patient-reported outcomes are completely subjective.

We are entering an era of new and exciting technologies for delivering treatment. Their names alone are exciting: microbubbles, implantable microchips, nanotechnology, and nanites. These technologies will potentially allow therapy to be targeted precisely to where it is needed, minimizing side effects and adherence issues while maximizing efficacy.

Most patients enter studies in hopes of improving their condition. How could a person know that he or she had received a microbubble or a nanite and not feel better? Given how markedly patients' beliefs can affect outcome, how can we assume that the results of our exciting new treatment are not affected by the subjects' and investigators' knowledge of the treatment received? If this is even a possibility, should not studies always be blinded, even if blinding may be imperfect? If a study has a contemporaneous control group, with treatment randomly allocated, why would we even think about not blinding the study as effectively as possible? Blinding is almost always simple to do—far simpler than recruiting the additional patients needed for the control group. Even if completely effective blind-

ing is impossible, how could one argue that a study design that does not attempt to incorporate blinding as effectively as possible is adequate and well-controlled? Would anyone believe the results?

The issue is not whether we should use blinding. The real issue is whether we are going to assess our new technologies using randomized controlled studies. Are we prepared to trust results without a contemporaneous control group, without random allocation to treatment, and with all the possible biases that such studies pose? Choosing not to carry out a randomized controlled study implies that the benefits of the new treatment are so overwhelming that rigorous proof is not needed. When a treatment shows overwhelming evidence of efficacy, the study would be stopped. Blinding is an additional safeguard to minimize bias once we have accepted that we need evidence from a randomized controlled study, even for our new, "obviously better" therapies. Blinding, even when imperfect, reduces potential biases—biases that may be stronger than the actual benefit of our new treatment.

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CONFLICT OF INTEREST

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How Well Does Blinding Work in Randomized Controlled Trials?: A Counterpoint

J Boehmer[1] and P Yong[2]

The blinding of patients and investigators to treatments in randomized controlled trials is a well-founded principle of study design. The blinding of clinical studies is not without its drawbacks, however. This article discusses these issues and raises questions as to whether blinding is truly effective. We support greater disclosure of blinding methods and success, follow-on studies to determine the effectiveness of therapies in actual practice, and the exploration of alternative approaches in cases where blinding may not be feasible.

It has long been known that patients who receive an inactive treatment may nevertheless report improvement. This response, referred to as the "placebo effect," may be generally described as a beneficial outcome that is attributed to the person's expectation that the treatment is effective rather than the treatment itself. Although the effect may be psychological in origin, there is a measurable physical response and placebos have been used in clinical practice in the past to produce symptomatic relief. In clinical studies, however, the placebo effect may bias the results. A strong placebo effect could potentially inflate the benefit of an experimental treatment and lead to a false conclusion about its true effectiveness.

Introduction of bias due to expectation is not restricted to patients. There may also be an "observer bias," in which an investigator may have preconceived notions about how well a new therapy will work and may be inclined to rate patients' improvement more highly if he or she knows which treatment is being administered. After all, investigators are unlikely to enroll their patients into clinical studies without some fundamentally sound basis that suggests the investigational treatment will be effective. It is entirely possible that a physician's enthusiasm about an intervention could bolster a patient's belief in it, thereby enhancing the placebo effect and confirming the benefit to the satisfaction of both the patient and the physician in a self-fulfilling prophecy.

Good clinical studies try to reduce or eliminate sources of bias, and randomized clinical trials are considered the gold standard. Clinical research scientists are also taught that good clinical studies incorporate "blinding" into their designs, in which knowledge about the

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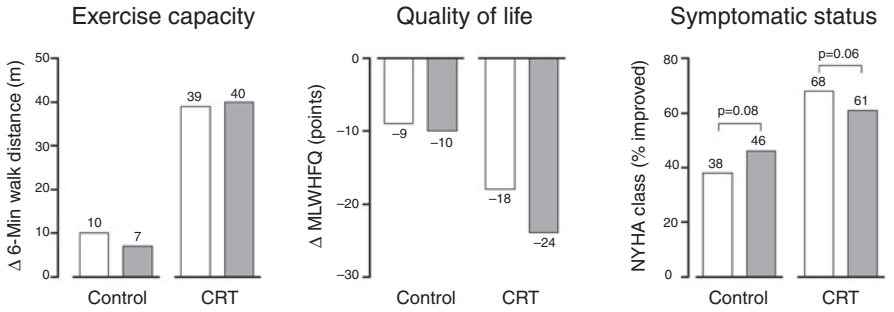

Figure 1 Magnitude of the placebo effect in randomized, double-blind, controlled clinical trials of cardiac resynchronization therapy (CRT). In randomized, double-blind, controlled clinical trials of CRT, there was a substantial improvement in exercise capacity, quality of life using the Minnesota Living With Heart Failure Questionnaire (MLWHFQ), and symptomatic status measured with the New York Heart Association (NYHA) functional-class categories in the control groups. Anywhere from 1/4 to nearly 1/2 of the benefit apparent with active pacing was seen in patients who received a pacemaker that was inactive over the 6-month study period. Black bar, COMPANION; white bar, MIRACLE.

randomized therapy is withheld. In a single-blinded study, it is the patients who do not know which treatment is being administered; in a double-blinded study, both study subject and investigator are unaware of the therapy assignment.

Although we do not dispute the existence of these sources of bias, we wish to raise several questions about whether the efforts made to blind study subjects and investigators truly improve the quality of research.

Do we know whether blinding is really effective?

Many studies state that they are double-blinded, but rarely is it reported whether an effort was made to determine how effective the blinding actually was. In a 10-year review of medical and psychiatric journals from 1972 to 1981, Ney *et al.*[1] found that an evaluation of the effectiveness of blinding was carried out in less than 5% of studies. Two decades later, the situation had not improved. In 2007, Hrobjartsson *et al.*[2] identified 1,599 blinded randomized studies and found that only 31 (2%) reported tests for the success of blinding. Even then, only 14 of 31 (45%) reported that blinding was successful.

The Consolidated Standards of Reporting Trials (CONSORT) guidelines recommend not only that blinding be performed but that the methods used to analyze the success of the blinding measures and outcome be reported as well.[3] Whether the CONSORT guidelines have been widely adopted has not yet been assessed. Thus far, there has been no large-scale systematic literature review of compliance with this recommendation in the post-CONSORT era.

Can the results of a successfully blinded study be generalized into clinical practice?

The US Food and Drug Administration (FDA) requires that labeling for pharmaceuticals and medical devices include the results of the clinical trials that led to their approval. Labeling is intended to help physicians better understand how to use these products. However, the placebo effect is not limited to placebos; well-established treatments may also have a placebo effect. Placebo effects are not driven by psychology alone; other factors may contribute to net benefit within a control group. Patients in clinical trials may receive more frequent contact, find themselves in the care of highly qualified specialists and their staff, or have access to protocol-required tests such as extensive blood analysis, exercise testing, or imaging that are beyond the standard of care but may provide a better level of understanding of their underlying condition. Even if there were no such thing as a placebo effect, it should be no surprise that patients randomized to a control group improve.

How should practicing physicians who do not routinely participate in clinical trials interpret the findings of double-blind studies when the patients they treat are not blinded and do not receive the rigorous follow-up demanded by a clinical trial? Although there are postmarketing studies mandated by the FDA, there may be an additional place for unblinded studies that are specifically designed to quantify the interaction of placebo effect and treatment effect once a new therapy has been integrated into clinical practice.

Does blinding inadvertently introduce bias in study outcome?

To expand on the previous question, clinical studies sometimes use extraordinary means to blind patients. For example, an implantable pressure-monitoring system was evaluated in a randomized trial called Chronicle Offers Management to Patients with Advanced Signs and Symptoms of Heart Failure (COMPASS-HF) in which blood pressure data were assessed in heart failure patients. Availability of these data could lead to early detection of congestion, potentially foretell future heart failure decompensation, and permit early intervention to prevent hospitalization.[4] In this single-blind study, all patients were implanted with the device. Randomization was to one of two groups. In one group, physicians had full access to the blood pressure data, whereas in the other group, access was blocked. It was expected that physicians who had full data access would be in greater telephone contact with their patients to inform them to change their medications in accordance with an observed change in blood pressure. Because increased physician contact could reveal the randomization, a call schedule was implemented in which patients in the control group (with blocked access) were also contacted by telephone to ensure that both groups received an equal frequency of communication.

Ultimately, the COMPASS-HF trial failed to demonstrate a statistically significant benefit of the strategy of using hemodynamic measurements to improve outcomes in patients with heart failure. Could the blinding methodology have contributed to this outcome? The authors believed that one of the reasons for failure was that increased vigilance of the control group was above and beyond the standard of care. The study designers had anticipated this possibility and com-

pensated the expected control event rate accordingly. Nevertheless, the event rate in the control arm was still substantially less than expected even after adjustment, undermining the power of the study to detect a significant difference. An alternative study design might have incorporated a third arm in which data access was blocked and no additional contacts were made. However, such a design might have substantially increased the duration and complexity of the trial. Perhaps additional end points drawn from less subjective data, such as echocardiography, would have provided corroborative evidence of effectiveness.

In conclusion, we believe that blinding is generally worthwhile, because the biases associated with prior knowledge of treatment assignment are well known. However, many studies simply state that the study design was double-blind without providing specific details or revealing whether the blinding was successful. Readers may assume that blinding has appropriately controlled for biases without actually knowing how well it worked or even if it worked at all. Not every study must be blinded; once a new therapy receives FDA approval, there is potential value in uncontrolled studies to learn their effectiveness under real-world conditions in which the placebo effect and treatment effect intermingle. Finally, there may be situations in which blinding places an extraordinary burden on a study. Alternative end points that are based on objective data rather than subjective evaluation by a patient or physician may be used.

CONFLICT OF INTEREST

P.Y. is a full-time employee of Boston Scientific Corporation. J.B. is a paid consultant of Boston Scientific Corporation.

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OPINION

Nanomedicine and Society

M Ferrari[1], MA Philibert[2] and WR Sanhai[3]

Nanomedicine is a reality of medical research and clinical practice, and it offers new and promising approaches to fundamental problems in medicine. Most prominent are the early detection of neoplastic disease and the individualized treatment of metastases. These potentially transformational developments require careful scrutiny of the potential impact of nanomedicine on society, so that the community can guide its deployment in keeping with the fundamental tenets of medical ethics.

Definitions of nanotechnology abound, including several used by US and international agencies. The various definitions have three substantially common elements: nanotechnological devices are at least partly man-made, have dimensions on the scale of 1 to 1,000 nm, and possess novel "emerging" properties stemming from their nanoscale dimension—that is, properties not present in identical devices of smaller or larger dimensions. The existence of a mechanism-based, predictive proof of the causal necessity of the new properties is also required by some authors as part of the definition of nanotechnology. In the working definition we use in this article, nanomedicine is the application of nanotechnology to medicine, from basic research to disease diagnosis to therapeutic intervention, monitoring disease progression and patient management, including quality-of-life measures.

Although this article neither subscribes to a consensus definition nor provides one for full adoption, it is consistent with the ones in common use, such as the one used by the National Nanotechnology Initiative[1] (to reduce ambiguity and misinterpretation). Furthermore, in the context of regulatory issues, as the science evolves and the US Food and Drug Administration (FDA) learns more about the interaction of nanoscale materials with complex biological systems, the agency may develop formal, fixed definitions, appropriately tailored to the regulation of nanoscale materials in FDA-regulated products.[2]

The promise of personalized nanomedicine was based on the understanding that each individual possesses a unique genetic profile predisposing him or her to respond to therapies differently. Now, armed with the predictive power of *in silico* models of patient populations, whole-genome testing, clinically qualified biomarkers that can assess individual responses to therapies, and other tools, the biomedical field is poised for significant advances and benefits to individuals rather than to their population mean. Nanomedicine, like no other field in science, has the potential of interpretative flexibility—flexibility in how scientists design, analyze, and interpret outcomes. The marriage of genomics, postgenomics, and nanomedicine offers the potential for disruptive innovation and therapies targeted to an individual.

"Nanomedicine" is a broad term that encompasses the development of sensors for single-molecule detection, identification of biomarkers, nanoparticles and nanocarriers for the detection and imaging of cancers, and the delivery of therapeutic molecules. Nanoparticle-based vehicles have also been used to deliver recombinant proteins as vaccines. In the area of therapeutic and imaging modalities, the overwhelming majority of recent developments have been aimed at cancer. Materials that have been investigated for their utility include soft and liquid polymers, polymers based on sugars or amino acids or both, liposomes, dendrimers, and metalloid/metallic and solid/hollow native or organically modified silicas. Such nanomaterials may be either persistent or biodegradable.

Despite the wide range of materials and potential applications, most medicinal nanoparticles are essentially targeted carriers or "smart" polymers that deliver drugs, imaging agents, and/or chemicals that disrupt growth of cancer cells.[3] Some nanomaterials use peptides and other ligands to actively target the appropriate site,[4] and others are passively targeted by exploiting the pathophysiological changes that occur in increased permeability of blood vessels or other cellular phenomena. The use of targeting antibodies, peptides, and other targeting moieties on the surface lends itself to personalized medicine. The fast-approaching capability to quickly identify unique surface markers on tumor cells and to design or fabricate a tailored nanomaterial that will attach

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itself with high affinity and selectivity will further enhance the attractiveness of using nanoparticles in imaging and therapeutic modalities. Moreover, several of these nanostructures lend themselves to screening *in situ*. Because many of the imaging nanomaterials, and all the therapeutic ones, are designed for launching to various compartments within the body, several consequent physiological challenges arise. Chief among these challenges is the somewhat predictable interplay between a complex bionanoparticle and the immune system.

All the advantages of this approach notwithstanding—and precisely because most of the useful therapeutic and nanoparticles (5–150 nm diameter) range in size from that of a typical rhinovirus (~20 nm diameter) to that of the pox virus *Molluscum contagiosum* (~200 nm diameter)—they are targeted by the immune system of the host. The potential for accumulation of complex, bioactive mixtures of nanomaterial and adherent peptides raises questions about acute or chronic idiopathic adverse reactions that may constitute a rare or underdiagnosed condition. Certainly, the era of nanomedicine will require extensive characterization of the therapeutic index during development and vigilant aftermarket surveillance.

On the side of the potential beneficial impact on society, considerations about the importance of nanomedicine must be placed in the context of the health problems they address. Focusing attention on oncology, it is recalled that in the United States a person dies of cancer approximately every minute, and the worldwide number is more than five times that. The dimensions of the tragedy have not decreased in recent years in the United States and Europe, despite extraordinary advances in the molecular-level understanding of the disease. Two crucial reasons for this failure to translate to basic progress in the clinic are: (i) the current inability to detect the disease at its earliest stages, when treatments are most effective and have the fewest adverse side effects, and (ii) the current inability to treat metastatic disease—in particular, due to the extraordinary, time-variable diversity of molecular connotations with which it manifests itself, even in a single person, in the course of neoplastic disease.

These are exactly the two fronts on which nanomedicine provides its most promising weaponry.[5] On the side of early detection, nanomedicine is evolving a host of new approaches that will allow for the rapid, non- or minimally invasive and inexpensive testing of a very large number of molecular signatures from biological fluids. Together with the development of new, molecularly targeted nanoscale contrast agents for radiological imaging, these are envisioned to aid in the birth of a new era of medical care, in which cancer screening is available to all and affordable for all. In view of the variability and diversity of the molecular presentations of metastatic disease, not only is the notion of "personalizing" treatment a good idea; it is a probably a necessary route to the eradication of death and suffering due to cancer. The need is even more radical: beyond personalization, what is required in many cases is treatment individualization, at the level of the individual lesion. Here nanotechnology offers the opportunity to develop new approaches, individualizing treatment simultaneously on three fronts: (i) the bioactive payload, which can be, e.g., a "molecularly targeted" drug, short interfering RNA, or microRNA; (ii) the design of the carrier nanoparticle, which could be tailored to the immune responses of a patient; and, again, (iii) the design of the carrier particulate system, which can be tailored so as to optimize the likelihood of selective concentration in the target lesion.[6]

Research with the intent of translation into the clinic may be viewed within the classical medical ethics framework of Childress and Beauchamp. This perspective was used by the first author of this article in his presentation to the US President's Council on Bioethics on 29 June 2007, in the first (and so far only) session dedicated by the council to matters of nanotechnology and nanomedicine.[7] The presentation reflected the four cardinal dimensions of analysis in the Beauchamp–Childress system: beneficence (the utilitarian perspective of maximizing community benefit), nonmaleficence (the Hippocratic mandate of "First, do no harm"), respect (including autonomy, or the patient's right to decide), and justice (including fair access to health care).

The main conclusions of the presentation are summarized as follows. Nanotechnology offers extraordinary opportunities for medical advances and, most important, offers new hope for early detection and individualized therapy of disease. Environmental risks from nanomedicine are very modest. Nanotechnology-enabled personalized medicine poses ethical questions of autonomy and privacy that must be discussed in the broadest community context for proper policy decisions to be made.

Nanomedicine is at risk of being available only to privileged societies, at least initially, but it offers unexplored opportunities for medical advances that would benefit underprivileged populations. The final, overarching conclusion is that careful scrutiny of potential safety risks is absolutely necessary—yet the greatest risk in nanomedicine may well be in letting our concerns paralyze our action and not taking advantage of the full, revolutionary potential that nanotechnology in medicine can offer humankind. We fully echo this conclusion.

ACKNOWLEDGMENT

The views expressed herein are those of the authors and may not reflect policies of the FDA.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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Combination Products Regulation at the FDA

KJ Lauritsen[1] and T Nguyen[1]

The US Food and Drug Administration (FDA) is responsible for protecting the public health by assuring the safety, efficacy, and security of drugs, biological products, and medical devices. As single-entity products, drugs are generally regulated by the Center for Drug Evaluation and Research (CDER), devices by the Center for Devices and Radiological Health (CDRH), and biologics by the Center for Biologics Evaluation and Research (CBER). In recent years, technological advances have led to a blurring of the historical lines of separation between the centers.

Compared with single-entity devices such as drugs and biologics, combination products have the potential to provide enhanced therapeutic advantages. More and more combination products are incorporating cutting-edge technologies that hold great promise for advancing patient care. Combination products range in complexity from drug-eluting stents to gene therapy systems and from chemotherapeutic drugs combined with monoclonal antibodies to novel nanotechnology-based drug delivery systems. They include innovative products for diagnostic and therapeutic treatments of cardiovascular, metabolic, and oncologic disorders, among others. Some estimates forecast that the combination-products market could increase from approximately US$6 billion in 2004 to nearly $10 billion by 2009.[1] Furthermore, some estimate that the total global value of the drug–device combination-products market will increase from $5.4 billion in 2004 to $11.5 billion in 2010.[2] Coincident with these estimates, the Food and Drug Administration (FDA) has seen a steady rise in the number of marketing applications for combination products (**Figure 1**). Combination products also raise challenging regulatory, policy, scientific, and review management issues. The FDA's Office of Combination Products (OCP) addresses these issues by classifying combination products, determining where they are reviewed, and developing regulations, policy, and guidance for industry and review staff.

What is a combination product?

As their name implies, combination products combine two or more different single-entity products—a drug combined with a device, a drug combined with a biologic, a device combined with a biologic, or a device combined with both a drug and a biologic. They may also be combined in multiple ways: physically, chemically, or otherwise; packaged together (co-packaged); or provided separately but specifically labeled for use together. These products are defined in Title 21 of the Code of Federal Regulations (CFR) 3.2(e).

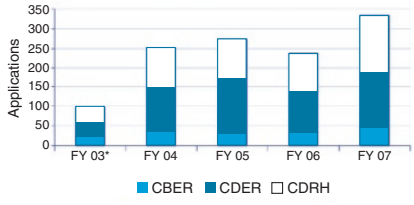

Figure 1 The FDA has seen a steady increase in the number of combination product applications, reaching a 5-year high in fiscal year (FY) 2007, the most recent year for which data are available. The FDA began data collection on 1 April 2003. *Numbers do not represent all of FY 2003. CBER, Center for Biologics Evaluation and Research; CDER , Center for Drug Evaluation and Research; CDRH, Center for Devices and Radiological Health.

Examples of combination products whose components are physically, chemically, or otherwise combined (21 CFR 3.2(e)(1)) include:

- A monoclonal antibody conjugated to a drug
- A device coated/impregnated with a drug or biologic
- A drug-eluting stent, a pacing lead with a steroid-coated tip, an antimicrobial-coated catheter, a condom with spermicide
- An orthopedic implant with growth factors
- Prefilled syringes, insulin injector pens, metered-dose inhalers, transdermal patches

Examples of combination products whose components are packaged together (21 CFR 3.2(e)(2)) include:

- A drug or biological product packaged with a delivery device
- A surgical tray with surgical instruments and antimicrobial swabs

Examples of combination products whose components are separately provided but labeled for use together (21 CFR 3.2(e)(3) or (e)(4)) include:

- A photosensitizing drug and activating laser/light source
- An iontophoretic drug-delivery patch and controller

Where are combination products reviewed and regulated?

Combination products are reviewed and regulated by the CBER, the CDER, or the CDRH. The FDA is required (by Section 503(g) of the Federal Food, Drug, and Cosmetic Act) to assign the primary jurisdiction for the regulation of a combination product to a center based on the primary mode of action (PMOA) of the product.

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The FDA defines mode of action as "the means by which a product achieves its intended therapeutic effect or action," in which "therapeutic" action or effect includes any effect or action of the combination product intended to diagnose, cure, mitigate, treat, or prevent disease or affect the structure or any function of the body (21 CFR 3.2(k)). Because combination products are composed of more than one type of regulated product (biological product, device, or drug) and each part contributes a biological product, device, or drug mode of action, combination products will have more than one mode of action. The FDA defines the PMOA as "the single mode of action of a combination product that provides the most important therapeutic action of the combination product." The most important therapeutic action is the one expected to make the greatest contribution to the overall intended therapeutic effects of the product (21 CFR 3.2(m)).

Typically, combination products with a PMOA attributable to the drug component are assigned to the CDER, a device PMOA to the CDRH, and a biologic PMOA to the CBER (21 CFR 3.4). The assigned center will consult or collaborate with the other centers during the review of the combination product. Once assigned to a lead center, the combination product will typically follow that center's application type for premarket review (**Figure 2**). For example, most drug delivery systems are combination products consisting of both a drug and a device. They may be simply a syringe prefilled with a drug or a complex iontophoretic drug-delivery patch. Usually, the most important therapeutic action of such a product is attributable to the drug component's role in treating a disease or other condition; the device plays a secondary role in delivering the drug. These types of products are reviewed by the CDER, with the CDRH consulting on the device aspects of the product.

The FDA recognizes that there may be times when the PMOA of a combination product cannot be identified with certainty or when a product has two (or more) distinct modes of action and neither is subordinate to the other. In those cases, a decision algorithm is used to assign the combination product to a lead center (21 CFR 3.4(b)). The FDA first assesses whether there is a center that regulates other combination products that present similar questions of safety and effectiveness with regard to the combination product as a whole and, if so, assigns the product accordingly. When there are no other combination products that present similar questions (e.g., it is the first such combination product or differences in its intended use, design, formulation, or other aspects present different safety and effectiveness questions), the agency assigns the combination product to the center with the most expertise in evaluating the most significant safety and effectiveness questions presented by the combination product.

Once assigned to a lead center, only one investigational application (an investigational new drug or an investigational device exemption application; see **Figure 2**) is necessary. Following the investigational period, a single marketing application should also be sufficient to ensure product safety and effectiveness, as well as to ensure consistent and appropriate postmarket regulation for most combination products. For combination products being developed by more than one manufacturer, there may be a desire to provide information to the FDA while maintaining the confidentiality of each manufacturer's intellectual property. The application holder can accomplish this by submitting to the FDA a letter of authorized cross-reference from the owner of the referenced material. In some instances, two marketing applications may also be appropriate. The product developers should discuss with the FDA which approach would be best for their specific product.

What process does the FDA use to determine the PMOA and the lead center?

The regulatory identity of a product as a drug, device, biological product, or combination product is often clear. Similarly, the PMOA of a combination product is often clear. However, there may be times when the jurisdiction of a product is unclear or in dispute. In these cases, sponsors may request a determination of the classification and assignment of a product through the Request for Designation process.[3] Sponsors may also request an informal determination of jurisdiction by e-mail (combination@fda.gov). Informal jurisdictional determinations are not binding on the FDA.

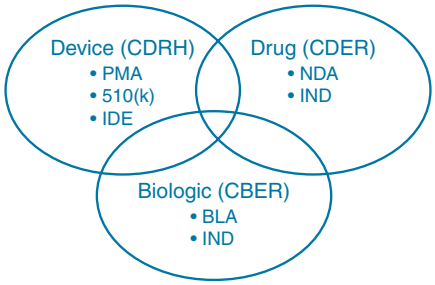

Figure 2 Regulatory approaches. Combination products combine drugs, devices, and biologics. Once assigned to a lead center, the product is typically reviewed following that center's application type (e.g., a primary mode of action attributable to a device component may reach market through a PMA or 510(k) submission), although some centers have regulatory oversight over more than one type of product (e.g., certain categories of biological products are assigned to the CDER). 510(k), premarket notification; BLA, biologics licensing application; IDE, investigational device exemption; IND, investigational new drug; NDA, new drug application; PMA, premarket approval.

What is the role of the Office of Combination Products?

The Medical Device User Fee and Modernization Act of 2002 mandated the creation of the Office of Combination Products to address the regulatory, policy, scientific, and review management issues raised by combination products. On 24 December 2002, the OCP was established by the Commissioner's Office of International and Special Programs.

The OCP's broad responsibilities cover the regulatory life cycle of combination products and include making product jurisdiction decisions; coordinating premarket reviews among the centers; ensuring consistent and appropriate postmarket regulation; developing policy, guidance, and regulations; and serving as a resource for industry and review staff. However, the primary responsibilities for scientific review and regulation of combination products remain in three product centers—CBER, CDER, and CDRH—to which the products are assigned by the OCP.

In the next several years, combination products will probably become more complicated as new technologies (e.g., nanotechnology) emerge and existing technologies mature. Therefore, the OCP will continue to focus on the most important and challenging issues relating to the regulation of combination products, including issues such as adverse-event reporting and good manufacturing practices. The OCP is committed to actively assisting industry and FDA reviewers in understanding this complex regulatory area. An updated list of FDA guidance documents that developers may find helpful in the development of their products is available on the OCP website at http://www.fda.gov/oc/combination. The OCP is always available as a resource to developers and review staff throughout the life cycle of a combination product (assignment, development, premarket review, and postmarket regulation) and can be reached at 301-427-1934 or at combination@fda.gov.

CONFLICT OF INTEREST

The author declared no conflict of interest.

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LETTERS TO THE EDITOR

Inappropriate Medications and Adverse Drug Reactions in the Elderly

P Gallagher[1] and D O'Mahony[1]

To the Editor: Laroche *et al.* reported in their article, "Inappropriate Medications in the Elderly," that "although the relationship between Beers criteria and ADR [adverse drug reaction] occurrence is frequently assumed, it has not been clearly demonstrated."[1] The authors also "question the validity of a list resting on an expert consensus that does not take into account to a sufficient extent the clinical setting and the overall medication burden."[1] While we agree with these statements, it must be acknowledged that the studies referenced in support of these statements did not use all the Beers "independent of diagnosis" criteria, and none used Beers "considering diagnosis" criteria, thereby possibly underestimating the association between inappropriate medicines (IMs) and ADRs in older patients. Furthermore, the studies used various systems to assess ADR causality, ranging from spontaneous, independent reporting of ADRs by emergency department physicians[2] to the Naranjo algorithm.[3] In order to enable an accurate assessment of the relationship between IMs and ADRs, the IM criteria must be valid in terms of content, and ADR causality assessment must be robust and reliable.

Drug–disease interactions are common in older patients and frequently result in hospital admissions for events such as cognitive decline secondary to anticholinergic or psychotropic medications, falls, excessive sedation secondary to benzodiazepines, and gastrointestinal bleeding secondary to nonsteroidal anti-inflammatory drugs in patients with peptic ulcer disease. We recently reported that serious ADRs related to the Screening Tool of Older Persons' Prescriptions (STOPP) criteria IMs contribute to almost twice as many hospitalizations of older people as serious ADRs resulting from *all* Beers criteria IMs, both independent of and considering diagnosis.[4] We believe that STOPP criteria enable assessment of more domains of prescribing appropriateness, and are therefore more relevant to everyday clinical geriatric medicine than Beers criteria are.

Apart from the deficiencies of Beers criteria, one must also consider the practical applicability of mainstream ADR causality criteria to older people, principally the Naranjo algorithm.[5] The Naranjo algorithm requires consideration of placebo response, drug concentration, and drug dechallenge/rechallenge. Clearly, these measurements are seldom practical or appropriate in frail, older patients. Furthermore, ADR causality may be weakened in older patients with multiple comorbidities and polypharmacy. For example, a patient with osteoarthritis and visual impairment, already at risk for falls, may be prescribed a benzodiazepine; the latter could indeed contribute to the patient's having a major fall, but it might not be the sole cause of it.

Finally, IM screening tools need to be tested in prospective randomized controlled trials in order to assess their true impact on negative outcomes such as ADRs and health-care expenditure.

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[1]Department of Geriatric Medicine, Cork University Hospital, Cork, Ireland. Correspondence: P Gallagher (pfgallagher77@eircom.net)

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Response to "Inappropriate Medications and Adverse Drug Reactions in the Elderly"

M.-L. Laroche[1], J.-P. Charmes[2], F. Bouthier[2] and L. Merle[1]

To the Editor: We thank Drs Gallagher and O'Mahony for their letter entitled "Inappropriate Medications and Adverse Drug Reactions in the Elderly" adding to the debate on the relationship between inappropriate medication (IM) criteria and the occurrence of adverse drug reactions (ADRs).[1] The authors indicate that this relationship should rely both on the quality of the criteria used and on the method of causality assessment.

We acknowledge the quality of the Screening Tool of Older Persons' criteria that the authors propose; the criteria are based on classifications made according to physiological systems and allow the possibility of assessing various domains of prescribing. These criteria seem to be

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better adapted for improved detection of ADR occurrence induced by IMs than Beers Criteria are.[2] However, we think that screening tools for IM use are validated only if their application tends to significantly decrease ADR occurrence. Some interventional studies to reduce IM use have shown a small decrease in ADRs.[3] This decrease is statistically significant, but its clinical importance is unknown as there have been no studies to evaluate their effect on the outcomes. This ultimate evaluation should be based on a randomized trial involving a control group. Moreover, if a tool is to be used on a regular basis and is to be of great help, it must remain simple and not time-consuming.

We agree with the authors that methods for causality assessment of ADRs must be reliable and adapted to geriatric applications. Unfortunately, up to now, no method has been universally accepted for causality assessment of ADRs, whatever the age of the patients.[4] In any event, an evaluation of the techniques currently available was outside the scope of this article.

Published lists of IMs, including the most recent one (from Australia), focus on fairly similar conditions but are tailored to the geriatric therapy norms of different countries.[5] We hope that promoting the use of all these lists will improve routine treatments in the elderly and allow a validation of the criteria used.

CONFLICT OF INTEREST

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A Critical Appraisal of the Safety and Efficacy of Drug-Eluting Stents

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Drug-eluting stents (DESs) have largely demonstrated their superiority to bare-metal stents (BMSs) with respect to in-stent restenosis. Since the US Food and Drug Administration (FDA) approved the first DES in 2003, there has been a significant increase in the use of these devices. They are used in 70–80% of all stent procedures worldwide. Nevertheless, safety concerns stemming from reports of increased risk of late stent thrombosis (ST) and myocardial infarction (MI) have tempered the enthusiasm that the advent of these stents originally generated. New-generation DESs with novel polymers, antiproliferative drugs, and improved platforms are now approved and available for use. In this review we provide a critical appraisal, based on published clinical data, of the safety and efficacy of various DESs.

Drug-eluting stents (DESs) have revolutionized the field of interventional cardiology. It has been nearly a decade since they were introduced in preclinical and phase I clinical trials in which, with single-digit target-lesion revascularization (TLR), they demonstrated outstanding results in regard to restenosis. Since their approval for marketing by the US Food and Drug Administration (FDA) in 2003, the pattern of their clinical use in the United States has been somewhat of a roller-coaster ride. Initially there was a rapid adoption, and within a few months the penetration of DES use in the United States accounted for nearly 90% of all coronary stent procedures. The enthusiasm subsided with accumulating reports of subacute and late stent thrombosis (ST) requiring prolonged dual antiplatelet therapy. In September 2006, at the European Society of Cardiology Congress in Barcelona, a meta-analysis of all available published studies on DESs suggested that sirolimus-eluting stents (SESs) are associated with increased mortality as compared with bare-metal stents (BMSs). This disturbing information, along with other reports on the incidence of very late ST (developing more than 1 year after implantation), decreased the use of DESs in the United States to <60%. An advisory panel of experts met with FDA officials in December 2006 in Rockville, MD. The panel concluded that the safety issues of DESs are comparable to those of BMSs, and that DESs have the advantage of inducing less revascularization when used for on-label indications. However, there is an increase in major adverse cardiac events when DESs are used off label. In addition, the FDA recommended dual antiplatelet therapy (aspirin and clopidogrel therapy) for ≥1 year and aspirin use to be continued indefinitely in patients who had received DESs. Over the past 2 years, more studies based on "real-world" registries have reported on the issue of safety and efficacy of DESs as compared with BMSs for off-label indications; few of them suggest that DESs save lives. Concurrently, reports surfaced regarding the lack of healing and the incidence of very late ST (with a constant hazard ratio (HR) of 0.6% per year) associated with the use of DESs. These reports are in addition to those dealing with chronic inflammation, impaired vasoreactivity of the DES-implanted blood vessel, and acquired stent malposition. In 2008, DES use in the United States was once again on the rise, and penetration of the use of these devices is estimated to reach 75% of all stent procedures by the first quarter of 2009. The purpose of this review is to critically appraise the safety profile of DESs using published data and to determine the magnitude of the risk/benefit ratio of DES use in clinical practice.

SAFETY OF DESs

Mortality

Despite the major benefit of DESs in regard to the reduction of restenosis rates and repeated revascularization, some studies have reported adverse outcomes, indicating a possible increase in mortality associated with DESs as compared with BMSs in long-term follow-up. The safety of DESs was questioned for the first time in September 2006 at the meeting of the European Society of Cardiology (World Congress of Cardiology in Barcelona), at which a meta-analysis reported a statistically significant increase in mortality at 2–3 years in patients who had received SESs. An analysis of the Swedish Coronary Angiography and Angioplasty Registry observational study published in 2007 also showed an increase in mortality associated with the use of

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DESs, one that became apparent 6 months after the procedure.[1] It is important to note that detailed data on the duration of dual antiplatelet therapy, a major point of concern related to the risk of ST, were not available in these reports.

In contrast, important and robust evidence from randomized control trials and several meta-analyses have shown that DESs do not result in excess mortality after 4–5 years of follow-up.[2–5] Of these, Stettler *et al.*[2] have reported outcomes of 38 DES trials in more than 18,000 patients with >4 years of follow-up. They reported that mortality and risk of ST were similar for DESs and BMSs. Death due to acute myocardial infarction (AMI), congestive heart failure, and ST occurred only infrequently after DES implantation[2] (**Table 1**). Results from multicenter registries such as REAL (Registro Angoplastiche dell'Emilia Romagna)[6] and a recently published article by Shishehbor *et al.*,[7] both of which represent "real-world" practice, did not show any difference in terms of safety when DES and BMS were compared in patients with off-label indications for DESs. In addition, Shishehbor *et al.* have reported, with a study carried out in a large population (8,032 patients; 6,053 DESs and 1,983 BMSs), that all-cause mortality was significantly lower with DESs in unadjusted and adjusted Cox proportional models when compared with BMSs (HR: 0.62, 95% confidence interval (CI): 0.53–0.73; $P < 0.001$) at 4½-year follow-up and that this remained significant after propensity-score matching (HR: 0.54, 95% CI: 0.45–0.66; $P < 0.001$).

ST

Patients who develop ST have a poor prognosis. Ten to thirty percent of patients presenting with definite ST die in the hospital.[8,9] Moreover, ST can lead to unexplained sudden death. In the past, this was not often considered, but it is now included in the new Academic Research Consortium definition.[10] However, nonfatal AMI is the most frequent clinical presentation of ST (70–80%).[8,9]

Early ST. Early definite ST was a common complication following BMS implantation in the early 1990s.[11] With the advent of thienopyridine, combined antiplatelet therapy greatly decreased the incidence of ST to around 1%, according to the most recent studies and meta-analyses.[12,13] To date, no difference has been found between DESs and BMSs in relation to early ST.[1,3,4,12]

Table 1 Sensitivity analyses summary of 29 DES trials with 13,677 patients

	Hazard ratio (95% credibility interval)			
	Death overall	MI	Definite ST	TLR
SES vs. BMS	1.00 (0.82–1.25)	0.81 (0.66–0.97)*	1.00 (0.68–1.63)	0.30 (0.24–0.37)*
PES vs. BMS	1.03 (0.84–1.22)	1.00 (0.81–1.23)	1.38 (0.96–2.24)	0.42 (0.33–0.53)*
SES vs. PES	0.96 (0.83–1.24)	0.83 (0.71–0.99)*	0.71 (0.48–1.13)	0.70 (0.56–0.84)*

BMS, bare-metal stent; MI, myocardial infarction; PES, paclitaxel-eluting stent; SES, sirolimus-eluting stent; ST, stent thrombosis; TLR, target-lesion revascularization.

*$P < 0.05$.

Late ST. Spaulding *et al.*,[4] in their meta-analysis of the four Cypher trials (RAVEL, SIRIUS, E-SIRIUS, and C-SIRIUS) that included 1,748 patients and used the Academic Research Consortium definition of ST, reported a cumulative rate of ST of 0.8% for BMSs vs. 1.8% for SESs ($P = 0.53$), at 1 year after the stent procedure. Using a per-protocol definition of ST, Stone *et al.*[3] reported, in a meta-analysis of nine trials (RAVEL, SIRIUS, E-SIRIUS, C-SIRIUS, and TAXUS I–V) that included 3,513 patients, a cumulative rate of ST of 0.6% for BMSs vs. 1.2% for SESs ($P = 0.2$) and 0.9% for BMSs vs. 1.3% for paclitaxel-eluting stents (PESs) ($P = 0.3$). Using the Academic Research Consortium definition, however, Mauri *et al.*,[13] in their meta-analysis of the same nine trials, reported a cumulative rate of ST of 1.7% for BMSs vs. 1.5% for SESs ($P = 0.7$) and 1.4% for BMSs vs. 1.8% for PESs ($P = 0.52$). In the Stettler *et al.*[2] meta-analysis (38 DES trials) using the Academic Research Consortium definition, ST incidence rates of 1% for BMSs, 1.1% for PESs, and 1.1% for SESs, ($P = 0.62$) were reported. Based on the findings from these recent studies and meta-analyses, the incidence rates of late ST for DESs and BMSs seem to be similar. However, the duration of dual antiplatelet therapy was usually longer with DESs than with BMSs in these studies (3 or 6 months vs. 1 month), thereby making conclusions more difficult.

Very late ST. The real incidence of very late ST is a poorly described, controversial topic, with little consensus on the potential increased risk of very late ST after DES implantation. Several authors have reported an incidence of very late ST of 0.4–0.6% per year after DES implantation.[4,14] Gruberg *et al.*, at the Transcatheter Cardiovascular Therapeutics conference in 2006 (Washington, DC), suggested that the incidence of very late ST seems to be higher in relation to the use of DESs than BMSs (0.6% vs. 0.2% per year). Additionally, Stettler *et al.*,[2] in their meta-analysis, reported similar rates of death for BMSs, SESs, and PESs, but with a significantly higher rate of very late ST in the PES group. Consequently, according to these meta-analyses, it would appear that the use of DESs increases the risk of very late ST moderately but significantly.

Endothelial dysfunction

Endothelial nitric oxide–dependent vasodilatation is the normal coronary response to exercise or acetylcholine infusion. Endothelial dysfunction, occurring during the early stages of atherosclerosis, can be demonstrated when paradoxical vasoconstriction occurs in the presence of stimuli that normally trigger vasodilatation. Endothelial function after DES deployment has been studied in two series of patients; the SES group had a higher rate of dysfunctional response as compared with the BMS group.[15,16] Another study also suggests that PESs and SESs induce similar patterns of abnormal distal vasoconstriction responses after acetylcholine infusion as compared with BMSs.[17]

Mechanisms for endothelial dysfunction after stent implantation may be directly associated with the severity of arterial injury.[18] Furthermore, incomplete endothelialization after DES

deployment may also contribute to more severe endothelial dysfunction.[19] Finally, it has been postulated that direct toxic effects of sirolimus and a lack of release of nitric oxide or other vasodilator from the endothelium within the stented segment could prevent downstream vasodilatation.[20] The newer generation of DESs has shown better endothelium-dependent coronary vasomotor response than SESs, but it is still unknown whether this finding is related to stent polymer or to drug class effect.[21]

Late-acquired incomplete stent apposition

Incomplete stent apposition (ISA) is defined as the clear separation of stent struts from the vessel wall as detected by intravascular ultrasound, with evidence of blood speckle behind the struts. Late-acquired ISA refers to ISA that is discovered at follow-up even though there had been complete apposition at the time of the stent implantation. This seems to be related to positive vessel remodeling after stent deployment.[22] Late-acquired ISA has been reported in 5.4 % of patients after BMS implantation at 6-month follow-up, but this figure did not affect the long-term incidence of cardiovascular events.[23] Higher rates of late-acquired ISA have been reported after DES implantation as compared with those after BMS implantation, with the former ranging from 8 to 12% at 6-month follow-up. But once again, no difference in mortality rates has been demonstrated.[24,25] Using intravascular ultrasound, Siqueira *et al.*[26] examined 195 DES patients at the time of stent implantation and 6 months later. Among these patients, 5.1% had evidence of late-acquired ISA. At 29 ± 15 months, no patient without ISA had presented with very late ST, whereas two of the patients with late-acquired ISA (20%) did. A possible association between the two phenomena must be investigated with a larger and longer intravascular ultrasound follow-up.

Hypersensitivity reactions associated with DESs

Although rare, several hypersensitivity-related adverse reaction symptoms associated with DESs have been reported. Nebeker *et al.*,[27] extracting cases from three databases, reported 17 cases (14 SESs and 3 PESs) of probable or certain DES-induced hypersensitivity syndromes. Clinical manifestations included nonurticarial rash ($n = 8$), hives ($n = 5$), dyspnea ($n = 6$), myalgia/arthralgia ($n = 3$), itching ($n = 2$), and blisters ($n = 1$). All urticarial eruptions began within 10 days of the implantation. Laboratory findings included hypereosinophilia and elevated IgE titers more than five times the normal value. According to this report, intrastent eosinophilic infiltrates and poor intimal healing as long as 18 months after stent implantation were observed in four patients who died of late ST.

An autopsy series reported five cases of late ST in SES patients secondary to hypersensitivity reaction. All of them were associated with positive vessel remodeling with extensive diffuse intimal, medial, and adventitial inflammation.[28] Hypersensitivity reactions seem to be a late phenomenon involving the entire stented segment, and they appear to be associated with the presence of eosinophil and T-lymphocyte infiltrates. It has been hypothesized that the Cypher nonerodible polymer, consisting of poly(ethylene co-vinyl acetate) and poly(n-butyl methacrylate), is the underlying cause of hypersensitivity reactions. Indeed, both components have been associated with allergic and toxic reactions in other territories.[29]

EFFICACY OF DESs

Restenosis

The main advantage of DESs over BMSs is the lower rate of restenosis (**Figure 1**).[2,3] Long-term follow-ups from the SIRIUS (sirolimus-eluting stent in coronary lesions)[30,31] and TAXUS IV[32] trials show yearly rates of angiographic restenosis

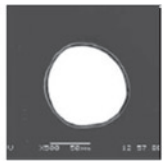
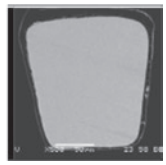
	Xience V	Endeavor	Taxus Express	Cypher
Picture of strut cross-section (×500)				
Strut thickness	81 μm	91 μm	132 μm	140 μm
Polymer thickness	7 μm	6 μm	16 μm	14 μm
Stent material	Co-Chr	Co-Chr	Stainless steel	Stainless steel
Chemical nature of the polymer	Biocompatible fluoropolymer	Hydrophilic phosphorylcholine	Hydrocarbon-based elastomer	PEVA and PBMA
Angiographic late loss (mm)	0.12 ± 0.29 mm	0.61 ± 0.46 mm	0.41 ± 0.54 mm	0.14 ± 0.42 mm
Binary restenosis	1.3%	9.4%	10.1%	2.6%

Figure 1 Comparison of strut cross-section thicknesses (microphotography at similar ×500 magnification levels), stent platforms, polymer characteristics, and efficacies of the different types of DESs. Co-Chr, cobalt–chromium alloy; PEVA, poly(ethylene co-vinyl acetate); PBMA, poly(n-butyl methacrylate).

of 6.8–7.9% for DESs. It is already known, from the pre-DES era, that more complex lesions such as long lesions, smaller-diameter lesions, saphenous vein grafts, bifurcations, and ostial locations predict the occurrence of restenosis. The presence of diabetes has also been strongly associated with restenosis.[33] It is therefore accepted that the use of DESs in these settings improves clinical outcomes as compared with the use of BMSs. Unfortunately, DESs have not been tested in most of these lesion types in randomized controlled trials (RCTs) against BMSs. Consequently, their use currently remains off label for these indications. Another hot topic in which significant controversies persist is the continued use of BMSs in the treatment of AMI, even though the first RCT Horizon AMI trial (Transcatheter Cardiovascular Therapeutics conference, Washington, DC, 2008) showed that it is beneficial to use DESs in this setting.

First-generation PES

Currently, nearly 5 million PESs (Boston Scientific, Natick, MA) have been implanted in patients worldwide. The platforms for both the first-generation Express stent and the second-generation Liberté stent consist of a polymer-based stent. The Liberté platform has smaller, more uniform open cells and thinner struts, thereby conferring more flexibility and navigability. Both stent platforms contain paclitaxel, an antiproliferative agent that stabilizes microtubules and blocks intracellular signaling, inhibiting smooth-muscle-cell migration and trophism.[34]

The Taxus stent is made with poly(styrene-b-isobutylene-b-styrene) (TransLute), which is a hydrocarbon-based elastomer with paclitaxel embedded in it. This diffusion-based controlled-release matrix system allows a slow and very specific delivery of the drug.[35] Data from the TAXUS IV, V, and VI RCTs[32,36,37] and a meta-analysis of the TAXUS RCT[3] showed that the Taxus Express stent significantly reduced the rates of TLR and binary restenosis as compared with the BMS Express stent, with no significant difference in death and MI rates in patients at standard risk.

Higher-risk populations have been studied in other trials. In the TAXUS Express meta-analysis, the use of PESs was associated with a reduction in restenosis rates without affecting safety in diabetic patients.[38] And in the TAXUS ATLAS trial, patients with smaller-diameter (<2.5 mm) vessel lesions treated with Taxus Liberté implantation presented a decrease in the rates of TLR and late loss up to the 9-month follow-up.[39]

The expected results of the Horizon AMI trial, which tested the safety and efficacy of PESs in the setting of AMI, were presented by Stone *et al.* at the 2008 Transcatheter Cardiovascular Therapeutics conference (Washington, DC). This trial showed that, at 1 year after the stent implantation procedure, the duration of ischemia-driven TLR was significantly shorter with Express Taxus (7.5%, $n = 2,257$) than with Express BMS (4.5%, $n = 746$) ($P = 0.002$). Angiographic follow-up was available for 1,204 patients, and the finding was that the incidence of binary restenosis was significantly lower in the Taxus group than in the Express BMS group (10.0% vs. 22.9%; $P < 0.0001$).

The recently published ARRIVE-1 Registry[40] consists of a detailed 2-year follow-up of 2,487 patients representing "real world" Taxus performance. Of these, the on-label group (35%) presented similar rates of death and MI as compared with the pooled data from the four TAXUS RCTs (death 3.5% vs. 3.4%, $P = 0.78$; MI 0.7% vs. 0.9%, $P = 0.72$) but lower rates of target vessel revascularization (TVR) (5.8% vs. 13.4%, $P < 0.0001$). The "expanded-use" group (65%), consisting of patients with conditions associated with greater risk and more complications, showed higher rates of death (7.4% vs. 3.5%, $P = 0.0003$) and TLR (9.4% vs. 5.8%, $P = 0.0031$) as compared with the on-label group.

First-generation SES

The Cypher stent is made with poly(ethylene co-vinyl acetate) and poly(n-butyl methacrylate) and has a stainless steel platform. It contains sirolimus, an antiproliferative drug that inhibits the G1 phase of the cell cycle. Most of the drug is released in ~3 weeks; thereafter, the concentration in the base coat decreases, resulting in decreased release rates.[35] Approved in April 2003 by the FDA, the Cypher stent has been the most widely used DES in the world and is considered to be the standard of comparison for all DESs. Indeed, the Cypher stent is currently the most extensively evaluated DES, tested in various trial designs and in diverse populations, and it has been studied for follow-up periods that are longer that those for other DESs.

Several multicenter RCTs have evaluated the safety and efficacy of SESs as compared with BMSs. In an analysis of pooled data from RAVEL, SIRIUS, E-SIRIUS, and C-SIRIUS trials ($n = 1,748$), at 5-year follow-up the SES group had a rate of death similar to that of the BMS group (8.9% for SES vs. 8.2% for BMS, $P = 0.57$) and an incidence of MI similar to that of the BMS group (7.9% for SES vs. 6.8% for BMS, $P = 0.44$). However, like for PESs, a sustained and significant reduction in the TLR rate was observed (9.8% for SES vs. 23.9% for BMS, $P < 0.0001$).[4]

In addition, the safety and efficacy of SESs have been demonstrated for off-label indications in higher-risk patients. In a systematic analysis of 14 RCTs comparing SES with BMS, the overall risk of death (HR: 1.03, 95% CI: 0.80–1.30) and the combined risk of death or MI (HR: 0.97, 95% CI: 0.81–1.16) were not significantly different between the groups.[12] There was a significantly lower combined outcome of death-MI-TVR (HR: 0.43, 95% CI: 0.34–0.54) associated with the use of SESs. This benefit was driven by a reduction in the TVR rate.

Furthermore, a recent analysis (which implemented risk adjustment and propensity-score matching) of data from 76,525 Medicare beneficiaries treated with implantation of Cypher or BMS showed that the use of SESs was associated with a significant reduction in both mortality and repeat revascularization.[41]

Second-generation everolimus-eluting stents (Xience V, Promus)

The platform is the Multi-Link Vision stent (Abbott Vascular, Markham, Ontario, Canada), which is made of a cobalt–chromium alloy and has very thin struts (81 μm). The open cells and a nonlinear design make the stent quite flexible. This stent is assembled on a semi-compliant balloon with short tapers that are intended to minimize injury outside the stent area. The drug, everolimus, has a high potency and high lipophilicity and

is an antiproliferative agent that inhibits the G1 phase of the cell cycle.[42] The everolimus-eluting stent (EES) has a nonadhesive, durable, and biocompatible fluoropolymer composed of an outer layer (poly(n-butyl methacrylate)) and a drug-reservoir layer (poly(vinylidene fluoride co-hexaflu-oropropylene)) that releases the drug slowly. This DES system releases ~80% of the drug in the first month and nearly 100% of it by 4 months.

The first pivotal RCT was SPIRIT I,[43] which demonstrated the safety and accuracy of EES as compared with BMS (Multi-Link Vision). The SPIRIT II trial,[44] conducted in Europe, had 300 subjects; 223 of these were randomized to receive EESs and 77 to receive PESs (3:1 EES:PES randomization). The primary end point—late loss at 6 months—was lower for EES (0.11 mm for EES vs. 0.36 mm for PES, 69% relative risk, $P < 0.0001$). Also, lower rates of ischemia-driven TLR (2.7% vs. 6.5%) and protocol-defined late ST (0.5% vs. 1.3%) were observed for EES as compared with PES (**Figure 1**).

The SPIRIT III RCT,[45] aimed at evaluating noninferiority of EESs in comparison with PESs, was carried out in 65 sites in the United States, had 1,002 subjects (2:1 EES:PES randomization). In-segment late loss at 240 days was significantly lower in EESs than in PESs (mean 0.14 mm vs. 0.28 mm; $P < 0.004$). At the 9-month follow-up, EESs were noninferior to PESs with respect to the major secondary end point, ischemia-driven target vessel failure (TVF) (7.2% vs. 9.0%; risk ratio: 0.79, $P <0.001$ for noninferiority); this held true at the 1-year follow-up as well (6.0% vs. 10.3%; risk ratio: 0.58, $P = 0.02$ for noninferiority). On the basis of these results, the FDA approved the use of this new DES in 2008. However, the long-term safety of EESs is a pending issue and the assessment of patient-reported outcomes in real-world settings should be followed for longer durations.

Second-generation zotarolimus-eluting stent (Endeavor)

The Endeavor zotarolimus-eluting stent (ZES) system (Medtronic CardioVascular, Minneapolis, MN) uses a cobalt-based alloy stent (Driver) coated with the sirolimus analogue zotarolimus, delivered via a phosphorylcholine polymer-based coating. The hydrophilic phosphorylcholine polymer of the ZES was designed to be biocompatible. The release kinetics of zotarolimus enables nearly complete drug delivery within the first month after stent placement.

The ENDEAVOR I[46] study was a single-arm, prospective, multicenter, first-in-human trial evaluating the performance and safety of the ZES in 100 patients with symptomatic coronary artery disease. At 12 months, in-stent late lumen loss was 0.61 ± 0.44 mm (corresponding to a percentage volume obstruction of $9.7 \pm 8.5\%$ as determined using intravascular ultrasound). The cumulative incidence of major adverse cardiac events (death, MI, emergent cardiac surgery, and repeat revascularization of the index lesion), was 1% at 30 days and 2% at 12 months.

The ENDEAVOR II[47] RCT was designed to examine the efficacy and safety of the ZES as compared with the Medtronic Driver BMS. A total of 1,197 patients with a single coronary artery stenosis were enrolled and randomly assigned to receive the ZES ($n = 598$) or the BMS ($n = 599$). At the 9-month follow-up, the primary end point, TVF, was lower in the Endeavor group (7.9%) as compared with the BMS group (15.1%) ($P = 0.0001$). The rate of occurrence of major adverse cardiac events decreased from 14.4% with the BMS to 7.3% with the ZES ($P = 0.0001$). The rate of ST was 0.5% with the ZES, which was not significantly different from the value of 1.2% associated with the BMS. In the 531 patients who submitted themselves to the angiographic follow-up, in-stent late loss had reduced from 1.03 ± 0.58 to 0.61 ± 0.46 ($P < 0.001$) (**Figure 1**). At 4 years, the ZES maintained an advantage over the BMS with respect to rate of TVF (13.6% for ZES vs. 22.6% for BMS; $P < 0.001$), primarily through a persistent reduction in TVR (9.8% vs. 18.8%; $P < 0.001$), as presented by Fajadet *et al.* at the EuroPCR Meeting (May 2008).

ENDEAVOR III[48] was a prospective, randomized, single-blinded multicenter angiographic trial designed to show the noninferiority of the ZES as compared with the SES. In this study, 436 patients with *de novo* native coronary lesions were randomized in a 3:1 ratio for treatment with the ZES ($n = 323$) or the SES ($n = 113$). At 8 months, the rate of in-segment late loss was higher with the ZES as compared with the SES (0.34 vs. 0.13; $P < 0.001$ for superiority of SES; $P = 0.65$ for noninferiority). However, at the 9-month follow-up, there were no significant differences between the ZES and the SES with respect to the occurrence of major adverse cardiac events (7.6% vs. 7.1%) and TVF (12.0% vs. 11.5%).

Unpublished data of the ENDEAVOR IV study, which was a randomized, single-blind, prospective, multicenter trial, were presented by Leon *et al.* at the Transcatheter Cardiovascular Therapeutics conference (Washington, DC, 2007). This trial randomized patients with single *de novo* native artery lesions for implantation of the ZES ($n = 774$) or the PES ($n = 775$). The rate of TVF, the primary study end point, was similar in both arms at 9 months (6.6% vs. 7.2%; $P = 0.685$ for superiority; $P < 0.001$ for noninferiority). In summary, the ZES appears to be safe and effective in treating single *de novo* coronary artery lesions. Despite the association of the ZES with a higher rate of late loss as determined by coronary angiogram, the clinically assessed outcomes of TVR and TLR with the ZES are similar to those with the SES and the PES. The issues that remain to be clarified with the Endeavor ZES relate to its efficacy and safety in more diverse patient populations with longer follow-up terms.

Comments regarding off-label use of DESs

According to several registries, off-label use occurred in nearly 60% of patients undergoing DES implantation procedures. These patients are a high-risk population with numerous comorbidities, unfavorable lesion morphology, and unstable clinical presentations, and they are expected to have higher rates of adverse clinical outcomes.[49]

Although randomized trials of off-label use of the DES as compared with the BMS have not yet been reported, numerous registries and randomized trials suggest that DESs are safe and effective under such circumstances.[6,7,50] The original Swedish Coronary Angiography and Angioplasty Registry publication suggested that there is a higher rate of late events in DES-treated

patients,[1] but a later, more comprehensive, unpublished report presented by James *et al.* at the European Society of Cardiology Congress (Vienna, Austria, 2007) showed that restenosis is 50% less frequent with DES use than with BMS use and that long-term mortality rates (at 4-year follow-up) were comparable in the two procedures. In the meta-analysis of 38 trials (18,023 patients) conducted by Stettler *et al.*[2], which included trials of primary percutaneous coronary intervention and off-label indications, mortality and the risk of ST were similar for DESs and BMSs at 4-year follow-up. Interestingly, SESs were associated with a significant reduction in the risk of MI (HR: 0.81, 95% CI: 0.66–0.91, $P = 0.03$) as compared with BMSs. Although additional trials are needed, it appears that DESs are safe and effective both for FDA-approved indications and for several off-label indications. However, physicians must use their clinical judgment in order to select the best device, keeping in mind the risk of restenosis and ST and the expected level of compliance with dual antiplatelet therapy.

Conclusion

In 2008, the consensus was that there is an ongoing risk associated with DESs; the main challenge with the next generation of DESs is to ensure safety with less dependence on prolonged dual antiplatelet therapy. New scientific approaches such as using biocompatible or bioabsorbable polymers or no polymer at all, lowering the dose of the drug, and promoting healing with progenitor cells are intriguing but do not necessarily guarantee translation into improved clinical outcome. The motivation in the first 30 years of interventional cardiology was to reduce restenosis; it is now time to focus on improving the safety performance of DESs in a wider range of patient populations.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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Heart Rhythm Disturbances Associated With Rupatadine: A Case Series From the Spanish and Portuguese Pharmacovigilance Systems

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We searched the Spanish and Portuguese pharmacovigilance databases for spontaneous case reports of heart rhythm disturbances associated with rupatadine and other new H1 antihistamines. Five cases were found involving patients treated with rupatadine (13.9% of all reports relating to this drug). In all five cases, the reaction started after exposure and resolved when the drug was discontinued. In two cases, rupatadine was the only medication being taken by the patient, and no other condition that could explain the heart rhythm disturbances was diagnosed. The reporting odds ratio was 3.2 (95% confidence interval, 1.0–10.5). The reporting rate was 2 cases per 100,000 patients treated per year (95% confidence interval, 0.4–6.0). These results suggest a causal relationship between rupatadine and heart rhythm disturbances.

Since the mid-1980s, a number of histamine H1 receptor antagonists (antihistamines) have been introduced onto the market worldwide. These second-generation antihistamines have fewer sedating effects than older antihistamines. All have similar chemical structures. Some are active metabolites of other antihistamine compounds; for example, fexofenadine, levocetirizine, and desloratadine are active metabolites of terfenadine, cetirizine, and loratadine, respectively. Rupatadine, introduced on 1 March 2003, inhibits both peripheral H1 histamine receptors and the platelet-activation factor. This may explain why rupatadine is more effective in treating allergic disorders than antihistamines that do not inhibit the platelet-activation factor.[1,2]

Most of the antihistamines in use have been associated with cardiotoxicity.[3,4] Astemizole and terfenadine were removed from the market for this reason.[5,6] Some of the newer antihistamines have been termed "third generation" because they are thought to have no cardiotoxic effect.[7]

In 2007, our pharmacovigilance center in Valladolid had knowledge of some cases, reported to the Spanish pharmacovigilance system, of heart rhythm disturbance presumably associated with rupatadine. This prompted us to investigate the possibility of a causal relationship between heart rhythm disturbances and rupatadine and other new antihistamines using data from the Portuguese and Spanish pharmacovigilance systems.

RESULTS

From 1 March 2003 until 12 December 2007, 36 reports in which rupatadine was associated with adverse reactions were collected by the Spanish (n = 32) and Portuguese (n = 4) pharmacovigilance systems. Five of these reports (13.9%) related to heart rhythm disturbances. **Table 1** summarizes these five cases, which occurred in four men and one woman. For four patients, the dose of rupatadine was 10 mg/day; one patient received 20 mg/day. The time between the patient first taking the drug and the onset of heart rhythm disturbances ranged from 1 day to 1 year. In three cases, rupatadine was the only drug the patient was taking; patients 2 and 5 were also being treated for other conditions. In addition to heart palpitations, patient 4 developed dry mouth, malaise, nausea, sweating, swelling, and weakness.

The estimated strength of association between heart rhythm disturbances of any type and rupatadine, astemizole, desloratadine, ebastine, loratadine, terfenadine, and levocetirizine was statistically significant (**Table 2**). The reported rates of heart rhythm disturbances for various H1 antihistamines are shown in **Table 3**. For azelastine and fexofenadine, there were no cases of

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Table 1 Case series of heart rhythm disturbances associated with rupatadine reported to the Spanish and Portuguese pharmacovigilance systems

Case	Age (years)/sex	Heart rhythm disturbance	Induction period (days)	Duration (days)	Other drugs or conditions	Indication	Outcome
Spanish Pharmacovigilance System							
1	69/Male	Ventricular tachycardia	365	3	Coronary ischemia	Allergy	Recovered
2	73/Male	Long QT/torsade de pointes	14	2	Sertraline[a]	Allergic rhinitis	Recovered/ sequelae
3	30/Female	Tachycardia/palpitations	ND	7	—	Not stated	Recovered
Portuguese Pharmacovigilance System							
4	32/Male	Palpitations	Months	ND	—	Eczema	Recovered
5	58/Male	Extrasystoles	3	ND	Prostatic hyperplasia; aspirin, serenoa repens, atorvastatin	Rhinitis	Recovered

Total number of reports from Spain for rupatadine was 32 during the period studied; from Portugal the corresponding figure was 4.

ND, no data.

[a]Patient with depression, diabetes, hypertension, and prostate cancer, also treated with amlodipine, glimepiride, aspirin, chlortalidone, ezetimibe, insulin, pentoxifilin, lormetazepam, omeprazol, and eprosartan. The patient had several episodes of syncope.

Table 2 Rupatadine and heart rhythm disturbances

Antihistamine	Heart rhythm disturbances (%)	ROR
Astemizole	8 (8.3)	2.8 (1.4–5.9)
Azelastine	1 (3.5)	1.1 (0.1–7.9)
Cetirizine	8 (3.6)	1.1 (0.6–2.3)
Desloratadine	10 (10.9)	3.8 (1.9–7.3)
Ebastine	15 (8.0)	2.7 (1.6–4.5)
Fexofenadine	1 (1.8)	0.5 (0.1–4.0)
Loratadine	15 (8.4)	2.8 (1.6–4.8)
Mizolastine	3 (5.1)	1.6 (0.5–5.3)
Rupatadine	3 (9.4)	3.2 (1.0–10.5)
Terfenadine	7 (7.2)	2.4 (1.1–5.2)
Levocetirizine	4 (16.7)	6.2 (2.1–18.1)

% Refers to the percent of the total number of reports for each particular drug. For all drugs, the total number of reports of heart rhythm disturbances since rupatadine was first marketed on 1 March 2003 and until the date of data collection for this study was 924. The total number of reports for all adverse reactions in general in the corresponding period was 29,330. For the antihistamines, the corresponding total number of reports of heart rhythm disturbances since they were first marketed were: astemizole (withdrawn on 1 April 2003), 2,237 (1 October 1985); azelastine, 2,583 (1 April 1993); cetirizine, 2,963 (1 November 1989); desloratadine, 959 (1 January 2003); ebastine, 2,958 (1 December 1989); fexofenadine, 1,764 (1 September 1998); loratadine, 2,958 (1 December 1989); mizolastine, 1,829 (1 March 1998); tefenadine, 3,146 (1 April 1985); and levocetirizine, 904 (1 April 2003). The total numbers of reports in the corresponding periods for all reactions were 72,027, 79,890, 93,621, 30,445, 93,458, 55,584, 93,458, 58,024, 100,877, and 28,694.

Reporting odds ratio (ROR) compared with estimates for other antihistamines.

Data from the Spanish Pharmacovigilance System until December 2007.

cardiotoxicity; for the other drugs, the rates range from 0.4 cases per 100,000 patients treated per year for mizolastine to 3.1 for desloratadine, with overlapping confidence intervals.

DISCUSSION

We examined data from spontaneous reporting schemes to identify cases of heart rhythm disturbances in patients treated with rupatadine. The same adverse reactions have been observed for most of the other newer antihistamines and are well established for the older ones. The case of torsade de pointes included in this study has been described in detail elsewhere.[8] Some of these adverse reactions are cause for concern because these drugs are commonly used for prolonged periods to treat a great number of patients with a wide range of conditions who are taking other medications at the same time. Such adverse reactions have prompted withdrawal of the antihistamines terfenadine and astemizole from the market.[5,6]

Although heart rhythm disturbances vary in their origin and severity, they can have similar consequences: blood flow to the brain is impaired, which can result in loss of consciousness (e.g., syncope) or even sudden death.

No causal relationship can be definitively established on a case-report basis. However, in all cases, the heart rhythm disturbances began after exposure to rupatadine and resolved when the drug was discontinued. In two cases, rupatadine was the only medication the patients were taking, and no other condition was diagnosed that could explain the heart rhythm disturbances. Because heart rhythm disturbances associated with antihistamines—particularly the most serious reactions— have a very low incidence, these rare but potentially fatal events can be identified only in a large population of patients with a broad range of conditions. In our series, one patient had a history of cardiovascular disease, and another was also taking sertraline, which may prolong the QT interval.

Heart rhythm disturbances have been reported for almost all antihistamines on the market.[3–6] In the published data from clinical trials of rupatadine, however, no cardiotoxicity has been identified.[9] In a "thorough QT/QTc" study conducted in 160 healthy volunteers, no significant increase in QT intervals was observed with doses of 10 and 100 mg,[10,11] even though peak plasma concentration and area under the curve increased and systemic clearance of the drug decreased after 7 days of once-daily treatment with rupatadine in healthy older volunteers compared with younger individuals. These data come from a small number of individuals and do not cover all of the clinical circumstances that

Table 3 Rupatadine and heart rhythm disturbances

Antihistamine	Cases of heart rhythm disturbances, *n*	DDDs[a]	Patients, *n*	Reporting rate per 100,000 patients treated per year (95% confidence interval)
Astemizole	8	194,600,456	532,787	1.5 (0.6–3.0)
Azelastine	1	68,301,500	186,999	0.5 (0.0–3.0)
Cetirizine	8	513,350,785	1,405,478	0.6 (0.2–1.1)
Desloratadine	10	115,705,740	316,785	3.1 (1.5–5.8)
Ebastine	15	645,620,000	1,767,611	0.8 (0.5–1.4)
Fexofenadine	1	96,529,290	264,283	0.4 (0.0–2.1)
Loratadine	15	441,030,292	1,207,475	1.2 (0.7–2.0)
Mizolastine	3	90,636,480	248,149	1.2 (0.2–3.5)
Rupatadine	3	53,650,180	146,886	2.0 (0.4–6.0)
Terfenadine	7	94,724,751	259,342	2.7 (1.1–5.6)
Levocetirizine	4	78,244,320	214,221	1.9 (0.5–4.8)

Reporting rate compared with that for the other antihistamines.

Data from the Spanish Pharmacovigilance System and from consumption through the Spanish National Health System until December 2007. Rupatadine was first marketed on 1 March 2003; astemizole on 1 October 1985 (withdrawn on 1 April 2003); azelastine on 1 April 1993; cetirizine on 1 November 1989; desloratadine on 1 January 2003; ebastine on 1 December 1989; fexofenadine on 1 September 1998; loratadine on 1 December 1989; mizolastine on 1 March 1998; terfenadine on 1 April 1985; levocetirizine on April 2003. All searches were conducted between the date of first marketed until December 12th, except for astemizole, which was conducted until date of withdrawal (1 April 2003); the last item is stated in the Methods section.

DDDs, defined daily doses.

[a]http://www.whocc.no/atcddd.

can account for higher susceptibility to arrhythmias, including hypokalemia, preexisting cardiac conditions, and coadministration of drugs that prolong the QT interval or inhibit metabolism. On the other hand, experiments with cloned human myocytes have shown that rupatadine can cause concentration-, time-, and voltage-dependent blockade of hKv1.5 channels involved in the duration of the action potentials in the human heart.[12] The peak plasma concentration in healthy volunteers after oral administration of 20 mg/day of rupatadine (the highest therapeutic dose) was 5.5 nmol/l.[13] This concentration is <1/400 of the K_D for hKv1.5 blockade (2,400 nmol/l). As K_D is the concentration that produces 50% of the maximum inhibitory effect and the inhibitory effect is evident at concentrations of 10 nmol/l, some degree of blockade might be expected for concentrations between 5.5 and 10 nmol/l. This range of concentrations is close to those reached with therapeutic doses of rupatadine and could be more readily reached in patients taking cytochrome P450 3A4 inhibitors at the same time. It is also possible that a mutated hKv1.5 was more sensitive to the drug than expected.[14]

Despite underreporting,[15] the data from Spain show a statistical association between rupatadine use and heart rhythm disturbances. The estimated association is similar to those for antihistamines known to induce such reactions. Because this is the first reported case series of heart rhythm disturbances thought to be induced by rupatadine, it is unlikely that a "notoriety bias" accounts for this association. The absence of such bias is supported by the fact that both the reporting odds ratio and the reporting rate point to a risk similar to those associated with other antihistamines. It is possible that some patients who had problems with other antihistamines or who were thought to be at risk of developing cardiotoxicity were switched to rupatadine (channeling). This would account for a higher estimate, but it would not indicate an absence of risk.

Torsade de pointes, the most serious antihistamine-induced heart rhythm disturbance, is thought to result from prolonged ventricular repolarization. Patients with this condition show a marked prolongation of the QT interval on electrocardiograms.[16] Antihistamines that do not block cardiac voltage-gated potassium currents related to repolarization, particularly the rapid component of the delayed rectifier potassium current, would not induce torsade. Nevertheless, under certain circumstances, including drug–drug interactions and kidney or liver impairment, rupatadine may reach plasma concentrations far above the therapeutic range that can block those currents. Other mechanisms by which antihistamines could induce severe heart rhythm disturbances have been proposed, including muscarinic receptor inhibition in the heart,[17] calcium interference,[18] and histamine release.[19] For example, desloratadine, the main metabolite of rupatadine, has been shown to release histamine from cardiac mast cells.[20]

The low incidence of drug-induced arrhythmia makes collaborations between countries, such as the one established for this study, particularly useful. As a system for "signal generation," the spontaneous reporting is considered key to the early identification of proarrhythmic newly marketed drugs.[21] The prevalence of allergic conditions has been increasing, and they are being treated for longer. Because H1 antihistamines are first-line medications for these conditions, they are among the most frequently prescribed drugs worldwide. It is a matter of debate whether arrhythmias are a class effect. Our results support the idea that antihistamines as a group are associated with an increased risk of heart rhythm disturbances. Thus, a statement should be included in the summary of product characteristics to indicate a possible association between rupatadine and cardiotoxicity. Physicians should be aware of the potential cardiotoxic effect of antihistamines and avoid prescribing them for patients who are prone

to these rare but potentially life-threatening heart rhythm disturbances. These include patients with hereditary long-QT syndrome, who are particularly sensitive to any factor that further prolongs the QT interval; patients taking cytochrome P450 3A4 inhibitors; and patients with kidney or liver impairment.

METHODS

The Spanish and Portuguese pharmacovigilance system databases used in this study include all adverse drug reactions reported to regional pharmacovigilance centers and coordinator centers in both countries. Physicians and pharmacists submit spontaneous reports of suspected adverse drug reactions. In particular, the centers request reports of severe events and of events associated with the use of recently marketed drugs. Ad hoc committees evaluate reports using an algorithm to determine whether a causal relationship exists. Reports are included in the databases regardless of causality and severity. Until July 2007, adverse drug reactions were coded according to the World Health Organization Adverse Reactions Terminology dictionary;[22] since then, the MedDRA dictionary has been used.[23]

We used a case/noncase approach to assess the strength of the association between rupatadine exposure and cardiotoxicity.[24] We took only the data from the Spanish database, as it included enough cases of interest. Cases were defined as reports of heart rhythm disturbances; noncases were defined as reports of all reactions other than heart rhythm disturbances. Exposure was defined as the mention of rupatadine in a report, whether or not it was suspected of causing the reaction. The search extended from the date each antihistamine came onto the market until 12 December 2007, except in the case of astemizole, which was withdrawn from the Spanish market in 2003.

The association between heart rhythm disturbances and rupatadine was estimated by calculating the reporting odds ratio with a 95% confidence interval.[25–27] The same ratio was calculated for other antihistamines on the market to compare the risks of heart rhythm disturbances. Drugs combining an antihistamine with one or more additional drugs (e.g., ebastine plus pseudoephedrine) were excluded from the study.

The reporting rate in Spain was calculated for all drugs studied as the number of reported cases divided by the number of person-years of treatment. To estimate the number of patients exposed, we used drug sales data from the Spanish National Health System extracted from the Especialidades Consumo de Medicamentos database of the Ministry of Health. This database contains information on community drug consumption throughout the Spanish National Health System, which covers 99% of the population. Drug consumption data were converted into defined daily doses and then into number of person-years of treatment. The reporting rate was estimated on the assumption that the exposed population was large and the number of cases few. On this assumption, reporting of suspected adverse reactions is expected to follow a Poisson distribution, and confidence limits can be calculated on the basis of the relation between Poisson and χ^2 distribution.[28,29]

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

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Pharmacogenetics-Based Population Pharmacokinetic Analysis of Efavirenz in HIV-1-Infected Individuals

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Besides CYP2B6, other polymorphic enzymes contribute to efavirenz (EFV) interindividual variability. This study was aimed at quantifying the impact of multiple alleles on EFV disposition. Plasma samples from 169 human immunodeficiency virus (HIV) patients characterized for *CYP2B6, CYP2A6*, and *CYP3A4/5* allelic diversity were used to build up a population pharmacokinetic model using NONMEM (non-linear mixed effects modeling), the aim being to seek a general approach combining genetic and demographic covariates. Average clearance (CL) was 11.3 l/h with a 65% interindividual variability that was explained largely by *CYP2B6* genetic variation (31%). *CYP2A6* and *CYP3A4* had a prominent influence on CL, mostly when *CYP2B6* was impaired. Pharmacogenetics fully accounted for ethnicity, leaving body weight as the only significant demographic factor influencing CL. Square roots of the numbers of functional alleles best described the influence of each gene, without interaction. Functional genetic variations in both principal and accessory metabolic pathways demonstrate a joint impact on EFV disposition. Therefore, dosage adjustment in accordance with the type of polymorphism (*CYP2B6*, *CYP2A6*, or *CYP3A4*) is required in order to maintain EFV within the therapeutic target levels.

Efavirenz (EFV), a non-nucleoside reverse transcriptase inhibitor, is widely used in combination with nucleoside inhibitors as first-line treatment of type I human immunodeficiency virus (HIV-1) infection. It is generally prescribed at a fixed dosage of 600 mg daily, despite the presence of a marked interindividual variability in tendency to produce elevated plasma drug concentration levels[1–3] that have been shown to be associated with central nervous system toxicity.[4–6]

EFV is metabolized primarily by *CYP2B6* and, to a lesser extent, by accessory pathways involving *CYP2A6*, *CYP3A4/3A5*, and uridine-glucuronyl-transferases.[7–9] Several studies have shown that *CYP2B6* is highly polymorphic and that genetic variations play an important part in EFV plasma concentration variability.[5,10–15] Genetic polymorphisms of *CYP3A4/3A5* have also been associated with higher EFV exposure,[6] but the influence of the *CYP2A6* polymorphism on EFV pharmacokinetics has not yet been characterized. Considering the increasing number of allelic variants that are being described and the resulting complexity of allele combinations that could influence EFV elimination, we conducted a population pharmacokinetic analysis in HIV-1-infected individuals fully characterized for *CYP2B6*, *CYP2A6*, and *CYP3A4/A5* genetic variations. Our main areas of focus were: (i) to assess the relative contributions of multiple functional alleles involved in EFV elimination along with other demographic or environmental factors, (ii) to characterize the nature of the relationship between individual allelic constitution and EFV disposition, and (iii) to explore models for gene–gene interactions that could lead to a better understanding of the interrelationships of specific enzymes involved in EFV elimination.

RESULTS

In total, 393 plasma samples were collected from 169 individuals. Concentration measurements ranged between 100 and 59,400 ng/ml. A one-compartment model with first-order absorption from the gastrointestinal tract fitted the data

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appropriately. Average clearance (CL) was 11.3 l/h with an interindividual variability of 65%, the volume of distribution (V) was 388 l, and the absorption constant (k_a) was 0.62 h^{-1}. The assignment of interindividual variability on either V or k_a did not improve the fit (change in objective function (ΔOF) = 0.0).

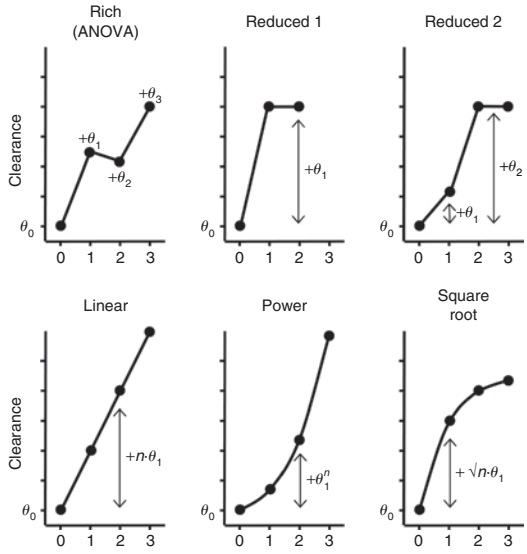

Figure 1 Various parameter models were tested to describe the level of oral clearance (*Y*-axis) as a function of the number of functional alleles of an enzyme (*X*-axis): 0 = Hom-LOF, 1 = Het-LOF, 2 = Hom-Ref, 3 = Het-GOF (a specific parameter for Het-GOF, is required only with *CYP2B6*). ANOVA, analysis of variance; GOF, gain of function; Het, heterozygous; Hom, homozygous; LOF, loss of function; Ref, reference allele.

Demographic analyses

Body weight and black ethnicity influenced CL, as did gender, age and, to a lesser extent, height. Co-medications were shown to have no significant influence on EFV pharmacokinetics. A multivariable combination of demographic factors revealed that body weight accounted for the effect of height, age, and gender while explaining 3% of CL variability, and it was the only demographic factor influencing CL besides black ethnicity, which remained statistically significant beyond body weight and reduced the variability in CL by another 3%.

Univariate genotype analyses

The influence of *CYP2B6*, *CYP2A6*, *CYP3A4/AA5* functional alleles on EFV CL was first tested in single-gene analyses, in which the allelic variants (Hom-LOF, Het-LOF, and Hom-Ref plus Het-GOF for *CYP2B6*) were entered into the model as covariates that partitioned individuals based on their genetic constitution.

Genetic variation of *CYP2B6* had by far the most salient impact on CL. Several competing models were tested, as depicted in Figure 1. The richest possible model, which assigned a separate fixed effect to each of the *CYP2B6* allelic variants (Eqs. 1/1a), markedly improved the fit and explained 31% of the 65% interindividual variability on CL. The average CL was 2.8 l/h in the Hom-LOF group and 10.8, 13.3, and 18.8 l/h in individuals carrying Het-LOF, Hom-Ref, and Het-GOF alleles, respectively. A series of reduced models showed CL to be statistically different among all *CYP2B6* groups (ΔOF = +9 and ΔOF = +10 for model reduced 1 and 2). Competing simplified models were tried to

Table 1 Functional alleles evaluated in the study and genotype-based activity score classification

Functional alleles					
Functional consequence	**CYP2B6 alleles**	**CYP2A6 alleles**	**CYP3A4 alleles**	**CYP3A5 alleles**	
Loss of function (LOF)	*11, *15, *28	*2, *4		*3, *6, *7, *10, *11	
Diminished function (DOF)	*6, *18, *27, *29	*1H, *1J, *5, *7, *9, *10, *12, *13, *15, *17, *19, *34	rs4646437 *1B	—	
>25%		*5, *7, *9, *10, *12, *13, *15, *17, *19, *34	—	—	
<25%		*1H, *1J	—	—	
Reference	*1, *2, *3, *5, *17	*1	*1	*1	
Gain of function (GOF)	*4, *22	*1X2	—	—	
Genotypes and activity score classification					
Score A	**Alleles (allele 1/allele 2)**	**Score B**	**Alleles (allele 1/allele 2)**	**Score C***	**Alleles (allele 1/allele 2)**
0	LOF/LOF LOF/DOF DOF/DOF	0	LOF/LOF	0	LOF/LOF
1	Ref/LOF Ref/DOF	0.25	LOF/DOF	0.25	LOF/DOF
2	Ref/Ref	0.5	Ref/LOF DOF/DOF	0.5	Ref/LOF
3	Ref/GOF	1	Ref/LOF	0.75	DOF/DOF
		1.5	Ref/DOF	1	Ref/DOF >25%
		2	Ref/Ref	1.5	Ref/DOF <25%
				2	Ref/Ref

DOF, diminished function; GOF, gain of function; LOF, loss of function; Ref, reference allele.

*Only for CYP2A6 alleles.

estimate CL as a function of the number of functional alleles, as defined by the activity score A (**Table 1**) and were compared with the richest model (Eqs. 1/1a); the use of a linear model (Eq. 2) or a power function model (Eq. 3) did not fit the data appropriately (ΔOF = +18.5 and +58.8, respectively). As interim explorations showed CL to be modestly reduced by ~25% in Het-LOF carriers but cut down by 75% in Hom-LOF individuals, a square root function model achieved the best fit using either an additive (Eq. 4) or a proportional model (ΔOF = −167.7). The recourse to alternative activity scores B for *CYP2B6*, allowing for the distinction between loss and decrease of function alleles, did not better characterize the genotype–phenotype relationships using Eq. 2, 3, or 4 (ΔOF >+8.2).

The assignment of *CYP2A6* allelic variants on CL using the richest model (Eqs. 1/1a) improved the fits (ΔOF = −7.9) and decreased by 1% the overall variability on CL. The average CL was 7.0, 10.8, and 12.1 l/h in Hom-LOF, Het-LOF, and Hom-Ref individuals, respectively. The difference in CL between Hom-Ref and Het-LOF individuals was not significant (ΔOF = −1.0). The description of the relationship between CL and the functional score, using either linear (Eq. 2) or power (Eq. 3) models, did not fit the data adequately when compared with the richest model (ΔOF >+6.8), whereas it was again best characterized using a square root function (Eq. 4) that provided a fit almost identical to that of the rich model (ΔOF = −7.8). Here too, the model integrating partial activity levels (scores B/C) did not improve data description (ΔOF = −0.4 for score B and −0.1 for score C).

The impact of *CYP3A4* on EFV CL was tested using two alleles associated with changes in function, *CYP3A4*1B* and *CYP3A4_* rs4646437. The rich model (Eqs. 1/1a) showed that both alleles influenced CL to a significant extent (ΔOF = −25.4 for *CYP3A4_* rs4646437, −10.4 for *CYP3A4*1B*). CL in Hom-LOF, Het-LOF, and Hom-Ref carriers were, respectively, 5.1, 10.3, and 11.9 l/h for *CYP3A4*1B* and 3.7, 10.7, and 12.3 l/h for *CYP3A4_* rs4646437 alleles. After inclusion of *CYP3A4*1B* and *CYP3A4_* rs4646437 alleles in the model, interindividual variability in CL dropped from 65 to 62 and 59%, respectively. The differences in CL between Hom-Ref and Het-LOF individuals were not significant, both for **1B* and for rs4646437 (ΔOF = −1.4 for *CYP3A4_* rs4646437 and −0.9 for *CYP3A4*1B*). When compared with the richest model, linear and power function models (Eqs. 2/3) did not fit the data adequately (ΔOF >+8.0); square root models (Eq. 4) described the data best (ΔOF = −10.3 for *CYP3A4*1B* and −24.8 for *CYP3A4_* rs4646437).

The influence of *CYP3A5* functional alleles on CL was shown to be small but significant, using the rich model (Eqs. 1/1a, ΔOF = −8.1), with a residual 64% interindividual variability. The difference in CL between individuals with Het-LOF allele and those with Hom-Ref allele was not significant (ΔOF = −0.0). None of the above models (Eqs. 2/3) could characterize the relationship between CL and *CYP3A5* allele variants better than the square root model could (Eq. 4, ΔOF = −8.2).

The effect of black ethnicity remained a statistically influencing covariate on CL in addition to genetic variation, causing an additional 25–40% decrease in CL when associated with functional alleles (ΔOF compared to *CYP2B6* = −8.1, *CYP2A6* = −9.9, *CYP3A4*1B* = −4.0, and *CYP3A5* = −7.8), except for *CYP3A4_* rs4646437 (ΔOF = −0.4), which was present in most of the black individuals, thus limiting the power to detect any association.

Gene–gene interaction analyses

The joint influence of functional alleles on EFV CL was first tested through the conjunction of *CYP2B6* with each of the other *CYP* alleles in dual-gene models, to finally build up the model including all genetic variables having influence on CL. The richest model (Eq. 5) characterizing the joint influence of *CYP2B6* and *CYP2A6*, using a fixed effect parameter for each allelic combination, suggested an additional contribution of *CYP2A6* in EFV elimination (ΔOF = −20 as compared to the final model for *CYP2B6*, Eq. 4). Competitive models were developed based on a functional score A (**Table 1**) using square root function models. The models evaluating the contribution of *CYP2A6* variation, whether on each *CYP2B6* genotypic group separately (Eq. 6) or through the use of a single parameter estimate across all *CYP2B6* allelic variants (Eq. 7), fitted the data with similar adequacy (ΔOF >−16), resulting in an absolute increase in CL of 1.2 and 1.7 l/h in *CYP2A6* Het-LOF and Hom-Ref individuals, respectively, as compared to Hom-LOF carriers. The contribution of *CYP2A6* functional alleles was more prominent in *CYP2B6* Hom-LOF carriers, in whom the relative change in CL was estimated to be 44% per active allele as compared to an 11% change in Hom-Ref individuals. The joint gene influence could be further described by simply adding *CYP2B6* and *CYP2A6* square root functions (Eq. 8, ΔOF = −12). This model was not considered statistically different from previous models (Eqs. 6/7), considering the reduced degrees of freedom and the loss of fit from the richest possible model (Eq. 5, ΔOF = +8). The introduction of a single interaction factor (Eq. 9) to evaluate some hyper- or hypo-additive trend did not produce any significant change (ΔOF = +3).

The same paradigm was successfully applied to characterize the other gene–gene interactions. The rich model (Eq. 5) characterizing the joint influence of *CYP2B6* with the *CYP3A4*1B* or *CYP3A4* rs4646437 alleles suggested an additive effect of both genes on EFV CL (ΔOF = −14 for **1B*, −25 for rs4646437). Reduced models integrating square root functions of *CYP3A4* activity scores on *CYP2B6* stratified by allelic variation (Eq. 6) described the data appropriately (ΔOF = −13 for **1B,* −18 for rs_4646437), and no deterioration of the fit was observed when allowing a single parameter estimate (Eq. 7) for the effect of *CYP3A4* across all *CYP2B6* allelic variants (ΔOF = +1.0 for **1B,* 0.0 for rs_4646437 as compared to Eq. 6). The additive contribution of *CYP3A4* on CL was 1.1 and 1.5 l/h in **1B* Het-LOF and Hom-Ref carriers, respectively, and 1.4 and 1.9 l/h in rs_4646437 Het-LOF and Hom-Ref carriers, respectively. CL increased by 40 (**1B*) and 48% (rs_4646437) per active allele of *CYP2B6* and *CYP3A4* as compared to the respective Hom-LOF. Further characterization of the joint contribution of *CYP2B6* and *CYP3A4* on EFV CL, using a mere addition of square root functions (Eq. 8), fitted the data appropriately, the loss of fit in comparison with previous models (Eqs. 5/6/7) being not significant (ΔOF = −9.0

Table 2 Summary of the key models used to examine the influence of demographic and genetic covariates on EFV clearance

Step 1	Demographic model	Model	θ_0	θ_1	θ_2	θ_3	ΔOF	*P*
	Body weight (BW)	$CL = \theta_0 \cdot (1 + \theta_1 \cdot BW)$	11.5	1.2			−25.6	**
	Height	$CL = \theta_0 \cdot (1 + \theta_1 \cdot Hgt)$	11.2	2.3			−4.2	*
	Age	$CL = \theta_0 \cdot (1 + \theta_1 \cdot age)$	12	0.7			−5.7	*
	Sex (M = 0, F = 1)	$CL = \theta_0 \cdot (1 + \theta_1 \cdot sex)$	12	0.2			−4.1	*
	Race	$CL = \theta_0(1 - q) + \theta_1 \cdot q$						
	Black (q = 1) vs. others (q = 0)		11.8	6.25			−13.2	**
	White (q = 1) vs. others (q = 0)		12	7.1			−7.7	**
	Hispanic (q = 1) vs. others (q = 0)		15.1	11.2			−0.9	NS
	Asian (q = 1) vs. others (q = 0)		11.3	9.8			−0.13	NS
	Other ARV							
	Ritonavir (RTV)	$\theta_0 \cdot (1 + \theta_1 \cdot RTV)$	11.3	0.01			0.2	NS
	Zidovudine (AZT)	$\theta_0 \cdot (1 + \theta_1 \cdot AZT)$	10.4	0.12			−1.1	NS
	Lamivudine	$\theta_0 \cdot (1 + \theta_1 \cdot 3TC)$	10.6	0.09			−0.6	NS
	NRTI in general	$\theta_0 \cdot (1 + \theta_1 \cdot NRTI)$	8.6	0.3			−1.8	NS
	PI in general	$\theta_0 \cdot (1 + \theta_1 \cdot PI)$	11.3	0.01			0.6	NS
Step 2	**Genotype-variant analysis**	**Model**	**CL_0**	**θ_1**	**θ_2**	**θ_3**	**ΔOF**	***P***
CYP2B6								
Rich: Eq. 1	I_1: Het-LOF, I_2: Hom-Ref, I_3: GOF	$CL = CL_0 + \theta_1 I_1 + \theta_2 I_2 + \theta_3 I_3$	2.8	10.8	13.3	18.8	−171	**
Reduced 1	I_1: Het-LOF or Hom-Ref, I_3: GOF	$CL = CL_0 + \theta_1 I_1 + \theta_3 I_3$	2.8	12.2	—	18.9	−162	**,[a]
Reduced 2	I_1: Het-LOF, I_2: Hom-Ref or GOF	$CL = CL_0 + \theta_1 I_1 + \theta_2 I_2$	2.8	10.8	14.2		−161	**,[a]
Eq. 2	n = 0, 1, 2, 3	$CL = CL_0 + \theta_1 \cdot n$	3.11	5.99			−152	**,[a]
Eq. 3		$CL = CL_0 + \theta_1^n$	5.03	2.9			−76	**,[a]
Eq. 4		$CL = CL_0 + \theta_1 \cdot \sqrt{n}$	2.8	7.8			−168	NS[a]
CYP2A6								
Rich: Eq. 1	I_1: Het-LOF, I_2: Hom-Ref	$CL = CL_0 + \theta_1 I_1 + \theta_2 I_2$	7.0	10.8	12.1		−7.9	*
Reduced 1	I_1: Het-LOF or Hom-Ref	$CL = CL_0 + \theta_1 I_1$	7.0	11.7			−6.9	NS[a]
Eq. 2	n = 0, 1, 2	$CL = CL_0 + \theta_1 \cdot n$	7.75	2.31			−6.8	NS[a]
Eq. 3		$CL = CL_0 + \theta_1^n$	7.8	2.11			−5.3	NS[a]
Eq. 4		$CL = CL_0 + \theta_1 \cdot \sqrt{n}$	7.0	3.63			−7.8	NS[a]
CYP3A4 rs4646437								
Rich: Eq. 1	I_1: Het-LOF, I_2: Hom-Ref	$CL = CL_0 + \theta_1 I_1 + \theta_2 I_2$	3.7	10.7	12.3		−25.4	**
Reduced 1 Eq. 1	I_1: Het-LOF or Hom-Ref	$CL = CL_0 + \theta_1 I_1$	3.7	11.9			−24	NS[a]
Eq. 2	n = 0, 1, 2	$CL = CL_0 + \theta_1 \cdot n$	4.84	4.06			−20	*,[a]
Eq. 3		$CL = CL_0 + \theta_1^n$	6.2	2.5			−12.9	*,[a]
Eq. 4		$CL = CL_0 + \theta_1 \cdot \sqrt{n}$	3.89	6.1			−24.8	NS[a]
*CYP3A4*1B*								
Rich: Eq. 1	I_1: Het-LOF, I_2: Hom-Ref	$CL = CL_0 + \theta_1 I_1 + \theta_2 I_2$	5.1	10.3	11.9		−10.4	**
Reduced 1 Eq. 1	I_1: Het-LOF or Hom-Ref	$CL = CL_0 + \theta_1 I_1$	5.1	11.7			−9.5	NS[a]
Eq. 2	n = 0, 1, 2	$CL = CL_0 + \theta_1 \cdot n$	6	3.6			−9.0	NS[a]
Eq. 3		$CL = CL_0 + \theta_1^n$	6.4	2.3			−7.0	NS[a]
Eq. 4		$CL = CL_0 + \theta_1 \cdot \sqrt{n}$	5.2	4.8			−10.4	NS[a]
CYP3A5								
Rich: Eq. 1	I_1: Het-LOF, I_2: Hom-LOF	$CL = CL_0 + \theta_1 I_1 + \theta_2 I_2$	4.4	10.3	11.8		−8.4	**
Reduced 1 Eq. 1	I_1: Het-LOF or Hom-LOF	$CL = CL_0 + \theta_1 I_1$	4.4	11.5			−7.4	NS[a]
Eq. 2	n = 0, 1, 2	$CL = CL_0 + \theta_1 \cdot n$	6.15	2.96			−6.4	NS[a]
Eq. 3		$CL = CL_0 + \theta_1^n$	7.0	2.22			−4.8	*,[a]
Eq. 4		$CL = CL_0 + \theta_1 \cdot \sqrt{n}$	4.7	5.2			−8.1	NS[a]

Table 2 Continued on next page

Table 2 (Continued)

Step 3	Gene–gene interaction analysis	*CYP* contribution	CL_0	θ_1	θ_2	θ_3	θ_4	ΔOF°
Eq. 5[b]	*CYP2B6/CYP2A6*	$CL = CL_0 + \theta_1 I_{01} + \theta_2 I_{02} + \theta_1 I_{10} + \theta_2 I_{11} + \theta_3 I_{12} + \theta_1 I_{20} + \theta_2 I_{21} + \theta_3 I_{22} + \theta_1 I_{30} + \theta_2 I_{31} + \theta_3 I_{32}$	1.8	2.59 7.42 9.74 14.6	3.15 11.6 11.9 19.6	10.9 14.3 18.8		−16
Eq. 6	*CYP2B6/CYP2A6*	*CYP2B6* $CYP2A6 \cdot \sqrt{q}$	1.75	8.15 0.95	2.42	9.21 3.44	19.3 2.49	−17
Eq. 7	*CYP2B6/CYP2A6*	*CYP2B6* $CYP2A6 \cdot \sqrt{q}$		2.02 1.21	7.7	9.45	13.6	−16
Eq. 8	*CYP2B6/CYP2A6*	$CYP2B6 \cdot \sqrt{p}$ $CYP2A6 \cdot \sqrt{q}$	1.5	7.7 1.2				−12
Eq. 5[b]	*CYP2B6/CYP3A4* _rs4646437	$CL = CL_0 + \theta_1 I_{01} + \theta_2 I_{02} + \theta_1 I_{10} + \theta_2 I_{11} + \theta_3 I_{12} + \theta_1 I_{20} + \theta_2 I_{21} + \theta_3 I_{22} + \theta_1 I_{30} + \theta_2 I_{31} + \theta_3 I_{32}$	1.66	2.46 10.5 13.3 16.3	3.9 11 12.3 17.0	10.6 13.5 19.3		−25
Eq. 6	*CYP2B6/CYP3A4* _rs4646437	*CYP2B6* $CYP3A4\_rs \cdot \sqrt{r}$		1.6 1.4	10.7 0.1	11.2 1.6	15.3 2.6	−22
Eq. 7	*CYP2B6/CYP3A4* _rs4646437	*CYP2B6* $CYP3A4\_rs \cdot \sqrt{r}$		1.62 1.36	8.99	11.5	16.9	−22
Eq. 8	*CYP2B6/CYP3A4* _rs4646437	$CYP2B6 \cdot \sqrt{p}$ $CYP3A4\_rs \cdot \sqrt{r}$	1.62	7.3 1.34				−18
Eq. 5[b]	*CYP2B6/CYP3A4*1B*	$CL = CL_0 + \theta_1 I_{01} + \theta_2 I_{02} + \theta_1 I_{10} + \theta_2 I_{11} + \theta_3 I_{12} + \theta_1 I_{20} + \theta_2 I_{21} + \theta_3 I_{22} + \theta_1 I_{30} + \theta_2 I_{31} + \theta_3 I_{32}$	1.69	2.33 10.6 13.4 16.4	3.23 10.3 11.7 17.2	10.8 13.6 19.4		−14
Eq. 6	*CYP2B6/CYP3A4*1B*	*CYP2B6* $CYP3A4*1B \cdot \sqrt{s}$		1.64 1.1	10.7 0.4	11.2 1.2	15.3 2.7	−13
Eq. 7	*CYP2B6/CYP3A4*1B*	*CYP2B6* $CYP3A4*1B \cdot \sqrt{s}$		1.62 1.1	8.99	11.5	16.9	−12
Eq. 8	*CYP2B6/CYP3A4*1B*	$CYP2B6 \cdot \sqrt{p}$ $CYP3A4*1B \cdot \sqrt{s}$	1.65	7.6 1.1				−9
Eq. 5[b]	*CYP2B6/CYP3A5*	$CL = CL_0 + \theta_1 I_{01} + \theta_2 I_{02} + \theta_1 I_{10} + \theta_2 I_{11} + \theta_3 I_{12} + \theta_1 I_{20} + \theta_2 I_{21} + \theta_3 I_{22} + \theta_1 I_{30} + \theta_2 I_{31} + \theta_3 I_{32}$	1.45	2.22 6.0 13.4 16.5	3.25 11.6 11.9 17.0	10.6 13.6 19.2		−16
Eq. 6	*CYP2B6/CYP3A5*	*CYP2B6* $CYP3A5 \cdot \sqrt{t}$		1.38 1.1	10.7 0.1	11 1.8	15 2.4	−14
Eq. 7	*CYP2B6/CYP3A5*	*CYP2B6* $CYP3A5 \cdot \sqrt{t}$		1.4 1.22	9.1	11.7	17.1	−13
Eq. 8	*CYP2B6/CYP3A5*	$CYP2B6 \cdot \sqrt{p}$ $CYP3A5 \cdot \sqrt{t}$	1.4	7.6 1.22				−10

ΔOF, difference in the objective function, compared to the final model structural model; ΔOF°, difference in the objective function compared to the model including CYP2B6 solely; ARV, antiretroviral medication; NRTI, non-nucleoside/nucleotide reverse transcriptase inhibitors; *n*, *p*, *q*, *r*, *s*, numbers of functional alleles (0 = Hom-LOF, 1 = Het-LOF, 2 = Hom-Ref, 3 = Het-GOF); NS, not statistically significant; PI, protease inhibitors.

[a]Differences in objective function compared to the rich model (Eq. 1). [b]I_{xy} represents the number of functional alleles for the x/y cytochromes. For clarity, θ have been numbered from 1 to 4. **P* < 0.05, ***P* < 0.01.

for **1B*, −18 for rs_4646437). No factor accounting for more than an additive interaction was observed (ΔOF = 0.0).

The joint assignment of *CYP2B6* and *CYP3A5* on CL also improved the fit as compared to *CYP2B6* alone (Eq. 5, ΔOF = −16). The successive nesting of models using functional scores successfully followed the paradigm previously described for other *CYPs*. The influence of *CYP3A5* on *CYP2B6* was appropriately described using Eqs. 6 or 7 (ΔOF >−13) or as a square root additive model (Eq. 8, ΔOF >−10). Again, an interaction between the two genes was not significant (ΔOF = 0.0).

A final joint model characterizing the cumulative influence of all the genetic variants on EFV CL was tested on the basis of the two-by-two combinations of genetic effects. The richest model that integrated the effects of all CYP alleles, using a generalization of Eqs. 6 and 7, improved the fit (ΔOF >−29 as compared to *CYP2B6* alone), but the impact of *CYP3A4*1B* and *CYP3A5* did not remain significant (ΔOF = 0.0 as compared to the rich model without those two alleles). The final model employed a single parameter estimate to quantify the influence of each of the alleles, *CYP2B6*, *CYP2A6*, and *CYP3A4* _rs4646437, using additive square root functions, which fitted the data appropriately (Eq. 10, ΔOF = −24). All models specified with proportional rather than additive effects gave very similar results (ΔOF <−0.5). In addition to genetic influences on CL, the influence of body weight remained the only significant demographic covariate (ΔOF = 19.2), while any ethnic influence vanished completely.

The final model estimated an average CL of 1.3 l/h in individuals carrying Hom-LOF *CYP2B6*, *CYP2A6*, and *CYP3A4_*rs4646437, which increased by 7.3, 0.7, and 1.03 l/h for the first active allele of those genes, respectively, and by another 0.41 times those factors for the second allele (where $0.41 = \sqrt{2} - 1$), with an additional 70% increase in CL associated with doubling of body weight. A summary of the model building procedure is presented in **Table 2** and the final population estimates in **Table 3.** A plot of the model-predicted concentration profile stratified by *CYP2B6* is shown in **Figure 2**. **Figure 3** represents the individual *post hoc* CL estimates with population average predictions for the different allelic combinations encountered.

Based on our final model, simulations show that, with the standard regimen of 600 mg of EFV daily, average trough concentrations are 1.19 (90% prediction interval: 0.6–2.35), 1.6 (0.8–2.5), and 8.1 mg/l (4.5–14.5) in *CYP2B6* Hom-Ref, Het-LOF, and Hom-LOF carriers, respectively, and 0.5 mg/l (0.2–1.0) in *CYP2B6* Het-GOF carriers. Taking into account the interindividual variability leads to the suggestion that most *CYP2B6* Hom-LOF individuals will exhibit concentrations exceeding the 1–4 mg/l range that is generally considered acceptable, and most individuals carrying a Het-GOF will have concentrations <1 mg/l. Predicted concentrations of 2.7 mg/ml (1.5–4.9) would be expected at a dosage regimen of 200 mg/day in *CYP2B6* Hom-LOF carriers. However, individuals having Hom-LOF of *CYP2A6*/*CYP3A4_* rs3434367 and *CYP2B6* will be exposed to considerably higher drug levels, yielding average predicted concentrations of 6.2 mg/ml (3.5–11.0) with a regimen of 200 mg/day.

Table 3 Final population pharmacokinetic parameter estimates of EFV

	Population mean		Interindividual variability	
Parameter	Estimate	SE (%)[a]	Estimate (%)[b]	SE (%)[c]
CL/F (l/h)[d]	1.3	18	27.8	43.8
θ_{2B6}[e]	7.3	7		
θ_{2A6}[e]	0.7	36		
$\theta_{3A4\_rs4646437}$[e]	1.03	27		
θ_{BW}[f]	0.7	42		
V_d/F (l)	332	16		
k_a (h^{-1})	0.6	38		
σ (CV%)[g]	30.8	44.2		

CL/F, mean apparent clearance; F, bioavailability; k_a, mean absorption rate constant; V_d/F, mean apparent volume of distribution.

[a]SEs of the estimates (SEE), defined as SE/estimate and expressed as percentages. [b]Estimate of variability is expressed as coefficient of variation (CV) (%). [c]SEs of the CV, taken as $\sqrt{(SE/estimate)}$ and expressed as percentage. [d]CL value in patients with Hom-LOF for *CYP2B6*, *CYP2A6,* and *CYP3A4_* rs4646437. [e]Contribution of *CYP2B6*, *CYP2A6,* and *CYP3A4* _rs4646437 to efavirenz (EFV) CL multiplied by $\sqrt{n}$ where $n = 0, 1, 2, 3$ for *CYP2A6,* $n = 0, 1, 2$ for *CYP2A6* and *CYP3A4* _rs4646437 (see text). [f]Relative influence of body weight on EFV clearance (see text). [g]Residual intrapatient variability, expressed as a CV (%).

DISCUSSION

This study enabled us to characterize and quantify for the first time the conjugated effects of major and minor metabolic pathways and their genetic variants on EFV pharmacokinetics in a population of HIV-1-infected patients. The average CL and variability of EFV plasma concentrations are in the range of values reported previously.[1–3] Among nongenetic covariates, we observed an impact of body weight on CL, as reported by others.[16–20] In our study, no differences could be observed between data from male and female subjects, but conflicting results exist in the literature.[20–23] The well-known influence of black ethnicity, which has been associated with *CYP2B6* and *CYP3A4* heterogeneity,[24,25] could

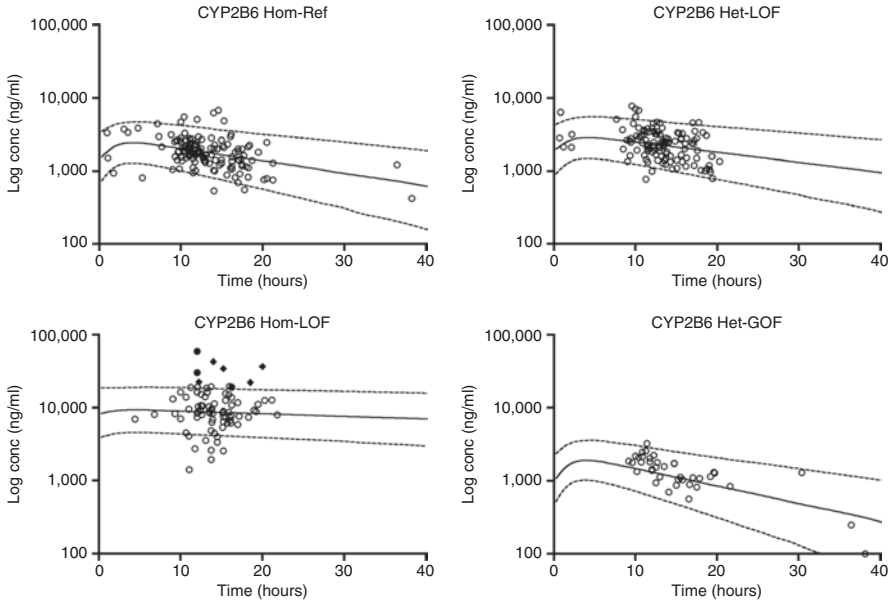

Figure 2 Efavirenz (EFV) plasma concentrations ($n = 393$) in 169 type I human immunodeficiency virus (HIV-1) individuals (open circles) in relation to *CYP2B6* polymorphism. Population predictions of the corresponding genotype are represented by black lines, and the 90% prediction interval is shown by gray dotted lines. Left lower panel: filled circles represent concentrations in individuals who are Hom-LOF for CYP2B6, CYP2A6, and CYP3A4_rs46464337, and filled diamonds represent concentrations in individuals who are Hom-LOF for CYP2B6 and Het-LOF for CYP3A4_rs4646437. Het, heterozygous; Hom, homozygous; LOF, loss of function.

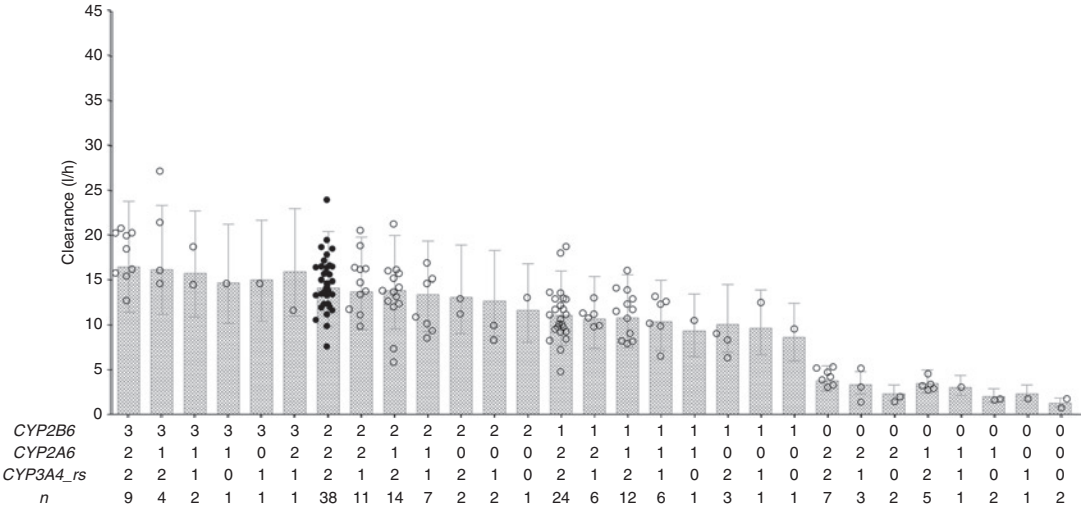

Figure 3 Individual predicted Bayesian clearances (open circles, the black circles correspond to individuals with reference alleles for all three cytochromes) and average predicted clearances (bars) with 90% population prediction interval for each *CYP2B6, CYP2A6*, and *CYP3A4* _rs4646437 allelic combination (according to score A categorization: 0 Hom-LOF/DOF, 1 Het-LOF/DOF, 2 Hom-Ref, and 3 Het-GOF; see text); *n* = number of individuals carrying the allelic combination (*n* = 0 for the eight combinations that are not represented). DOF, diminished function; GOF, gain of function; Het, heterozygous; Hom, homozygous; LOF, loss of function; Ref, reference allele.

be mostly explained by the joint influence of *CYP2B6*, *CYP2A6*, and *CYP3A4* variations, whereas its influence remained discernible when these *CYP* alleles were considered separately. Given the limited number of Hispanic ($n = 6$) and Asian ($n = 5$) individuals in our study, no clear effect on EFV levels could be attributed to these ethnic groups, although an influence has been reported previously.[1,3,10,20] Among genetic covariates, *CYP2B6* allelic variation accounted for most of the interindividual differences in EFV CL. The *CYP2A6* and *CYP3A4/A5* accessory pathways appeared to influence EFV elimination independent of *CYP2B6*. Among those, the overall impact of *CYP3A4_* rs4646437 was the largest; it accounted for 6% of CL variability. The unexpected lower CL in *CYP3A5* Hom-Ref carriers as compared to Hom-LOF carriers can be explained by the linked Hom/Het-LOF of *CYP2B6* and *CYP3A4_* rs4343437 observed in most individuals. This hypothesis is further confirmed by the lack of effect of *CYP3A5* when the influence of all cytochromes is assessed in a joint analysis. When information on *CYP2B6* was included along with *CYP2A6* and *CYP3A4* in joint analyses, an additive effect of accessory pathways was still present, both on fully functional *CYP2B6* and in the presence of reduced-function or gain-of-function alleles. However, the compensation ensured by these *CYP* alleles was small (1–2 l/h) and therefore more discernible on *CYP2B6* Hom-LOF. An evaluation of *CYP* genetic variations according to an activity score, as recently proposed by Gaedigk *et al.*[26] for *CYP2D6*, enabled us to quantify genetic influences of all alleles based on the same paradigm. A model of remarkable parsimony, which included only one parameter per allele and required no interaction term, could capture the nonlinear relationship between CL and the different genotypic groups. The use of such square root relationships in gene–dose effects was not described for other *CYP* isoenzymes, in particular for the noninducible CYP2D6,[24] which tend more toward linear effects.[27] This phenomenon suggests adaptive mechanisms that might be explained by the upregulation of extensive metabolizer alleles in response to an increase in concentration, possibly through the activation of nuclear receptors.[28,29] It is noteworthy that a similar pattern has already been reported for *CYP2B6*, not only in relation to EFV[30] but also in relation to S-methadone.[31] The use of different functional score classifications to implement partial activity levels failed to improve the model when compared with the traditional classification. The mechanisms by which allelic variants express a loss/diminished function could therefore not be translated in a "semiquantitative gene–dose system," as described by Steimer *et al.*[32] for amitriptyline and nortriptyline.

The cumulative influence of *CYP2B6*, *CYP2A6*, and *CYP3A4* _rs4646437 allelic variants on CL implies a critical 90% decrease of EFV elimination in triple-Hom-LOF individuals, in whom CL appeared reduced to 1.3 l/h as compared to the estimated 12.9 l/h in triple-Hom-Ref individuals. Of note, the only individual who exhibited extremely high EFV concentrations[33] was found to be triple-Hom-LOF; this emphasizes the importance of accessory pathways in EFV elimination. The interindividual variability in CL dropped from 65 to 27% in the final model, with *CYP2B6* genetic variants accounting for 31% and another 7% being explained by *CYP2A6, CYP3A4_* rs4646437, and body weight variations. The remaining variability might be attributed to adherence issues[34] or to variation in uridine-glucuronyltransferase metabolic pathways.

In conclusion, functional alleles of *CYP2B6* accounted for the major part of EFV interindividual variability. Genetic variations in EFV accessory metabolic pathways demonstrated their importance in EFV pharmacokinetics in addition to *CYP2B6*, in particular in individuals with limited *CYP2B6* function. The dosage is required to be reduced to 200 mg/day of EFV in individuals with impaired *CYP2B6* function so as to ensure drug levels within the therapeutic range. The expression of pharmacogenetic influences on EFV elimination could be characterized using a single common paradigm across all *CYPs*. This paradigm involves the addition of the contribution of each enzymatic pathway proportional to the square root of the number of

Table 4 Demographic and genetic characteristic of the population study

Characteristics	Value	% Of study population
Sex (no.)		
Men	124	73
Women	45	27
Age (years)		
Median (range)	47 (30–73)	—
Body weight (kg)		
Median (range)	77.5 (44–101)	—
Height (cm)		
Median (range)	179 (153–193)	—
Ethnicity (no.)		
White	142	83
Black	16	10
Hispanic	6	4
Asian	5	3
PIs (no.)		
Ritonavir	20	13
Saquinavir	4	3
Amprenavir	0	0
Lopinavir	15	9
Atazanavir	18	11
NRTIs (no.)		
Lamivudine	116	72
Stavudine	15	9
Didanosine	29	18
Abacavir	0	0
Tenofovir	29	18
Emtricitabine	4	2
Zidovudine	81	50
Entry inhibitors (no.)		
Enfuvirtide	4	2
CYP P450 inducers (no.)	2	1
CYP P450 inhibitors (no.)	4	2
CYP2B6 *genetic polymorphism (no.)*		
Hom-Ref	75	44
Het-LOF	53	33
Hom-LOF	23	14
Het-GOF	16	9
Het-LOF/Het-GOF[a]	2	1
CYP2A6 *genetic polymorphism (no.)*		
Hom-Ref	99	61
Het-LOF	55	30
Hom-LOF	13	8
Het-GOF	2	1
CYP3A4 *genetic polymorphism (no.)*		
Hom-Ref	138	82
Het-LOF *1B	24	14
Hom-LOF *1B	7	4
CYP3A4 *genetic polymorphism (no.)*		
Hom-Ref	118	70
Het-LOF_rs4646437	41	24
Hom-LOF_rs4646437	10	6
CY3A5 *genetic polymorphism (no.)*		
Hom-Ref	5	3
Het-LOF	30	18
Hom-LOF	134	79

GOF, gain of function; Het, heterozygous; Hom, homozygous; LOF, loss of function; NNRTIs, non-nucleoside reverse transcriptase inhibitors; NRTIs, nucleoside reverse transcriptase inhibitors; PIs, protease inhibitors; Ref, reference allele.

[a]Two individuals are LOF/GOF for *CYP2B6* and were considered to be Het-LOF.

functional alleles. Such a model appropriately predicts average CL for various allele combinations, and its mechanistic explanation warrants further investigation.

METHODS

Study population. A total of 169 HIV-1-infected individuals from the Swiss HIV Cohort Study were characterized with respect to *CYP2B6*, *CYP2A6*, and *CYP3A4/A5* genetic variations. EFV drug levels were measured during routine therapeutic drug monitoring according to local treatment guidelines. All participants gave their informed consent for genetic testing. A median of 1 concentration sample per individual (range 1–23) was collected between 0.6 and 38 h after the last drug intake under steady-state conditions.

Method of analysis. Blood samples (5 ml) were collected into lithium heparin or EDTA-K Monovette syringes (Sarstedt, Nümbrecht, Germany). Plasma was isolated by centrifugation, inactivated for virus at 60 °C for 60 min, and stored at −20 °C until analysis. Plasma EFV levels were determined by liquid chromatography coupled with tandem mass spectrometry in accordance with a validated method. The calibration curves were found to be linear up to 10 μg/ml, with a lower limit of quantification of 0.1 μg/ml.

Nomenclature and functional score. Alleles are designated in concordance with the *CYP* Allele Nomenclature Committee (http://www.cypalleles.ki.se). Proposed functional consequences for EFV pharmacokinetics have been reported for *CYP2B6*,[15] *CYP2A6*, and *CYP3A4/A5*.[35] The study participants were categorized into genotypic groups on the basis of the number of functional alleles (**Table 1**). The simplest scoring scheme (score A) assigned values of 2, 0, and 1 to the fully functional reference (Hom-Ref), homozygous (Hom-LOF), and heterozygous (Het-LOF) diminished/loss of function, respectively, and a value of 3 to *CYP2B6* gain-of-function alleles (Het-GOF). Two individuals having a single-gene duplication of *CYP2A6* were assimilated to the Hom-Ref group because they constituted only a small number. The classification was refined to distinguish between individuals with *CYP2B6* and *CYP2A6* loss/diminished function alleles, to reflect the predicted level of activity from *in vitro* studies (**Table 1**, scores B/C).

Pharmacokinetic structural model. EFV pharmacokinetics were characterized using a one-compartment model, as assessed previously.[2] Since EFV is administered only orally, CL and *V* represent apparent values.

Covariate model. The analyses of the covariate effects on CL were divided into three main sections for assessing: (i) the influence of demographic variables and concomitant medications, (ii) the impact of *CYP2B6*, *CYP2A6, CYP3A4*, and *CYP3A5* alleles based on univariate analyses, and (iii) the joint effect of *CYP2B6* with *CYP2A6, CYP3A4*, and *CYP3A5* alleles in multivariate analyses.

Demographic analyses. The typical value of CL was modeled to depend linearly on a covariate X (body weight, centered on the mean; categorical covariates coded as 0/1) as shown in the equation: $CL = \theta_a \cdot (1 + \theta_b \cdot X)$, where θ_a is the average estimate and θ_b is the relative deviation (positive or negative) from average attributed to the covariate X. The available demographic covariates were sex, ethnicity, age, body weight, and height. Only a few co-medications were recorded, and these were principally other antiretroviral drugs and known *CYP* inducers or inhibitors (**Table 4**).

Univariate genotype. In these analyses, each genotype was entered solo into the model. Several models relating CL with functional scores were tested using different methods (**Figure 1**) and compared with the richest possible model, which assigned a separate fixed effect to each score level as follows:

$$CL = CL_0 + \theta_1 I_1 + \theta_2 I_2 + \theta_3 I_3 \quad (1)$$

$$CL = CL_0 \cdot (1 + \theta_1 I_1) \cdot (1 + \theta_2 I_2) \cdot (1 + \theta_3 I_3) \quad (1a)$$

where CL_0 is the typical value of CL in Hom-LOF individuals (Hom-Ref for *CYP3A5*), I_i is an indicator variable that takes the value of 1 if an individual carries the ith genotypic score (i.e., I_1: Het-LOF, I_2: Hom-Ref, and I_3: Het-GOF) and 0 otherwise, and θ_i is the absolute or fractional (Eqs. 1/1a) change in CL relative to the Hom-LOF group. The impact of functional alleles on EFV CL was further explored to distinguish the difference between the genotypic groups, using two reduced models in which the same genotyping group was assigned to Hom-Ref and Het-LOF or to Hom-Ref and Het-GOF carriers (reduced 1 and 2, **Figure 1**). Competing models attempted to account for gene effect as a function of the number of functional alleles (**Table 1**, score A), using linear and power relationships with either additive or proportional (data not shown) impact, using the following models:

$$CL = CL_0 + \theta_1 \cdot n \quad (2)$$

$$CL = CL_0 + \theta_1^n \quad (3)$$

$$CL = CL_0 + \theta_1 \sqrt{n} \quad (4)$$

where $n = 1$, 2, or 3 represents the functional score, and θ_1 the average contribution per active allele above that of Hom-LOF CL (CL_0). The alternative activity scores B/C for *CYP2B6* and *CYP2A6* were explored using parameter models in Eqs. 2, 3, or 4 and compared with an extension of the rich model (Eq. 1).

Gene–gene interaction analyses. The joint influence of functional alleles on EFV CL was first tested using pairwise conjunction of *CYP2B6* with each of the other *CYP* alleles, so as to finally build up a model including all genetic variants that have an influence. The investigation of the joint influence of *CYP2B6* and *CYP2A6* alleles is shown as an example. The richest model that served as reference for the evaluation of reduced competing models was:

$$CL = CL_0 + \theta_{01} I_{01} + \theta_{02} I_{02} + \theta_{10} I_{10} + \theta_{11} I_{11} + \theta_{12} I_{12} + \theta_{20} I_{20} + \theta_{21} I_{21} + \theta_{22} I_{22} + \theta_{30} I_{30} + \theta_{31} I_{31} + \theta_{32} I_{32} \quad (5)$$

where CL_0 is the Hom-LOF CL for both genes and I_{ij} is an indicator variable that takes the value of 1 for the *CYP2B6* ith/*CYP2A6* jth genotype carrier and is 0 otherwise, and each θ_{ij} estimates the absolute change in CL among the different genotypic groups. The same model was parameterized for relative changes (data not shown). The following competing models were evaluated:

$$CL = CL_0 + \theta_{0\_0} \cdot \sqrt{q} + \left(\theta_1 I_1 + \theta_{1\_1} \cdot \sqrt{q}\right) + \left(\theta_2 I_2 + \theta_{2\_2} \cdot \sqrt{q}\right) + \left(\theta_3 I_3 + \theta_{3\_3} \cdot \sqrt{q}\right) \quad (6)$$

$$CL = CL_0 + (\theta_1 I_1 + \theta_2 I_2 + \theta_3 I_3) + \theta_4 \cdot \sqrt{q} \quad (7)$$

$$CL = CL_0 + \theta_1 \cdot \sqrt{p} + \theta_2 \cdot \sqrt{q} \quad (8)$$

$$CL = CL_0 + \theta_1 \cdot \sqrt{p} + \theta_2 \cdot \sqrt{q} + \theta_3 \cdot (p \cdot q) \quad (9)$$

where p indicates the functional score for *CYP2B6* and q the score for *CYP2A6*. In Eq. 6, the contribution of *CYP2A6* (θ_{0_0},...,θ_{3_3}) is investigated on *CYP2B6* stratified by genotypic groups; in Eq. 7 the influence of *CYP2A6* is characterized using a single fixed-effect parameter θ_4 across all *CYP2B6* genotypes; and in Eq. 8 square root functions are integrated for both genes. Finally, an interaction term was allowed so as to further check for some nonadditive interaction between *CYP* variants (Eq. 9). All these models used either additive or proportional (data not shown) effects.

All significant allelic groups were integrated into a final model, wherein the contribution of each genotypic group was estimated using a generalization of Eqs. 6–8 to be finally formulated using the following additive or proportional (data not shown) relationships:

$$CL = CL_0 + \theta_1 \cdot \sqrt{p} + \theta_2 \cdot \sqrt{q} + \theta_3 \cdot \sqrt{r} + \theta_4 \cdot \sqrt{s} + \theta_5 \cdot \sqrt{t} \quad (10)$$

where CL_0 is the Hom-LOF CL for all genes and θ_i values estimate the absolute or fractional change in CL as a function of score A for different combinations of *CYP2B6* (p), *CYP2A6* (q), *CYP3A4* _rs4646437 (r), *CYP3A4*1B* (s), and *CYP3A5* (t) alleles.

Variance model. The individual CL values were modeled assuming a log-normal distribution (mean zero and variance Ω). A proportional error model (mean zero and variance σ^2) was used the description of intraindividual variability.

Parameter estimation and selection. NONMEM (version VI; NM-TRAN, version II, GloboMax, Hanover, MD) was used with FOCE INTERACTION to fit the models.[36] As goodness-of-fit statistics, NONMEM uses the objective function, which is approximately equal to minus twice the logarithm of the maximum likelihood. The likelihood ratio test, based on the reduction in objective function (ΔOF), was used to carry out comparisons between any two models. A ΔOF (−2 log likelihood, approximate χ^2 distribution) of 3.84, 5.99, and 7.81 points for 1, 2, or 3 additional parameters, respectively, was used for determining statistical significance ($P < 0.05$) of the difference between two models. The reliability of the results was checked on diagnostic goodness-of-fit plots, along with the measure of the SEs. The identification of potential outlier values resulting from compliance issues or inadequacy in self-reporting information was explored using a sensitivity analysis. One individual had an EFV concentration of 59,400 ng/ml, and this value was at first excluded to prevent single outlier effect but was integrated at the end. Except for this, all data were considered reliable. Simulations based on the final pharmacokinetic estimates were performed with NONMEM using 1,000 individuals to calculate the 90% prediction intervals. The concentrations encompassing the range from 5th to 95th percentile at each time point were retrieved in order to construct the intervals. Further simulations were performed for a series of genotype combinations in 1,000 individuals undergoing various dosage regimens (200, 400, 600, and 800 mg), so as to suggest the dosages that would ensure trough levels falling within the 1–4 mg/ml therapeutic interval in 90% of the individuals. The figures were generated using GraphPad Prism (version 4.00 for Windows; GraphPad Software, San Diego, CA, http://www.graphpad.com).

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M.A.-A. contributed to the pharmacokinetic modeling and J.D.I. to the genetic testing.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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UGT Genotype May Contribute to Adverse Events Following Medication With Mycophenolate Mofetil in Pediatric Kidney Transplant Recipients

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Leukopenia and diarrhea are the predominant adverse events associated with mycophenolate mofetil (MMF), leading to dose reduction or discontinuation in children. Polymorphisms of the drug's main metabolizing enzyme, uridine diphosphate–glucuronosyl transferase (UGT), confer alteration in drug exposure. We studied the incidence of these polymorphisms in pediatric kidney transplant recipients experiencing MMF-associated leukopenia and diarrhea. UGT genotypes of 16 affected children who recovered after MMF dose reduction or discontinuation were compared with those of 22 children who tolerated the drug at standard doses. DNA was extracted and sequenced using standard procedures to detect polymorphisms associated with increased (e.g., *UGT1A9* –331T>C) or decreased drug exposure. All three patients who were homozygous for *UGT1A9* –331T>C developed leukopenia, and heterozygotes also had significantly more toxicity ($P = 0.04$). A weaker association ($P = 0.08$) existed in *UGT2B7* –900G>A carriers. Our data implicate UGT polymorphisms associated with altered drug exposure as potential predictors of MMF adverse events.

Mycophenolate mofetil (MMF) is an immunosuppressive prodrug commonly used in organ (especially kidney) transplantation.[1–3] After oral administration, MMF undergoes rapid and almost complete hydrolysis to its active form, mycophenolic acid (MPA). MPA overexposure may be associated with adverse events (AEs).[2] Major drug-related AEs reported in kidney transplant recipients are gastrointestinal (GI) complications (e.g., diarrhea) and leukopenia. In a European multicenter trial involving 325 adult recipients, those taking MMF as part of their immunosuppressive therapy experienced 40% more GI AEs than subjects taking placebo, and leukopenia was noted more often in the MMF treatment groups than in the placebo group.[3]

These AEs have a negative impact on the quality of life of patients, and they may necessitate dose reduction or discontinuation of MMF therapy. In a review carried out by Hardinger and colleagues[4] of 6,400 adult kidney transplant recipients on MMF therapy, GI complications were identified in 1,753 patients (27.3%), and MMF was discontinued in 1,117 patients (17.5%) in the first post-transplant year. Additionally, the frequency of MMF discontinuation was significantly higher in patients with GI complications (21.3%) than in those without such complications (16.0%) (odds ratio 1.33; $P < 0.0001$).[4]

Such MMF dose reductions put transplant recipients at an increased risk of rejection and, consequently, possible graft loss. A retrospective review of kidney transplant recipients by Knoll and colleagues[5] assessed dose reduction of MMF and subsequent risk of acute graft rejection. In their cohort of 213 adult kidney transplant recipients, 126 (59%) required a total of 176 dose reductions due to MPA-associated AEs. The relative risk of rejection increased by 4% for each week that the MMF dose was reduced below full dose ($P = 0.02$).[5]

Pediatric kidney transplant recipients on MMF therapy have an even higher likelihood of experiencing drug-related AEs than adults do. In a study comparing findings from pediatric kidney

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transplant patients ($n = 22$) with adult patients ($n = 37$), all of whom started with the recommended dose of MMF, the incidence of GI symptoms was significantly higher in the pediatric patient population than in the adults (54.5% vs. 21.6%; $P = 0.02$).[6] Moreover, among the subjects who experienced significant GI AEs, the need for MMF discontinuation was reported in 75% of the pediatric subjects vs. 50% of the adults.

We observed similar responses to MMF at our pediatric center.[7] A retrospective chart review evaluated the clinical variability of MMF in 31 pediatric patients with newly transplanted kidneys. All subjects began MMF therapy at the recommended dose based on body surface area. Clinically significant AEs leading to an MMF dose decrease or discontinuation occurred in 15 (48%) of the 31 subjects (GI: 6; bone marrow suppression: 8; infection: 2; other: 2).

In kidney transplantation settings, relationships have been identified between MPA exposure, measured as area under the concentration–time curve (AUC), and drug efficacy as well as toxicity. A target range for the MPA AUC of 30–60 mg·h/l has been suggested, given that individuals with AUCs >60 mg·h/l are more likely to experience drug-related AEs,[8] whereas individuals with AUCs <30 mg·h/l are at higher risk of kidney allograft rejection.[9,10] While these data were generated in adult patients, similar relationships have been suggested in children.[11]

Of note, large interindividual variability in MPA exposure exists. Factors contributing to this variability continue to be identified; they include genetic polymorphisms in the uridine diphosphate–glucuronosyl transferases (UGTs), the enzyme family responsible for MPA glucuronidation.[12,13] MPA is glucuronidated to MPA glucuronide (MPAG), the acyl glucuronide of MPA (AcMPAG). Both UGT1A8 and UGT1A9 are involved in MPAG formation,[14] while the polymorphic UGT2B7 enzyme facilitates the metabolism of MPA to the AcMPAG.[15]

UGT1A9 is the enzyme that is primarily responsible for the glucuronidation of MPA to MPAG. UGT1A9 is expressed mainly in the liver[14] but also in the GI tract[16] and kidneys.[17] Two known single-nucleotide polymorphisms (SNPs) found in the promoter region of the gene, *UGT1A9* –275T>A and –2152C>T, result in higher glucuronidation of MPA to MPAG. This, in turn, results in a lower MPA AUC and therefore lower MPA exposure. In human liver microsomes containing one or both of these SNPs, higher glucuronidation of MPA was observed in comparison with liver microsomes not possessing these SNPs.[9,18] In a clinical study of 95 kidney transplant recipients, individuals with one or both of these SNPs had significantly lower MPA exposure and higher estimated MPA

Table 1 Clinical characteristics of study subjects

Characteristics	Control group	Adverse events group	*P* value
Number of subjects	22 (58%)	16 (42%)	
Age at transplantation (years; 50% percentile (25%, 75%))	13.0 (4.9, 16.0)	14.0 (6.3, 15.8)	0.71
Gender			
Male	13 (59%)	12 (75%)	0.49
Female	9 (41%)	4 (25%)	
Calcineurin inhibitor			
Tacrolimus	16 (73%)	12 (75%)	1.0
Cyclosporine	2 (9%)	1 (6%)	
None	4 (18%)	3 (19%)	
Corticosteroid therapy			
Yes	17 (77%)	13 (81%)	1.0
No	5 (23%)	3 (19%)	
Transplant type			
Living donor	14 (64%)	9 (56%)	0.74
Deceased donor	8 (36%)	7 (44%)	
Number of transplants			
First transplant	20 (91%)	13 (81%)	0.63
Retransplant	2 (9%)	3 (19%)	

P values were calculated from exact χ^2-test for categorical variables and Wilcoxon–Mann–Whitney test for continuous variable.

Table 2 Genotype distributions of the uridine diphosphate–glucuronosyl transferase (UGT) enzymes of all subjects ($n = 38$)

	Genotype		Control group	Adverse event group	*P* value
UGT1A8 830 (**3*)	G/G	Major homozygotes	20 (91%)	15 (94%)	1.0
	G/A	Heterozygotes	2 (9%)	1 (6%)	
UGT1A9 –275	T/T	Major homozygotes	18 (82%)	12 (75%)	0.70
	T/A	Heterozygotes	4 (18%)	4 (25%)	
UGT1A9 –331	T/T	Major homozygotes	14 (64%)	6 (38%)	0.04
	T/C	Heterozygotes	8 (36%)	7 (44%)	
	C/C	Minor homozygotes	0 (0%)	3 (19%)	
UGT1A9 –2152	C/C	Major homozygotes	18 (82%)	13 (81%)	1.0
	C/T	Heterozygotes	4 (18%)	3 (19%)	
UGT1A9 98 (**3*)	T/T	—	22 (100%)	16 (100%)	—
UGT2B7 –900	A/A	Major homozygotes	8 (36%)	2 (13%)	0.08
	G/A	Heterozygotes	12 (55%)	10 (63%)	
	G/G	Minor homozygotes	2 (9%)	4 (25%)	

P values were computed using the Cochran–Armitage trend test.

clearance as compared to the "wild-type" recipients (clearance: 40.3 ± 20.1 l/h vs. 19.5 ± 10.7 l/h, exposure: 31.7 ± 17.6 mg·h/l vs. 63.6 ± 30.9 mg·h/l, *P* for both = 0.009).[9] In contrast, two other strongly linked SNPs found in the promoter region of the *UGT* gene, *UGT1A9* –440C>T and –331T>C, were recently found to confer decreased enzymatic activity. This may, in turn, increase MPA exposure and consequently the likelihood of MPA-related AEs.[19] Somewhat along these lines, in a study of 40 adult kidney transplant recipients, substantial interpatient variability of MPA exposure was significantly associated with the presence of the *UGT1A9* –440/331 genotype (*P* = 0.005).[19]

UGT1A9 also has an SNP, *UGT1A9*3* (98T>C), located in the coding region and resulting in decreased glucuronidation. This leads to an increase MPA AUC and therefore increased MPA exposure, as shown in a clinical study in which subjects with the *UGT1A9*3* SNP were shown to have higher MPA exposure as compared to those without the SNP (78.7 ± 18.7 mg·h/l vs. 42.5 ± 23.4 mg·h/l, *P* = 0.04).[9]

The other UGT family member responsible for the metabolism of MPA to MPAG is UGT1A8. UGT1A8 is involved in MPA glucuronidation and is expressed in the kidney and throughout the GI tract.[20] *In vitro* studies have also shown that SNPs in the *UGT1A8* gene, specifically *UGT1A8*3* (C-277Y), confer decreased enzyme activity,[17,21] which may result in increased MPA exposure. However, no *in vivo* data evaluating the potential clinical relevance of this SNP had been published at the time we designed our study.

UGT2B7 facilitates the metabolism of MPA to AcMPAG. This polymorphic enzyme is expressed in the liver and intestines. In an *in vitro* study by Bernard and colleagues, the promoter region SNP *UGT2B7* –900G>A (formerly –842 or –840) was associated with increased enzymatic activity and higher AcMPAG plasma concentrations. The AcMPAG production was 1.24- and 1.56-fold higher in the presence of –900GA and –900AA, respectively, as compared to wild type (*P* = 0.01).[15] AcMPAG, in turn, has been suggested to be responsible for MPA-associated GI toxicities;[22] however, recent *in vivo* data indicate that AcMPAG plasma concentrations and MPA-related side effects in patients undergoing renal transplantation are not related to the *UGT2B7* –900G>A gene polymorphism,[23] and AcMPAG plasma concentrations were not found to be associated with diarrhea.[24]

The primary objectives of this study were to obtain preliminary data on the frequencies of specific UGT variants in MMF-treated pediatric kidney transplant recipients and to compare their distribution between children who tolerated the drug at full dose and children who experienced MMF-related AEs.

Table 3 Observed frequencies of 1A8 830 and 1A9 –275 genotypes in study patients

		1A9 –275		
		T/T	T/A	A/A
1A8 830	G/G	30	5	0
	G/A	0	3	0
	A/A	0	0	0

RESULTS

Characteristics of patients and controls

A total of 38 pediatric renal transplant recipients were included in our analysis. Clinical characteristics of the AE group (*n* = 16) and the controls (*n* = 22) are summarized in **Table 1**.

Genotype frequencies between groups

All SNPs follow Hardy–Weinberg equilibrium (data not shown). Tests for linear trend in UGT genotype distributions in the AE and control groups are summarized in **Table 2**. There was a distinct linear trend in the frequency distributions of the three genotypes of *UGT1A9* –331 SNP (*P* = 0.04 by Cochran–Armitage trend test), indicating an increased probability for carriers of this SNP to be in the AE group. A similar, but weaker, trend was observed for the *UGT2B7* –900 SNP (*P* = 0.08).

As shown in **Table 3**, all three study patients who were heterozygous for the *UGT1A8* 830A allele possessed the T/A genotype for *UGT1A9* –275. Consistent with this observation, haplotype analysis of *UGT1A8* and *UGT1A9* showed that the *UGT1A8* 830A (**3*) allele was accompanied by *UGT1A9* –275A (**Table 4**). In view of the fact that these two polymorphisms may have opposite effects on UGT activity and consequently on MPA exposure,[19,21] we focused our analysis on the 30 patients who were carriers of the wild-type *UGT1A8* 830G (**1*) and *UGT1A9* –275 T alleles, thereby concentrating on the effects of *UGT1A9* –331 SNPs. This analysis, shown in **Table 5**, stratifies patients according to whether they experienced leukopenia or diarrhea. A significant greater frequency of occurrence of *UGT1A9* –331 SNP is evident in the AE group as compared to the control group (*P* = 0.04).

We next explored the combined effects of *UGT1A9* –331 and *UGT2B7* –900 SNPs on the incidence of AEs. Because MPA is more extensively metabolized by UGT1A9 than by UGT2B7,[1] it was assumed that the presence of the UGT1A9 –331 SNP would

Table 4 Estimated haplotype frequencies of genotypes

		UGT1A8	*UGT1A9*						95% Confidence interval	
Expected haplotype		830 (**3*)	–2152	–331	–275	98 (**3*)	Predicted frequency	SE	Lower limit	Upper limit
I	G-C-T-T-T	G	C	T	T	T	0.63	0.06	0.52	0.74
II	G-C-C-T-T	G	C	C	T	T	0.26	0.05	0.16	0.36
III	G-T-T-A-T	G	T	T	A	T	0.05	0.03	0.002	0.10
IV	A-T-T-A-T	A	T	T	A	T	0.04	0.02	<0.001	0.08
V	G-C-C-A-T	G	C	C	A	T	0.01	0.01	<0.001	0.04

UGT, uridine diphosphate–glucuronosyl transferase.

Table 5 UGT1A9 –331 genotype frequencies

	UGT1A9 -331[a,b]			
Group	T/T	C/T	C/C	Total
Control group	10 (56%)	8 (44%)	0 (0%)	18
Adverse events group	3 (25%)	6 (50%)	3 (25%)	12
Total	13	14	3	30

UGT, uridine diphosphate–glucuronosyl transferase.

[a]An exact Cochran–Armitage trend test for this contingency table gave a *P* value of 0.04. [b]Patients were classified into three groups (T/T, C/T, and C/C) according to the presence of *UGT1A9* –331 single-nucleotide polymorphism. T/T, C/T, and C/C groups were identical to the combinations of haplotypes (diplotypes) I/I, I/II, and II/II, respectively in **Table 4**.

Table 6 Classification of combined genotypic status (based on predicted capacity of UGT for MPA elimination)

UGT1A9 –331	*UGT2B7* –900	Predicted capacity of UGT for MPA elimination
T/T	A/A	Normal
T/T	G/A	Normal
C/T	A/A	Normal
T/T	G/G	Intermediate
C/T	G/A	Intermediate
C/T	G/G	Poor
C/C	A/A	Poor
C/C	G/A	Poor
C/C	G/G	Poor

Subjects homozygous for "T" at –331 in the promoter region of *UGT1A9* were considered to have higher metabolic activity for MPA than those homozygous for "C." Subjects homozygous for "A" at –900 in the promoter region of *UGT2B7* were considered to have higher metabolic activity for MPA than those homozygous for "G." Subjects heterozygous for each polymorphism were considered to have intermediate activity.

MPA, mycophenolic acid; UGT, uridine diphosphate–glucuronosyl transferase.

Table 7 Frequency distribution of predicted capacity of UGT for MPA elimination in the study groups

	Predicted capacity of UGT for MPA elimination[a]		
Group	Normal	Intermediate	Poor
Control	11 (61%)	7 (39%)	0 (0%)
AE	4 (33%)	4 (33%)	4 (33%)

AE, adverse event; MPA, mycophenolic acid; UGT, uridine diphosphate–glucuronosyl transferase.

[a]One-sided Cochran–Armitage trend test showed a linear relationship between the predicted metabolizer phenotypes and MPA-related AEs ($P = 0.02$).

have a greater influence on MPA elimination than *UGT2B7* –900 SNP status. Accordingly, we combined alleles that provide normal enzymatic activity with alleles yielding lower enzymatic activity in order to predict metabolic phenotypes (**Table 6**). The detailed distribution of the predicted capacities of UGT for MPA elimination in the two groups is shown in **Table 7**.

One-sided Cochran–Armitage trend test showed that the probability of being in the AE group increases as the presumed metabolic capacity decreases ($P = 0.02$, **Figure 1**). Poor metabolizers had a 13-fold increase in the odds ratio of developing an AE as compared to "normal" patients (odds ratio of 13.3 with 1.5

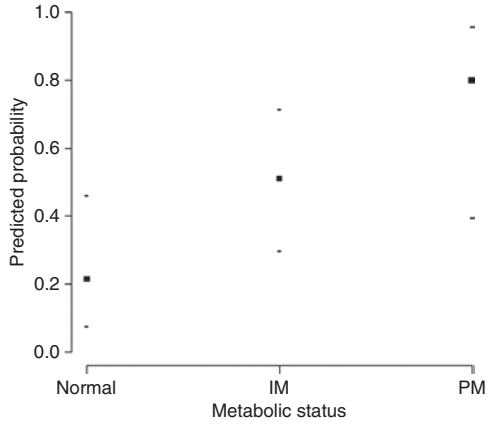

Figure 1 Predicted probability of adverse events (AEs) as a function of presumed poor, intermediate, or normal mycophenolic acid metabolizer status (PM, IM, and normal; see text) based on uridine diphosphate–glucuronosyl transferase single-nucleotide polymorphisms. Note the increase ($P = 0.02$, one-sided Cochran–Armitage trend test) in the likelihood of the occurrence of AEs with presumed decreases in metabolic activity, assuming that this capacity changes linearly.

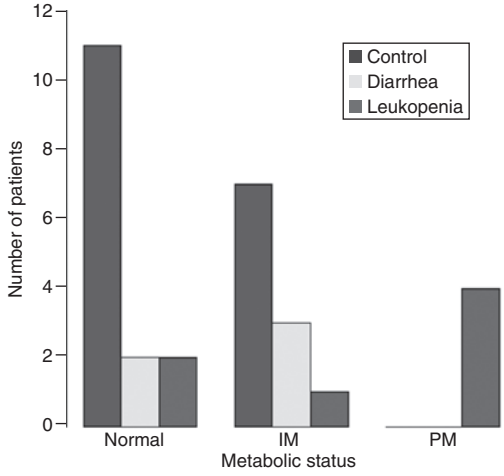

Figure 2 Frequency distribution of presumed poor, intermediate, and normal metabolizers (PM, IM, and normal) in the groups of control patients and patients who developed leukopenia or diarrhea. Note the exclusive appearance of predicted "poor metabolizers" in the leukopenia group, in which these individuals comprise more than 50% of all affected patients.

as the 95% confidence interval lower bound), and intermediate metabolizers had an almost fourfold increase in odds ratio (3.6 with 1.2 as the 95% confidence interval lower bound).

Of note, and as shown in **Figure 2**, the four subjects classified as likely to have UGT with low metabolic capacity did indeed experience leukopenia as their dose-limiting AE, as did two patients classified as likely to have normal UGT metabolic capacity. The MMF doses for these two patients were decreased as a result of a downward trend in their white blood cell counts (which had not, however, reached 2,500/μl). This observation suggests a relationship between low predicted UGT metabolic capacity (and therefore increased MPA exposure) and leukopenia. In contrast, no such relationship was apparent between predicted UGT metabolic capacity and diarrhea.

DISCUSSION

Our study demonstrates a difference in the distribution of polymorphic *UGT1A9* variants between pediatric kidney transplant recipients who experienced MMF-related AEs and comparable patients who tolerated MMF without problems. Specifically, a significantly higher incidence of the *UGT1A9* –331 variant allele was observed in patients who experienced AEs, as compared to controls. Given that the presence of the *UGT1A9* –331 SNP has been shown to confer increased MPA exposure,[19] we propose that such increased exposure is the main cause of MMF-related AEs in individuals with *UGT1A9* –331 SNP. A recent study provides evidence for the contribution of UGT2B7 genotype to MPA exposure.[25] Accordingly, we have initiated pharmacokinetic studies to correlate UGT polymorphisms and MPA exposure in pediatric kidney transplant recipients.

Moreover, we found that all patients who were homozygous carriers of the *UGT1A9* –331 SNP experienced clinically significant leukopenia, whereas there was no clear association between this (or other UGT polymorphisms) and the other important MMF-associated AE, diarrhea. Therefore, we speculate that leukopenia related to MMF therapy is indeed a result of increased systemic MPA exposure, whereas diarrhea as an AE of such therapy may be the result of other effects. Along these lines, it has been suggested that MMF causes diarrhea through local action in the GI tract and therefore in a manner independent of its absorption and systemic metabolism.[13] Alternatively, it has also been proposed that MMF-associated diarrhea is not related to increased systemic exposure to MPA or to one of its metabolites.[15,23,24] Both of these findings indicate the potential involvement of other genetic polymorphisms, affecting either transporters responsible for the drug's absorption or, possibly, other local mechanisms in MMF-related GI toxicity.

Similarly, we also found that polymorphisms other than *UGT1A9* –331 can contribute to MMF-related AEs. Specifically, the presence of *UGT2B7* –900 SNP also appeared to confer increased susceptibility to leukopenia. Our inability to detect additional AE–pharmacogenomic relationships may be, at least in part, a function of the relative rarity of the other SNPs included in our study and of the small size of our pediatric sample.

In conclusion, this pilot study identifies pediatric kidney transplant recipients who are carriers of the polymorphisms in the *UGT1A9* –331 and, possibly, of *UGT2B7* –900 SNPs as being at increased risk for developing an AE when receiving MMF. If pharmacokinetic studies in this pediatric population confirm data that demonstrate increased MPA exposure in adult individuals with these polymorphisms, novel (i.e., personalized and prospective) MMF dosing and monitoring strategies may be feasible. Such strategies could reduce the high incidence of MMF toxicity in children without compromising the drug's protective effects against graft rejection.

METHODS

Patients. In this study, we used extreme discordant phenotype methodology, which focuses on the most sensitive and most resistant subpopulations of patients receiving MMF treatment as part of their immunosuppressive therapy.[26] The study was approved by the institutional review boards of the participating institutions. Parents/guardians provided written consent, and patients' assent was obtained when applicable.

After a review of data from kidney transplant recipients followed at our institution at the time of the study ($n = 214$) and the data from patients enrolled in a pharmacokinetic/pharmacogenomic study ($n = 31$) conducted by the Pediatric Pharmacology Research Unit network, we were able to enroll 16 eligible patients who experienced one of the two cardinal AEs associated with MMF—diarrhea (several loose stools/day, described by the patient/family as a significant change) or leukopenia (white blood cell count <2,500/μl or steadily decreasing toward this cutoff)—and resolution of the AE after MMF dose reduction or discontinuation, both in the absence of other apparent causes for the AE or its disappearance. Only patients who were ≤18 years of age when they experienced their AE, and were not recipients of additional organ transplants, i.e., liver or small bowel, were included in the AE group. Sixteen patients who met these criteria were identified, and they and/or their guardians consented to genetic analysis. A cohort of subjects taking MMF without experiencing diarrhea or leukopenia but meeting all other inclusion criteria served as the control group ($n = 22$). To ascertain our extreme discordant phenotype design, we included in the AE group only patients whose chart review indicated an unequivocal relationship between MMF therapy, the AE, and its resolution after MMF dose reduction or discontinuation. Patients who had leukopenia or diarrhea leading to MMF dose reduction or discontinuation, but without an obvious beneficial response, were not included, nor were patients in whom other possible contributors to their leukopenia or diarrhea (e.g., symptoms of gastroenteritis, evidence of concomitant viral infection, or therapy with other medications associated with leukopenia, such as valganciclovir) could be identified.

Data collection. The data collected included gender, age, date of transplantation, age at transplantation, weight, height, body surface area, underlying disease state, MMF dose, MMF dose adjustments or discontinuation, concomitant medications and serum drug levels, white blood cell count with differential, documentation of AE, physician's rationale for MMF dose adjustment, and response to the change in MMF dose or discontinuation. Blood samples for genotyping were obtained during clinic visits requiring blood work for routine monitoring, and buccal swabs were obtained from patients who had no scheduled blood work during the study period.

Genotyping. All the patients ($n = 38$) were genotyped for *UGT1A8* 830G>A (*3), *UGT1A9* 98T>C (*3), *UGT1A9* –2152C>T, *UGT1A9* –331T>C, *UGT1A9* –275T>A, and *UGT2B7* –900G>A (formerly –842 or –840) polymorphisms. Genomic DNA was extracted from blood or buccal swab using standard procedures. Mutations were determined using the direct sequencing method. Forward and reverse primers were 5′-ttgagacagagtcgtgctgttt-3′ and 5′-aggtcaaggtgggcgtatc-3′, respectively, for *UGT1A9* –2152C>T; 5′-gacagagagtatttggttgc-3′ and 5′-cttatggtctttgccttg-3′ for –331T>C; –275T>A, 5′-gttctctgatggcttgcaca-3′ and 5′-atgccccctgagaatgagtt-3′ for 98T>C; 5′-ttcgccaggggaatag-3′ and 5′-atttgctctaggggtc-3′ for *UGT1A8* 830G>A; and 5′-ctgcataattctaggacaac-3′ and 5′-ctaccataacaatcagttgg-3′ for *UGT2B7* –900G>A. The PCR conditions for *UGT1A9* and *UGT2B7* SNPs consisted of denaturation at 95 °C for 7 min, followed by 35 cycles for denaturation at 95 °C for 45 s, annealing at 55.5 °C for 45 s, and extension at 72 °C for 30 s. These amplicons were generated with a Taq DNA polymerase (Invitrogen, Carlsbad, CA). The PCR product for UGT1A8 830G>A was amplified using AmpliTaq Gold (Applied Biosystems, Foster City, CA) under the following conditions: denaturation at 95 °C for 10 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 30 s. After incubation with ExoSAP-IT (USB, Cleveland, OH), the PCR products were directly sequenced using the ABI PRISM 3730 DNA autosequencer (Applied Biosystems) with a ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit in accordance with the manufacturer's protocol (Applied Biosystems).

Statistical analysis. Descriptive statistics were used to characterize the demographics of the AE and control groups. Quartiles were used to describe noncategorical variables, and Wilcoxon–Mann–Whitney rank sum tests were used to detect shifts in the locations of the distributions. Categorical variables were reported using frequency and proportions, and exact χ^2-test was used because of small counts.

General information about each SNP, such as allele frequency, genotype frequency, and Hardy–Weinberg equilibrium, was gathered. For each of the two study groups, the genotypes of each SNP were tested for differences, using exact χ^2-tests and exact Cochran–Armitage trend tests. In total, five genetic polymorphisms of the UGT1A family and one genetic polymorphism of the UGT2B family were analyzed. Unphased haplotypes for UGT1A were also estimated. PROC ALLELE and PROC HAPLOTYPE in SAS/Genetics software, version 9.1 (SAS Institute, Cary, NC), were used for the analyses. Statistical significance was defined as $P < 0.05$. A significant trend was defined as $P < 0.10$. Two-sided or one-sided statistical tests were used according to the study purpose. Because of the exploratory nature of this study, no adjustments were made for multiple testing.

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

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The Effect of Activated Charcoal on Drug Exposure in Healthy Volunteers: A Meta-Analysis

G Jürgens[1,2], LC Groth Hoegberg[2,3] and NA Graudal[4]

The objective of the study was to estimate the effect of activated charcoal (AC) administered during the first 6 h after drug intake and the effect of drug properties on drug exposure. Sixty-four controlled studies were integrated in a meta-analysis. AC administered 0–5 min after administration of a drug reduced median drug exposure by 88.4% (25–75 percentile: 65.0–96.8) ($P < 0.00001$). The effect of AC continued to be statistically significant when administered up to 4 h after drug intake (median reduction in drug exposure 27.4% (range 21.3–31.5%, $P = 0.0006$). The reduction in drug exposure was correlated with the AC/drug ratio ($\rho = 0.69$, $P < 0.0001$), the volume of distribution (V_d) ($\rho = 0.46$, $P = 0.0001$), and time to peak concentration ($\rho = 0.40$, $P = 0.02$). We found that AC is most effective when given immediately after drug ingestion but has statistically significant effects even when given as long as 4 h after drug intake. AC appears to be most effective when given in a large dose.

Activated charcoal (AC) given in connection with drug intake reduces drug exposure and is used in the routine management of oral drug overdose.[1] The effect of AC is based primarily on a prevention of drug absorption and is greatest within the first hour after drug ingestion. This led to the recommendation in the position statement of the American Academy of Clinical Toxicology/European Association of Poison Centres and Clinical Toxicologists that the benefit of AC is limited primarily to the first hour after drug ingestion.[1] This, however, excludes the majority of patients from treatment, as only a small subgroup of patients present this early.[2,3] But AC also has a "late" effect, which is caused not only by delayed absorption (sustained-release preparations, ventricular retention, and decreased gut mobility) but also by an enhancement of drug elimination through the interruption of enterohepatic and/or enterovascular drug re-circulation.[4]

Although the pharmacological effect of AC is well documented in a large number of volunteer studies involving subtoxic drug ingestion, its impact on the clinical outcome of drug poisoning is still controversial. A randomized controlled trial of multiple-dose AC conducted in an Asian population of 401 persons poisoned with oleander seeds (cardiac glycosides) showed a mortality reduction from 8 to 2% in the multiple-dose AC-treated group as compared to the placebo/single-dose AC-treated group.[5] However, a recently published larger study ($N = 4{,}632$) in a similar population showed no effect on mortality of either single- or multiple-dose AC treatment as compared with placebo.[3] Another randomized clinical trial, conducted in an Australian population of 327 patients who had been admitted to an emergency department with oral drug overdose, failed to show any beneficial effect of AC on length of hospital stay, the incidence of vomiting, or the need to be put on a ventilator.[2]

The reason for the discrepancy between the outcomes of these studies is unclear. However, the results of these large trials question the clinical use of AC. Given that the clinical studies of drug intoxications are few and the results are controversial, this meta-analysis of pharmacological studies was performed in order to improve the foundation for decision making concerning the use of AC in the treatment of drug intoxications.

The aim of this review was to analyze the effect on drug exposure of single-dose AC administered during the first 6 h after drug intake, and the impact of physical and pharmacological drug properties on this effect, in order to suggest criteria for the use of AC in the treatment of drug intoxications.

RESULTS

No studies were identified involving intoxicated patients. Consequently, all the included studies were conducted in healthy volunteers. In 84 comparisons (41 studies), AC was administered 0–5 min after administration of drug. The

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Table 1 Results of seven meta-analyses of the effect on drug exposure of activated charcoal (AC) administered at different time points after drug intake

Time to AC after drug intake	0–5 min	30 min	60 min	120 min	180 min	240 min	360 min
Standardized mean difference	−3.67	−1.27	−1.54	−0.80	−0.82	−0.86	−0.41
Median reduction of drug exposure (%)	88.4	48.5	38.4	24.4	13.6	27.4	11
25–75 percentile	65.0–96.8	35.4–56.6	25.4–61.7	13.6–31.5	7.9–38.1	21.3–31.5	7.4–14.6
P (*Z*)	<0.00001 (17.57)	<0.00001 (7.91)	<0.00001 (9.06)	<0.00001 (4.75)	0.03 (2.13)	0.0006 (3.42)	0.29 (1.07)
df	83	15	29	8	4	3	1
Studies, *n*[a]	41	8 (5)	20 (16)	7 (0)	5 (2)	4 (1)	2 (0)
Comparisons, *n*	84	16	30	9	5	4	2
Different drugs, *n*[b]	43	12 (4)	14 (3)	4 (0)	2 (0)	3 (0)	2 (0)
AUC, *n*[c]	59	10	24	9	2	4	2
C_{max}, *n*[c]	3	3	1	0	2	0	0
Urine recovery, *n*[c]	22	3	5	0	1	0	0
Participants, *n*	605	110	266	106	69	44	22
Individual drug measurements, *n*	1,046	218	508	163	92	71	28

AUC, area under the curve; C_{max}, peak blood concentration; df, degrees of freedom; *Z*, test for overall treatment effect, from which the *P* value is derived.

[a]New studies in addition to the 41 studies included in the 0–5 min meta-analysis. [b]New drugs in addition to the 43 different drugs included in the 0–5 min meta-analysis. [c]Number of comparisons.

standardized mean difference was −3.67 ($Z = 17.57$, $P < 0.00001$) (**Supplementary Figure S1** online). This corresponds to a median reduction of drug exposure by 88.4% (65.0–96.8) as compared to no treatment (Table 1). A separate analysis excluding 25 studies based on peak blood concentration and urine recovery did not change standardized mean difference (−3.47, $Z = 14.77$, $P < 0.00001$).

Table 1 shows the results of the seven meta-analyses. When AC was administered 30 min after drug intake, the effect on drug exposure was reduced to 48.5%; when administered 60–240 min after drug intake, the reduction in drug exposure of the body was still statistically significant and stable at ~25%. Even when AC was administered after 360 min, there seemed to be a reduction in drug exposure although this was not statistically significant.

The percentage reduction in drug exposure was correlated with the AC/drug ratio ($\rho = 0.69$, $P < 0.0001$). This relationship was described by means of a sigmoid dose–response curve (Figure 1).

There was a correlation between the effect of early administration of AC on drug exposure and volume of distribution (V_d) ($\rho = 0.46$, $P = 0.0001$). In a multiple regression analysis, this correlation was found to be independent of the AC/drug ratio.

There was a better effect on drugs that were nondialyzable ($n = 17$) than in those that were dialyzable ($n = 40$), the mean reduction in drug exposure (±SD) being 84% (±18) vs. 66% (±30), $P < 0.03$.

A correlation was also demonstrated between the effect of AC administered late (>1 h after drug ingestion) and time to peak concentration of the individual drugs ($\rho = 0.40$, $P = 0.02$).

Of the 84 comparisons measuring the early effect of AC (≤5 min), 20 involved drugs with anticholinergic effects. The median effect in these comparisons did not differ significantly from the 64 comparisons that involved nonanticholinergic drugs (95% vs. 85%, $P = 0.10$). Among the 47 comparisons (≥60 min) made for measuring the effects of late administration of AC, 8 involved drugs with anticholinergic effects. The median effect in these 8 comparisons did not differ significantly from the 39 involving nonanticholinergic drugs (35% vs. 30%, $P = 0.42$).

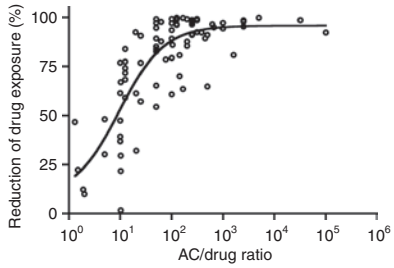

Figure 1 Effect (reduction of drug exposure (%)) of activated charcoal (AC) administered 0–5 min after drug intake, expressed as a function of log AC/drug ratio. Equation of the sigmoid dose–response relationship: reduction of drug exposure (%) = 8.95 + (86.79/(1 + 10^(0.9861 − log AC/drug))).

DISCUSSION

This is the first meta-analysis of the effect of AC on drug exposure. It confirms the position paper of the American Academy of Clinical Toxicology/European Association of Poison Centres and Clinical Toxicologists[1] in concluding that the effect of AC is highly variable and has its major effect (reduction of drug level to near-zero in the body) when given immediately after drug intake. In addition, our data show that, even if administered 1 h after drug ingestion, AC reduces the median drug exposure in the body by at least 62% in one-quarter of all the comparisons and achieves at least a 32% median reduction in drug exposure in one-quarter of the comparisons when administered at a time point up to 4 h after drug intake.

These effects of later administration are within the range of what can be achieved by administering AC within the first hour of drug ingestion (**Table 1**). This contradicts the statement of the American Academy of Clinical Toxicology/European Association of Poison Centres and Clinical Toxicologists that the benefit of AC is limited to the first hour after drug ingestion.

In spite of this finding, data on the beneficial effects of AC in clinical studies of drug-intoxicated patients have been conflicting. It has been argued that the lack of clinical effect in some studies is due to the possibility that the pharmacological effects of AC reported in studies of healthy volunteers using subtoxic drug doses cannot be extrapolated to patients suffering from drug poisoning, because of the concomitant ingestion of drugs or ethanol, differences in fasting state, or gastric pH. In experimental studies, these factors have been shown to interfere with the binding capacity of AC.[6] However, the data in this meta-analysis are based on studies involving the administration of single drugs (47 studies) and also on those involving up to five drugs in combination (17 studies), and, although most volunteers were fasting (127 comparisons), there were nonfasting volunteers involved in 20 comparisons. Furthermore, pharmacokinetic data on the effect of AC over time in patients with drug overdose[7–9] show results within the limits of our meta-analysis, thereby suggesting that results obtained in healthy volunteers can very well be extrapolated to patients suffering from poisoning. Therefore, it is difficult to accept that such large and statistically highly significant effects should have no clinical impact.

When evaluating the results of clinical studies on the effect of AC in drug-overdose patients, one must consider that the overall mortality in such patients is small. Furthermore, all existing evidence indicates that drug intoxication events should ideally be treated with AC as early as possible. Deliberate self-poisoning is often impulsive and associated with poor accuracy of reported dose history. To wait for information on the identity of the drug or for verifying the dose is to lose important time during which AC may work if given straight away. Therefore, the clinical decision must be made rapidly and often on the basis of insufficient information, leading to overtreatment in a considerable number of patients. Furthermore, all clinical studies included several different drug exposures, thereby increasing the risk of introducing factors that could confound the outcome. Severely poisoned patients were not included in these studies, but all were treated with gastric decontamination and/or specific antidotes.[2,3,10] This is understandable for ethical reasons; nevertheless, it is problematic, because it is reasonable to expect that severely intoxicated patients would benefit the most from the treatment. Therefore, even large-scale studies do not have the statistical power to show hard outcomes such as death.

Because the ingestion of AC is related to discomfort such as nausea and vomiting,[10] which, in many cases, leads to a period of observation, studies using soft outcomes such as vomiting and length of hospital stay will have difficulty in showing statistical differences.[2] We are therefore of the opinion that none of the existing clinical studies rejects the effect of AC given within the first hour of poisoning, while a large number of studies in healthy volunteers support its pharmacological effect.

AC is usually considered a safe treatment. This opinion is supported by the data from randomized studies of drug intoxications,[2,3,10] which found that the side effects in AC-treated patients were no more numerous than in placebo-treated patients. Furthermore, a large retrospective analysis of aspiration pneumonia in more than 4,000 intoxicated patients found that AC treatment was not a risk factor for aspiration pneumonia.[11] However, there have been a few case reports of aspiration pneumonia associated with AC treatment resulting in death in patients with impaired consciousness or in small children.[11,12] Consequently, the risk of AC aspiration, although small, does exist and is especially unacceptable when it involves patients that have been treated unnecessarily. This leaves us in a dilemma: if we withhold treatment in most patients so as to minimize the risk of side effects such as AC aspiration, we may also be withholding potentially life-saving treatment from a minority of these patients. In order to promote more selective use of AC, especially when it is to be administered at a later time point (>1 h), we tried to identify factors that could explain the large variation in the effects of AC on drug exposure.

First, we looked at the AC/drug ratio. *In vitro* studies show that the adsorption of drug by AC improves with increasing AC/drug ratios, from <20% (ratio 1) to >90% (ratio 10).[13] This has lead to the recommendation of an AC/drug ratio of 10 in the treatment of drug-intoxicated patients.[1] In practice, this means that 50 g AC is expected to bind 5 g of drug, and additional doses of AC are considered if larger amounts of drug have been ingested. To identify the optimal dose of AC, we correlated the effect of AC (reduction of drug exposure) to the AC/drug ratio. Our analysis of this dose–response relationship shows that the sigmoid dose–response curve reaches its plateau at a considerably higher ratio than 10. This finding implies that the adsorptive capacity of AC can be improved by increasing the AC/drug ratio to ~40 (**Figure 1**). In patients who are intoxicated by ingestion of low-potency drugs such as paracetamol and nonsteroidal anti-inflammatory drugs, this ratio is impossible to achieve, but the effect of AC in toxic ingestions of high-potency drugs such as tricyclic antidepressants, digoxin, and other antiarrhythmics could be improved by aiming at AC/drug ratios much higher than 10.

Second, we looked at V_d of the single drugs. Experimental studies showed that the binding capacity of AC is influenced by physicochemical drug properties. V_d is usually easily available and reflects a large number of drug properties. It is related to a decreased effect of other drug eliminating procedures, such as dialysis. Our analysis showed a direct relationship between increase in V_d of the study drugs and the early effect of AC. It is interesting to see that the same drug property that limits dialysis clearance renders agents more likely to exhibit enhanced adsorption by AC. Therefore these drug characteristics could be used to better identify which patients would benefit most from AC therapy. Although our data cannot distinguish between the mechanisms of absorption by AC and increased elimination, it is tempting to believe that elimination plays an increasing role as the time interval between drug intake and AC administration increases, and this may explain the apparent stabilization of the

effect when AC is administered between 2 and 4 h after drug ingestion (**Table 1**).

Third, we looked for the time point at which the highest drug concentration occurs after oral administration (time to peak concentration), as an expression of a slow absorption and a larger amount of drug being present in the gut. As expected, this parameter correlated with the effect of later-administered AC (>1 h). A subsequent analysis of the influence of anticholinergic side effects (reduction of gut motility and increase in the gut transit time[14]) on the effect of early (within 5 min) or late (>1 h) administration did not show an increased effect of AC. However, this could very well be a consequence of the often very low doses (always subtoxic and sometimes subtherapeutic) that were used in this population of healthy volunteers.

In our opinion, data from our meta-analysis support the use of AC in drug-poisoned patients. AC is an inexpensive treatment and does not usually require invasive procedures. It has the advantage that the effect is based primarily on the reduction of absorption, while other options to remove the drug from the body, such as hemodialysis, are based solely on increasing the elimination, which means that the drug has already been absorbed and has possibly done harm.

This means that AC should be given in situations of potentially dangerous drug intoxication, especially in patients in whom other treatment options, such as hemodialysis, are limited. AC/drug ratios that are much higher than the usually recommended ratio of 10 can be used, especially in cases of poisoning with high-potency drugs.

METHODS

Criteria for considering studies for this review

Types of studies. Controlled clinical trials with a parallel design or a crossover design.

Types of participants. Healthy volunteers or patients with a suspected history of oral drug overdose.

Type of intervention. All comparisons of the effect of single-dose AC with those of placebo, water, or no treatment, after administration of a drug.

Type of outcome measure. Reduction of drug exposure as estimated by area under the curve (AUC) calculations, peak blood concentrations, or drug recovery in urine after administration of a drug in subtoxic doses. Intervention groups were categorized according to the time elapsed between drug intake and treatment with AC (or no AC treatment) into the following seven groups: 0–5, 30, 60, 120, 180, 240, and 360 min.

Search strategy for identification of studies. The reference list of the most recently published position paper on the effect of single-dose AC[1] was searched, and 55 studies within the field of this meta-analysis were identified. Four studies did not fulfill our criteria and were excluded. A search of the electronic databases PubMed (1950s until November 2008) and EMBASE (1980 until November 2008), and a subsequent review of the abstracts, did lead to the identification of another 14 studies. A search of the reference lists of included studies did not lead to the identification of further studies.

Two review authors assessed the trials for quality of methodology without consideration of the results and extracted the data. A meta-analysis was performed for each of the seven intervention groups.

In addition, meta-regression analyses were performed to determine the relationship between the percentage reduction of drug exposure calculated from comparisons involving the administration of AC within 5 min after drug ingestion (primary outcome) and (i) the AC/drug ratio (i.e., dose–response relationship) and (ii) the V_d.[15,16] V_d was chosen because the binding capacity of AC is influenced by physicochemical drug properties such as polarity and lipophilicity. Nonpolar substances with high lipid solubility are better absorbed by AC than polar substances with low lipid solubility.[17] Both properties are important for the permeability through lipoid membranes and are therefore important determinants of V_d.[18] Furthermore, the percentage reduction of drug exposure, calculated from each comparison involving AC administration between 60 and 360 min after drug ingestion, was correlated with the time to peak concentration values of the ingested drugs (mean values from the control groups of the studies included). The ingested drugs were considered as belonging to two groups: drugs with anticholinergic effect and those without.[19] The effects on drug exposure of early (≤5 min) and late (>1 h) administration of AC were compared between these two groups. Furthermore, the ingested drugs were categorized as either dialyzable or nondialyzable drugs,[15,16] and the effects on drug exposure after early (≤5 min) administration of AC were compared between these two groups. Because of the strong correlation between AC/drug ratio and effect (reduction of drug exposure), all regression analyses vs. effect were also performed as multiple regression analyses including AC/drug ratio so as to assess the contribution of covariates. Because the effect of AC was calculated on the basis of blood concentrations and drug recovery in urine with respect to many different drugs, a weighted estimate of the mean difference would be meaningless. Instead, a standardized mean difference was calculated by dividing the difference in mean outcome between groups with the SD of outcome among participants. Standardized mean difference is difficult to interpret as it is reported in units of SD rather than in units of any of the measurement scales used. Therefore, we also calculated the median percentage reduction of drug exposure. This was done by dividing the effect in the AC-treated group with the effect in the control group, for each comparison. Next the median and 25–75 percentile of the individual comparisons were calculated.

Statistical tests. The seven meta-analyses were performed using Review Manager 4.2 (The Nordic Cochrane Centre, Copenhagen, Denmark). Heterogeneity between trial results was calculated using an I^2-test. In case of heterogeneity, a random-effect model was used. In case of homogeneity, a fixed-effect model was used.

The relationship between AC dose (milligrams) and drug dose (milligrams), i.e., the factor by which AC exceeds the drug, was defined as "the dose," and the effect size (percentage reduction in drug exposure) was defined as "the response." Because of the enormous variations in dose, it was log transformed. The response was depicted as a sigmoid function of log dose (GraphPad Prism 4.0; GraphPad Software, San Diego, CA).

The correlation coefficients in the meta-regression analyses were calculated by means of a distribution-free rank correlation method (Spearman's ρ) (StatView 5.0; SAS Institute, Cary, NC) because none of the scatter plots in the meta-regression analyses followed a Gaussian distribution. Similarly, groups were compared using an unpaired distribution-free Mann–Whitney test (StatView 5.0; SAS Institute, Cary, NC).

Description and methodological qualities of included studies. A total of 69 studies, including 154 comparisons, were identified. Four studies (four comparisons) were excluded from the meta-analyses, because these related to administration of AC before the drug under investigation. Consequently, 65 studies (references are given in the **Supplementary Data** online), including 150 comparisons, were included. The meta-analyses include 50 different drugs. Each drug given at one time point was considered to be one comparison; that is, one study could include more than one comparison, either because AC was administered at different time points or by including different drugs (**Supplementary Table S1** online).

Fifty-nine studies were randomized; 6 were not. In none of the 59 randomized studies was the method of randomization explained. Six studies were parallel studies, and 59 were crossover studies.

None of the studies included in the meta-analysis was double blind. However, the outcome measure was based solely on a laboratory variable measured by laboratory technicians who were blinded with respect to the category of the participant (AC treatment or no treatment). Consequently, the studies were all single blind. All the studies included healthy volunteers and lasted only a few hours. We therefore judged that the risk of a systematic bias in a nonrandomized control group would be negligible as compared to the disadvantage of losing the statistical information. Consequently, we did not exclude the few nonrandomized studies ($N = 6$), which all had results within the limits of the randomized studies.

The included studies used different variables to estimate the effect of AC on drug exposure. Forty-seven studies used AUC, 15 studies used urinary recovery, and 3 used peak blood concentration. Of these variables, AUC is assumed to be the most reliable method to estimate total drug exposure of the body. However, most studies were crossover studies, which minimize interindividual variability of factors that could influence the outcome of peak blood concentration or urinary recovery (e.g., rate of absorption or renal function). We decided to include non-AUC studies (i) because they add valuable information to our analysis, (ii) to increase the power of the meta-analysis, and (iii) to avoid systematic bias. In order to exclude a possible impact of these differences in estimation methods on the overall effect measure, a separate meta-analysis was performed of comparisons that used AUC to estimate the effect of AC given within 5 min after drug ingestion. For AC administered at later time points, there were very few comparisons that did not use AUC for estimation of drug exposure, and therefore no separate analyses of these comparisons were carried out (**Table 1**).

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at http://www.nature.com/cpt

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

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Assessment of MAO-B Occupancy in the Brain With PET and [^{11}C]-L-Deprenyl-D_2: A Dose-Finding Study With a Novel MAO-B Inhibitor, EVT 301

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Inhibition of monoamine oxidase type B (MAO-B) activity in the brain is a putative strategy for the treatment of Alzheimer's disease (AD). We performed a dose-selection and validation study of a novel, reversible MAO-B inhibitor, EVT 301. Sixteen healthy volunteers received selegiline (10 mg) or EVT 301 (25, 75, or 150 mg) daily for 7–8 days, and four subjects with AD received 75 mg of EVT 301. MAO-B occupancy in the brain was assessed using positron emission tomography (PET) with [^{11}C]-L-deprenyl-D_2. EVT 301 was found to dose-dependently occupy MAO-B in the human brain, with occupancy ranging from 58–78% at a dose of 25 mg to 73–90% at a dose of 150 mg. The corresponding occupancy after selegiline was 77–92%. Determination of MAO-B inhibition in blood platelets underestimated the actual brain occupancy achieved with EVT 301. A daily EVT 301 dose of 75 or 150 mg appears suitable for clinical efficacy studies in patients with AD.

Current medications for Alzheimer's disease (AD) are based on the inhibition of the enzyme acetylcholinesterase so as to increase synaptic acetylcholine concentrations in the brain (donepezil, rivastigmine, and galantamine), or on the antagonism of N-methyl-D-aspartic acid–type glutamate receptors (memantine). These medications provide only modest relief of symptoms and do not significantly slow disease progression.[1] Current therapeutic research in AD is focused mainly on preventing further neurodegeneration. Potential new treatments include inhibitors of β-amyloid aggregation, antiinflammatory drugs, neurotrophic factors, antioxidants, and inhibitors of the enzyme monoamine oxidase type B (MAO-B).

MAO-B catalyzes the oxidative deamination of catecholamine neurotransmitters, generating hydrogen peroxide. Hydrogen peroxide can react to form hydroxyl radicals, which are highly reactive oxygen species. In AD, there is evidence of increased oxidative stress, which is attributed to the aberrant generation of reactive oxygen species, leading to neurodegeneration.[2] In addition, several studies have shown an increase in the activity of MAO-B in the brain and also in blood platelets of AD patients.[3–5]

MAO-B is the predominant isoform of MAO in the human brain and constitutes up to ~70% of total brain MAO activity.[6] Cerebral MAO-B levels increase with age. MAO-B is further upregulated in the brains of AD patients; it is mostly localized in astrocytes surrounding senile plaques.[7] The inhibition of the enzyme prevents MAO-B-mediated formation of reactive oxygen species and has been shown to reduce oxidative stress. It can therefore be expected to slow the progression of AD.

The data from some clinical studies carried out with selegiline, an irreversible MAO-B inhibitor, have supported the utility of MAO-B as a therapeutic target in AD, but the results are inconsistent.[8] A meta-analysis showed that selegiline improved cognition and activities of daily living in the short term but had no sustained long-term effects.[8] In a 2-year placebo-controlled study in 341 patients with AD, subjects were randomized to receive selegiline, α-tocopherol (vitamin E), their combination, or placebo, with end points being institutionalization, deterioration in ability to perform basic activities of daily living, or death.[9] In subjects with moderately advanced AD, selegiline treatment, alone or in

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combination with α-tocopherol, appeared to delay the time to end point, suggesting slower disease progression.[9]

EVT 301 (N-[4-(3-fluoro-benzyloxy)-phenyl]-malonamide) is a reversible inhibitor of MAO-B. It has been in clinical development by Evotec for the treatment of AD. The results of an unpublished phase I single-dose study, carried out with positron emission tomography (PET) imaging, showed that EVT 301 is a reversible inhibitor of MAO-B and that it crosses the blood–brain barrier. In subjects who received 200 mg of EVT 301, the study demonstrated >90% occupancy of the MAO-B enzyme in the brain over a period of 48 h (data on file).

This study investigated the MAO-B occupancy in the brain resulting from different doses of EVT 301, using PET imaging with [^{11}C]-L-deprenyl-D_2 to identify a daily dose level of EVT 301 that produces near-complete inhibition of MAO-B enzyme activity in the brain. For comparison, the inhibition of MAO-B activity was also measured in blood platelets. To ensure that the observed results would be amenable to generalization to the AD patient population, patients with AD were studied at a preselected EVT 301 dose level (75 mg daily), and the results were compared with those of healthy elderly controls.

RESULTS

All treatments were well tolerated. The compliance checks indicated that all subjects had taken their medication according to the instructions. The pharmacokinetic analysis of EVT 301 plasma concentrations indicated that steady state had been achieved before the second PET scan, given that there were no significant differences in the EVT 301 concentrations in the 3 days preceding the second PET scan ($P = 0.248$ for the visit effect). After a daily dose of 75 mg, no differences were observed between the steady-state concentrations of EVT 301 in AD patients and those in healthy control subjects (**Table 1**). The steady-state concentrations were significantly dose-related, as was expected (**Table 1**).

[^{11}C]-L-deprenyl-D_2 binding in the brain

Because it is currently unknown which brain region is most relevant as a target of MAO-B inhibition in AD, we included a comprehensive set of brain regions in the analyses. The λk_3, an index of specific binding of radiotracer to MAO-B, values before and after treatment are summarized in **Table 2**. The AD patients had, on average, 9% higher baseline λk_3 values than the control subjects in all brain regions, with a statistically significant difference in the hippocampus (18%, $P = 0.047$). Average regional MAO-B occupancy values for the selegiline and EVT 301 regimens are given in **Table 2** and visualized in **Figure 1**. Treatment effects were highly significant ($P < 0.0001$, analysis of variance) in all brain regions investigated. In the control subjects on selegiline at 10 mg daily, the occupancy was 77–92% (range of mean regional values), confirming the utility of measuring MAO-B occupancy *in vivo* using PET and [^{11}C]-L-deprenyl-D_2. For control subjects on EVT 301 at 25 mg daily, the MAO-B occupancy in different brain regions was 58–78%; for control subjects on EVT 301 at 75 mg daily, it was 70–87%; for control subjects on EVT 301 at 150 mg daily, it was 73–90%; and for AD subjects on EVT 301 at 75 mg daily, it was 75–90%. Visualization of MAO-B occupancy in the brain in one control subject, induced by 150 mg of EVT 301 daily is shown in **Figure 2.** In general, the occupancy associated with 25 mg of EVT 301 was lower than that achieved with 75 or 150 mg of EVT 301 or with 10 mg of selegiline (data not shown), but no other

Table 1 Concentrations of EVT 301 in plasma

Subject number	Treatment (mg)	Baseline[a]	1. Pre-PET	2. Pre-PET	3. Pre-PET	2nd PET[b]
1	75	BLQ	1,660	1,890	1,980	1,710
2	150	BLQ	3,610	4,040	3,070	2,870
4	25	BLQ	323	319	418	295
5	75	BLQ	914	1,090	818	1,090
6	150	BLQ	3,480	3,670	3,780	2,900
7	25	BLQ	267	307	331	283
9	25	BLQ	410	469	582	406
11	150	BLQ	3,670	4,020	5,570	3,960
12	75	BLQ	1,860	1,850	2,220	2,000
16	150	BLQ	4,240	3,960	4,280	4,390
21 (AD)	75	BLQ	2,800	2,570	2,460	2,340
22 (AD)	75	BLQ	1,730	1,610	1,740	1,810
23 (AD)	75	BLQ	2,290	2,340	2,140	2,520
24 (AD)	75	BLQ	1,570	1,760	1,610	1,480
113	75	BLQ	1,920	2,230	1,770	1,450
115	25	BLQ	464	453	464	435

Results are expressed as ng/ml.

AD, Alzheimer's disease.

[a]Baseline samples were taken just before the first positron emission tomography (PET) scans. BLQ, below limit of quantitation, <1 ng/ml. [b]Last sampling was performed 22–24 h after the last drug administration.

statistically significant differences between the treatments or groups emerged (Figure 2).

The cerebellum was included in the analysis to explore the possibility of using it as a reference region, thereby making arterial blood sampling unnecessary. However, all drug treatments were associated with profound and significant decreases in cerebellar [^{11}C]-L-deprenyl-D_2 binding, ranging from −65% for a dose of 25 mg of EVT 301 to −83% for a dose of 10 mg of selegiline, suggesting specific binding of the tracer to MAO-B also in this brain region.

The concentration–response relationship of each of the three EVT 301 doses with MAO-B occupancy was studied by fitting a nonlinear regression model. A representative regression curve for the hippocampus is presented in Figure 3. Judging by the overlap in the confidence intervals for occupancy by selegiline and occupancy by EVT 301, a daily dose of 75 mg of

Table 2 Regional [^{11}C]-L-deprenyl-D_2 λk_3 values before and after treatment as well as respective occupancy values induced by the drug regimens

	Caudate nucleus	Frontal cortex	Hippocampus	Occipital cortex	Parietal cortex	Putamen	Temporal cortex	Thalamus
EVT 25 mg								
Pre-treatment	0.27 (0.16–0.32)	0.13 (0.08–0.16)	0.21 (0.16–0.24)	0.11 (0.06–0.13)	0.12 (0.07–0.13)	0.24 (0.12–0.29)	0.13 (0.07–0.15)	0.23 (0.13–0.28)
Post-treatment	0.06 (0.04–0.07)	0.04 (0.03–0.05)	0.05 (0.03–0.06)	0.04 (0.03–0.05)	0.04 (0.03–0.04)	0.06 (0.04–0.07)	0.04 (0.03–0.05)	0.05 (0.03–0.07)
Occupancy (%)	78.2 (70.2–88.1)	65.2 (47.1–79.6)	76.3 (71.6–87.0)	58.0 (31.7–75.8)	64.2 (42.6–79.7)	74.6 (61.9–87.4)	65.3 (45.7–80.1)	76.9 (67.9–88.0)
EVT 75 mg								
Pre-treatment	0.29 (0.24–0.36)	0.15 (0.12–0.16)	0.24 (0.20–0.26)	0.12 (0.09–0.14)	0.12 (0.11–0.14)	0.27 (0.22–0.34)	0.14 (0.11–0.16)	0.26 (0.21–0.33)
Post-treatment	0.04 (0.03–0.04)	0.04 (0.03–0.04)	0.03 (0.03–0.04)	0.03 (0.03–0.04)	0.03 (0.03–0.04)	0.04 (0.04–0.05)	0.03 (0.03–0.04)	0.03 (0.03–0.04)
Occupancy (%)	87.2 (84.9–89.3)	75.3 (68.7–82.5)	85.8 (83.6–89.2)	69.7 (60.4–76.8)	72.9 (64.0–78.8)	85.3 (83.4–87.8)	75.1 (67.7–81.5)	86.7 (84.1–89.9)
EVT 75 mg AD								
Pre-treatment	0.30 (0.27–0.33)	0.17 (0.15–0.19)	0.27 (0.22–0.30)	0.13 (0.12–0.15)	0.14 (0.12–0.16)	0.30 (0.28–0.31)	0.15 (0.13–0.18)	0.27 (0.23–0.31)
Post-treatment	0.03 (0.02–0.04)	0.03 (0.02–0.05)	0.03 (0.02–0.05)	0.03 (0.02–0.05)	0.03 (0.02–0.04)	0.04 (0.02–0.05)	0.03 (0.02–0.05)	0.03 (0.02–0.05)
Occupancy (%)	89.8 (86.4–92.9)	80.3 (74.2–85.4)	89.1 (83.7–93.7)	75.3 (69.4–82.3)	79.5 (74.5–84.6)	87.6 (82.4–91.7)	79.3 (74.4–84.9)	89.1 (84.6–93.7)
EVT 150 mg								
Pre-treatment	0.32 (0.28–0.35)	0.16 (0.14–0.18)	0.24 (0.19–0.26)	0.13 (0.11–0.14)	0.14 (0.12–0.15)	0.29 (0.25–0.32)	0.15 (0.12–0.17)	0.27 (0.24–0.30)
Post-treatment	0.03 (0.02–0.04)	0.03 (0.02–0.04)	0.03 (0.03–0.04)	0.03 (0.02–0.04)	0.03 (0.02–0.04)	0.04 (0.03–0.05)	0.03 (0.02–0.04)	0.03 (0.02–0.04)
Occupancy (%)	90.4 (87.4–95.0)	79.7 (74.7–86.4)	87.4 (84.6–90.6)	73.1 (67.7–80.5)	78.3 (73.5–84.9)	87.5 (84.8–91.6)	78.9 (73.7–86.2)	89.0 (87.1–92.2)
Selegiline 10 mg								
Pre-treatment	0.31 (0.28–0.42)	0.16 (0.13–0.20)	0.24 (0.21–0.30)	0.13 (0.11–0.17)	0.14 (0.12–0.16)	0.29 (0.23–0.39)	0.15 (0.12–0.19)	0.27 (0.23–0.34)
Post-treatment	0.03 (0.02–0.04)	0.03 (0.02–0.03)	0.03 (0.02–0.04)	0.03 (0.02–0.03)	0.03 (0.02–0.03)	0.03 (0.02–0.04)	0.03 (0.02–0.03)	0.03 (0.02–0.04)
Occupancy (%)	91.6 (89.0–93.6)	81.4 (77.7–84.0)	89.4 (86.5–90.7)	77.5 (74.7–81.1)	80.6 (77.5–82.5)	90.1 (91.1–89.0)	82.2 (78.9–84.5)	91.1 (89.7–92.2)

Values are means (range). The treatment effects were statistically highly significant in all brain regions ($P < 0.0001$, analysis of variance).

AD, Alzheimer's disease.

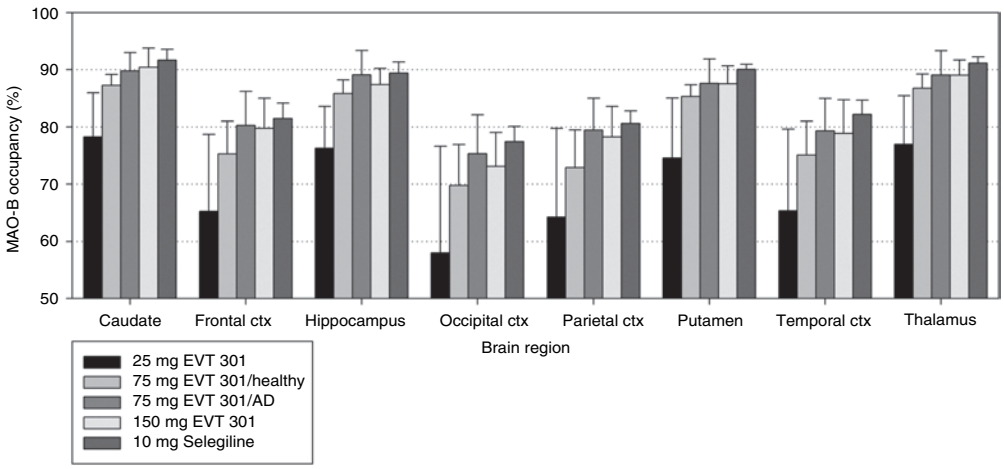

Figure 1 Regional monoamine oxidase type B (MAO-B) occupancy values associated with the various drug regimens. Error bars represent SDs. AD, patients with Alzheimer's disease; ctx, cortex.

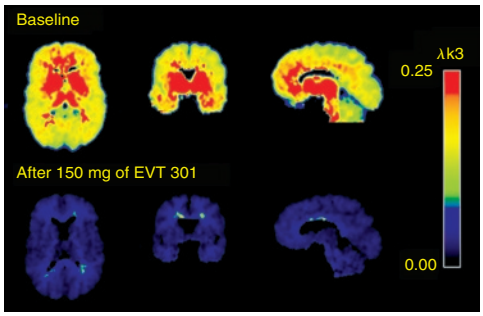

Figure 2 A visualization of monoamine oxidase type B occupancy in the brain, induced by 150 mg of EVT 301 daily. Axial, coronal, and sagittal slices of [^{11}C]-L-deprenyl-D_2 positron emission tomography (PET) images from subject 2 are presented before (top row) and after (bottom row) drug treatment. PET images are parametric λk_3 maps created by estimating λk_3 for each voxel separately using a model similar to that used in the region of interest–level analyses.

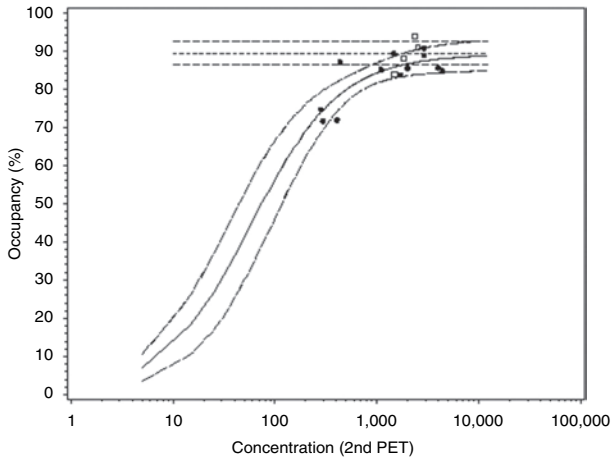

Figure 3 Nonlinear regression between the plasma concentration of EVT 301 during the second positron emission tomography (PET) scan and monoamine oxidase type B occupancy in the brain. Filled dots are from healthy subjects; open squares represent Alzheimer's disease patients. The solid line is the regression curve, and long-segment broken lines represent its 95% confidence interval. The horizontal lines represent the mean occupancy level induced by 10 mg of selegiline and its 95% confidence interval.

EVT 301 would be required to induce occupancy rates similar to those produced with a 10 mg daily dose of selegiline.

Regional cerebral blood flow

Regional cerebral blood flow (rCBF) was measured as a negative control variable to explicitly exclude the potential confounding effects of treatment-induced changes in rCBF. For one AD patient, valid [^{15}O]H_2O scan data were not acquired because of camera malfunction, and therefore rCBF results are presented for 19 subjects. As compared to baseline values, neither EVT 301 nor selegiline 10 mg changed the rCBF in any brain region (P = 0.091–0.976). Also, no differences in the change of rCBF were observed between the different daily doses of EVT 301 and selegiline 10 mg (data not shown).

As methodological validation, regression analysis was carried out for the rCBF values against the parameters K_1 (which should represent radiotracer delivery dependent on rCBF) and λk_3 (which should reflect specific binding and be independent of rCBF). As expected, rCBF values were not associated with λk_3 values in any brain region (all Ps >0.2) but were highly correlated with K_1 values (Pearson R = 0.68–0.84, all Ps < 0.001).

MAO-B inhibition in platelets

The determination of MAO-B inhibition in platelets was included to explore whether this variable can be used as an estimate of MAO-B occupancy in the brain, which would significantly reduce the costs of studying MAO-B occupancy in clinical drug trials. The mean extent of MAO-B inhibition in blood platelets was 44 ± 8% (33–51%) in control subjects on EVT 301 at a dose of 25 mg daily, 60 ± 26% (22–81%) in control subjects on EVT 301 at 75 mg daily, 72 ± 10% (58–80%) in control subjects on EVT 301 at 150 mg daily and 98 ± 2% (95–99%) in control subjects on selegiline at 10 mg daily. In AD subjects on EVT 301 at 75 mg daily, it was 63 ± 4% (57–67%). The extent of MAO-B inhibition in platelets was greater in control subjects receiving selegiline at a dose of 10 mg than in subjects receiving any dose level of EVT 301 (P = 0.016–0.001). At a dose of 75 mg of EVT 301, no difference in platelet MAO-B inhibition was observed between AD patients and control subjects. Control experiments carried out with blood platelets collected from nonmedicated individuals indicated that the MAO-B inhibition associated with EVT 301 is reversible, with a half-life of ~60 min, whereas inhibition by selegiline is irreversible (data not shown).

Plasma DHPG concentrations

Plasma 3,4-dihydroxyphenyl glycol (DHPG) was measured to exclude any effects of the drug treatments on MAO-A. These analyses are presented in **Supplementary Data** online.

DISCUSSION

The main aim of this study was to determine a daily dose level of EVT 301 that would be sufficient to produce near-complete occupancy of the drug target, the MAO-B enzyme in the brain. PET imaging with [^{11}C]-L-deprenyl-D_2 as tracer was used to assess MAO-B occupancy in the brain. Daily dosing with selegiline at 10 mg was used in a control group of four healthy elderly subjects to validate the assessment of MAO-B occupancy. MAO-B occupancy in the brain was considered the main outcome measure, and other variables were considered to be supplementary.

MAO-B occupancy in the brain, as determined using [^{11}C]-L-deprenyl-D_2-PET, was 77–92% (mean values) in various brain regions in control subjects on selegiline at 10 mg daily. This appears to be the practical upper limit of pharmacologically achievable inhibition of the uptake of the employed tracer into the brain, because this daily dosage of selegiline has previously been reported to cause nearly complete inhibition of the activity of this enzyme.[10]

In healthy elderly control subjects, MAO-B occupancy in the brain, as measured using [^{11}C]-L-deprenyl-D_2-PET, did not differ after at least 7 days of treatment with EVT 301 at doses of 75 or 150 mg daily from that observed after at least 7 days of treatment with the irreversible MAO-B inhibitor, selegiline (10 mg daily). EVT 301 at 25 mg daily was associated with lower levels of

MAO-B occupancy in the brain. In patients with AD, EVT 301 75 mg daily was associated with MAO-B occupancy in the brain similar to that observed in elderly control subjects treated with EVT 301 at 75 or 150 mg daily or with selegiline at 10 mg daily.

The [^{11}C]-L-deprenyl-D_2 binding capacity at baseline, reflecting MAO-B activity in the brain, was highest in the striatum and thalamus and lower in the cortical areas in the brains of healthy control subjects, consistent with earlier findings.[11] At baseline, AD patients had, on an average, 18% higher [^{11}C]-L-deprenyl-D_2 binding capacity in the hippocampus than control subjects did, and other brain regions showed a similar but statistically nonsignificant tendency. In previous studies conducted postmortem, increased MAO-B activity has been reported in several brain regions of AD patients, including the hippocampus.[5,12] However, this study was not primarily designed to compare MAO-B activity between AD patients and healthy control subjects, because the groups were small and the subjects with AD were older than the controls.

No significant drug-associated changes were observed in cerebral blood flow, as assessed using [^{15}O]H_2O. This suggests that the drug-related changes observed in [^{11}C]-L-deprenyl-D_2 binding were not confounded by concomitant alterations in cerebral blood flow. This notion is also supported by the lack of significant correlations between λk_3 and rCBF measures.

The extent of MAO-B inhibition in blood platelets was significantly greater in subjects receiving selegiline at 10 mg than in subjects receiving EVT 301. This finding is in disagreement with the PET results, in which MAO-B occupancy in the brain was similar after EVT 301 doses of 75 and 150 mg or of a selegiline dose of 10 mg daily. One possible explanation for this is that selegiline is an irreversible inhibitor of MAO-B, whereas EVT 301 is bound to the enzyme in a reversible manner with a dissociation half-life of ~60 min at 37 °C. Some dissociation of the drug from the enzyme may thus have taken place during the handling or storage of the blood platelets or during the assay procedure. This suggests that inhibition of platelet MAO-B activity is not a valid predictor of MAO-B occupancy in the brain by EVT 301, at least not when assessed using a conventional radioenzymatic assay method.

Arterial cannulation is invasive and should be avoided in clinical studies whenever possible. A useful reference region for PET analyses would obviate the need for arterial blood sampling.[13] In this study, we attempted to validate the use of the cerebellum as a reference region by observing the specific binding of [^{11}C]-L-deprenyl-D_2 after treatment with the MAO-B inhibitors. Both EVT 301 and selegiline induced profound decreases in the binding of [^{11}C]-L-deprenyl-D_2 in the cerebellum. This suggests the presence of non-negligible amounts of the MAO-B enzyme in the human cerebellum, invalidating the use of the cerebellum as a reference region for [^{11}C]-L-deprenyl-D_2 studies.

Limitations of this study include the small sample size per group and the inclusion of two irregular smokers. Future studies with larger sample sizes would provide more robust estimates of the mean values of MAO-B occupancy in the brain for the population as a whole.

In conclusion, 7 days of treatment with either 75 or 150 mg of EVT 301 daily resulted in a near-complete occupancy of the drug target in the brain, namely, the MAO-B enzyme. This study demonstrates and validates the usefulness of measuring drug-induced MAO-B occupancy in the living human brain using PET and [^{11}C]-L-deprenyl-D_2.

METHODS

The study was approved by the Ethics Committee of the Hospital District of Southwest Finland and was conducted in accordance with the Declaration of Helsinki. The National Agency for Medicines, as the competent regulatory authority in Finland, was notified before the commencement of the study. All subjects gave their written informed consent. Consent was also received from a caregiver of each AD patient.

Subjects and study design. Sixteen healthy control subjects (10 women and 6 men) and four subjects with AD (2 women and 2 men) were recruited for the study. Their demographic characteristics are summarized in **Table 3**. The most common concomitant treatment was an antihypertensive drug. All AD patients used stable anticholinesterase medication (rivastigmine 3 mg and 6 mg b.i.d., galantamine 16 mg q.d., and donepezil 10 mg q.d.). All subjects were instructed to follow a diet with low tyramine content.

This was a randomized, single-center, open-label, multiple-dose study. The control subjects were randomized into four groups to receive daily doses of 25, 75, or 150 mg of EVT 301 or 10 mg of selegiline for 7–14 days. Selegiline was administered as an established irreversible MAO-B inhibitor for comparison purposes. The AD patients were to receive daily doses of 75 mg of EVT 301 for 7–14 days. In practice, the treatment duration was 7 or 8 days in all subjects. Treatment compliance was assured by drug intake logs, daily phone calls to the caregivers of the AD subjects, and tablet counting.

Table 3 Demographic characteristics of the study subjects (means ± SD)

	EVT 301 (25 mg)	EVT 301 (75 mg)	EVT 301 (150 mg)	Selegiline (10 mg)	EVT 301 (75 mg AD)
N	4	4	4	4	4
Age (years)	58.1 ± 4.7	63.5 ± 8.9	62.5 ± 8.0	58.6 ± 5.4	71.3 ± 4.8
Females, *n* (%)	2 (50)	3 (75)	3 (75)	2 (50)	2 (50)
Height (cm)	171 ± 5	167 ± 7	162 ± 5	169 ± 7	166 ± 11
Weight (kg)	81 ± 8	71 ± 12	70 ± 11	65 ± 4	67 ± 10
Irregular nicotine users, *n* (%)	1 (25)	0 (0)	1 (25)	0 (0)	0 (0)
Concomitant medication, *n* (%)	3 (75)	3 (75)	2 (50)	3 (75)	4 (100)
MMSE score	NA	NA	NA	NA	23.5 ± 3.7

AD, Alzheimer's disease; MMSE, mini-mental state–examination; NA, not applicable.

At baseline, each subject underwent a PET scan with $[^{15}O]H_2O$ to evaluate rCBF and with [^{11}C]-L-deprenyl-D_2 to reflect MAO-B enzyme activity in the brain. Blood was sampled to determine the baseline activity of MAO-B in platelets and for plasma concentrations of the deaminated metabolite of noradrenaline, DHPG. DHPG plasma concentrations were used as an indicator of possible drug effects on the enzyme MAO-A.[14] The subjects started their allocated medication immediately after the first PET scan session, and the second PET scan session was performed 24 h after the last drug administration.

Assessment of pharmacokinetics. Plasma concentrations of the test drug EVT 301 were measured at baseline just before the first PET scan, on 3 subsequent days during the week at trough value time points (just before the next administration of the drug), and at 22–24 h after the final drug administration (i.e., just before the second PET scan). The samples were shipped to Huntingdon Life Sciences, Huntingdon, UK, for analysis by high-performance liquid chromatography with mass spectrometric detection. The pharmacokinetic variables (EVT 301 concentrations and EVT 301 concentration fluctuation data) were summarized according to treatment group. A repeated-measures analysis of variance model was used to determine whether pharmacokinetic steady state had been achieved before the second PET scan. Differences in values on different days were estimated individually for each subject; in addition, intra-group and inter-group variations were also estimated.

Preparation of [^{11}C]-L-deprenyl-D_2. The precursors N-desmethyl-deuterium deprenyl hydrochloride and deuterium deprenyl hydrochloride were obtained from PharmaSynth AS, Tartu, Estonia. The preparation of [^{11}C]-L-deprenyl-D_2 (*N*-[methyl-^{11}C]-(R)-(-)-*N*-methyl-α-methyl-*N*-(1,1-dideutero-2-propenyl)-benzeneethaneamine, *N*-[methyl-^{11}C]-(-)-deuterium-deprenyl) from [^{11}C] methyl triflate was performed essentially as described for [^{11}C] deprenyl.[15]

Preparation of $[^{15}O]H_2O$. The oxygen-15 isotope was produced using a Cyclone 3 cyclotron (IBA Molecular, Louvain-la-Neuve, Belgium) by the $^{14}N(d,n)^{15}O$ nuclear reaction on natural nitrogen gas. When the nitrogen target gas contains 1%[16,17] oxygen, oxygen-15 isotope is recovered at the target output as $[^{15}O]O_2$ gas. $[^{15}O]O_2$ and H_2 were processed in an oven at 700 °C to water vapor. Radiowater, $[^{15}O]H_2O$, was produced in a continuous-working water module (Hidex RWG, Turku, Finland) using a diffusion membrane technique to trap radioactive water vapor within sterile saline solution.[18]

PET procedures. PET studies were carried out using an ECAT EXACT HR+ scanner (Siemens/CTI, Knoxville, TN) in three-dimensional mode. This camera records 63 slices of 2.43-mm thickness (axial FOV 155 mm) and has a spatial resolution of 4.4 mm in the horizontal plane and 4.1 mm axially (full width at half maximum). $[^{15}O]H_2O$ and [^{11}C]-L-deprenyl-D_2 scans were performed ~10 min apart and were preceded by a single transmission scan. The left radial artery was cannulated for collection of blood samples. $[^{15}O]H_2O$ was administered, using an automated system, into a right antecubital vein as a 15 s bolus. Brain uptake was traced for 2 min after the injection with concomitant measurement of the arterial blood radioactivity concentration using an automated online analyzer. The dose of $[^{15}O]H_2O$ was 362 ± 57 MBq (mean value ± SD), with no differences in the injected doses between the first and the second scans or between the groups (data not shown). [^{11}C]-L-deprenyl-D_2 was administered into the same vein as the bolus. The dose, mass, and specific activity of the injected radioligand were 482 ± 30 MBq, 2.5 ± 1.5 μg, and 39.6 ± 9.9 MBq/nmol, respectively (mean values ± SD), with no differences in the injected doses between the first and the second scans or between the groups (data not shown). The uptake of [^{11}C]-L-deprenyl-D_2 in the brain was followed for 60 min using 19 time frames: 4 × 30 s, 8 × 60 s, 7 × 300 s. For blood sampling, an automated online analyzer was used for the first 5 min, and manual blood samples for radioactivity measurements were drawn at 8, 10, 15, 20, 30, 45, and 60 min; radioactive metabolites were analyzed at 4, 10, 20, 30, 45, and 60 min. PET images were reconstructed into 256 × 256 × 63 volumes.

Analysis of arterial blood samples. The concentration of total radioactivity in arterial plasma was measured using an automated gamma counter (1480 Wizard 3; EG&G Wallac, Turku, Finland) cross-calibrated with the PET scanner. For metabolite analysis, plasma proteins were precipitated with acetonitrile containing cold L-deprenyl as internal standard. The supernatant was analyzed using high-performance liquid chromatography with ultraviolet detection and online radioactivity monitoring (Radiomatic 150TR, Flow Scintillation Analyzer; Packard, Meriden, CT). High-performance liquid chromatography separation was achieved with a μBondapak C-18 column (Waters, Milford, MA) and a gradient of acetonitrile and 50 mmol/l phosphoric acid. Radioactivity peaks were integrated, and unchanged [^{11}C]-L-deprenyl-D_2 was identified based on the retention times of radioactive [^{11}C]-L-deprenyl-D_2 and cold L-deprenyl at 208 nm. A Hill-type function was fitted to the unchanged fraction data, and a metabolite-corrected arterial plasma curve was constructed.

Quantitation of [^{11}C]-L-deprenyl-D_2 binding and rCBF. Individual dynamic [^{11}C]-L-deprenyl-D_2 and $[^{15}O]H_2O$ PET images were realigned within-subject, and T1-weighted magnetic resonance images were co-registered with the mean images of PET–PET-realigned summated [^{11}C]-L-deprenyl-D_2 images, as previously described.[13] To obtain regional time–activity curves, regions of interest were drawn on co-registered magnetic resonance images using Imadeus software (version 1.2; Forima, Turku, Finland). Regions of interest were drawn bilaterally on three to six transaxial slices onto the caudate nucleus, putamen, thalamus, hippocampal complex (including amygdala, hippocampus, and parahippocampal gyrus), frontal cortex, temporal cortex, parietal cortex, occipital cortex, and cerebellum. Radioactivity concentrations in the left and right hemispheres were averaged. Time–activity curves were weighted on the basis of frame duration and counts per frame, a fixed 2.6% vascular component (whole blood) in the tissue was assumed and subtracted from regional time–activity curves, and a correction was made for the delay in data acquisition start times between PET and blood data.

The kinetics of [^{11}C]-L-deprenyl-D_2 was modeled using a two-tissue compartmental model in which the tissue compartments represent the free plus nonspecifically bound and specifically bound radiotracer, using the metabolite-corrected arterial plasma curve as the input function.[19] The parameter λk_3 (where $\lambda = K_1/k_2$) was solved using a nonlinear approach and was used as an estimate for specific binding to MAO-B because it is independent of perfusion and has better reproducibility than k_3 alone.[19–21] Occupancy of MAO-B in the brain by EVT 301 and selegiline was calculated from regional λk_3 values as follows:

$$\text{Occupancy} = 100\% \times \frac{\left|\lambda k_{3\text{after}} - \lambda k_{3\text{baseline}}\right|}{\lambda k_{3\text{baseline}}}.$$

Perfusion (ml (blood)/min/dl (tissue)) was calculated on the basis of a one-tissue compartmental model using arterial radioactivity concentration as the input function.[22] Blood data were corrected for delay, the partition coefficient was set to 0.8, and the vascular component of the region of interest was assumed to be 1.5% of total blood radioactivity (arterial component).

MAO-B inhibition in platelets and plasma DHPG concentrations. The extent of MAO-B inhibition in blood platelets was determined as the percentage difference in MAO-B activity in blood platelets collected at baseline and in a second sample taken 22–24 h the after final dose of test

drugs, i.e., just before the second PET scan. Samples (10 ml) of venous blood were collected into chilled evacuated tubes containing 0.1 ml of Na_2-EDTA solution. Platelets were isolated and stored at −70 °C until analysis. The MAO-B activity in human isolated platelets is stable for at least 2 years at this temperature. MAO-B activity was assayed radiochemically using [^{14}C]-phenylethylamine as the substrate. Protein content was measured with bovine serum albumin as the standard.[14] Some control experiments were carried out with blood platelets collected from nonmedicated individuals and pure reference substances (selegiline from Sigma-Aldrich, St. Louis, MO; EVT 301 from Evotec AG, Hamburg, Germany). Platelets were first incubated with the inhibitors (100 nmol/l of selegiline or 3 µmol/l of EVT 301) for 30 min at 37 °C to induce complete MAO-B inhibition, and the reversibility of the inhibition was then investigated by diluting the samples 1:100 with buffer.[23] The extent of MAO-B inhibition present in the diluted samples incubated at 37 °C was assessed at several time points to determine the reversibility of the drugs.

Statistical analyses. Regional MAO-B occupancy induced by the drug regimens was considered the primary outcome variable. Differences in MAO-B occupancy between the treatment groups were analyzed by means of analysis of variance, with MAO-B occupancy as the response variable and treatment group as the fixed factor, followed by *t*-tests for groupwise comparisons. MAO-B occupancy in the brain was related to the plasma levels of EVT 301. A nonlinear regression curve in the control subject group (EVT 301 groups) was fitted for MAO-B occupancy as a function of steady-state plasma drug concentrations. From this regression analysis, the plasma EVT 301 concentration required to reach the average extent of MAO-B occupancy observed in the selegiline group was estimated. Two-sided 95% confidence intervals were calculated both for the regression curve and for the average MAO-B occupancy in the selegiline group. MAO-B inhibition in blood platelets, plasma DHPG concentrations, and rCBF data were considered secondary outcome variables and were analyzed in a manner similar to that described earlier for the primary efficacy variable.

CONFLICT OF INTEREST

This study was financially supported by Evotec. The authors declared no other conflict of interest.

Supplementary Material is linked to the online version of the paper at http://www.nature.com/cpt

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Dapagliflozin, a Novel, Selective SGLT2 Inhibitor, Improved Glycemic Control Over 2 Weeks in Patients With Type 2 Diabetes Mellitus

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Dapagliflozin, administered to patients in once-daily oral doses, is a sodium–glucose cotransporter 2 (SGLT2) inhibitor that blocks the reabsorption of glucose from urine into the blood. This 14-day study randomized patients with type 2 diabetes mellitus (T2DM) to four treatment groups receiving daily oral doses of 5-, 25-, or 100-mg doses of dapagliflozin or placebo, in order to evaluate glucosuria and glycemic parameters. Significant reductions in fasting serum glucose (FSG) were observed on day 2 with 100 mg dapagliflozin (−9.3%, $P < 0.001$), and dose-dependent reductions were observed on day 13 with the 5-mg (−11.7%; $P < 0.05$), 25-mg (−13.3%; $P < 0.05$), and 100-mg (−21.8%; $P < 0.0001$) doses as compared with placebo. Significant improvements in oral glucose tolerance test (OGTT) were observed with all doses on days 2 and 13 ($P < 0.001$ as compared with placebo). On day 14, urine glucose values were 36.6, 70.1, and 69.9 g/day for the 5-, 25-, and 100-mg doses (as compared with no change for placebo), which were slightly lower than those on day 1. This was attributed to the decrease in filtered glucose load following improved glycemic control. Dapagliflozin produced dose-dependent increases in glucosuria and clinically meaningful changes in glycemic parameters in T2DM patients.

Type 2 diabetes mellitus (T2DM) is a disorder characterized by elevated serum glucose. Hyperglycemia is well established as a major risk factor for microvascular and potentially macrovascular complications of diabetes. In addition, there is strong evidence to suggest that hyperglycemia *per se* has a deleterious effect on insulin secretion and reduces insulin sensitivity—an effect referred to as glucotoxicity—and this, in turn, contributes to the progression of diabetes.[1,2] The majority of therapies that treat T2DM work on the insulin signaling pathway or on insulin secretion itself, and none lowers blood glucose through insulin-independent mechanisms.

A novel approach to reducing serum glucose in T2DM patients is by therapeutic inhibition of glucose reabsorption in the kidney.[3] The kidney plays an important role in the overall control of glucose, given that glucose is filtered through the glomeruli at the rate of ~8 g/h and almost completely reabsorbed in the proximal tubule via sodium–glucose cotransporters (SGLTs). This SGLT-mediated reabsorption of glucose is highly efficient, with <0.5 g of glucose being eliminated in the urine per day in healthy individuals.[4] SGLT2, 1 of 14 transmembrane-domain SGLTs,[4,5] is found primarily in the brush border membrane of the S1 segment of the proximal tubule in the kidney.[6] This specific isoform is responsible for reabsorbing the majority of glucose filtered at the glomerulus and has very little expression in other tissues, including the brain, liver, and heart.[4,7]

Inhibition of SGLT2 is a rational insulin-independent therapeutic approach to lowering blood glucose through renal glucose reabsorption,[8,9] resulting in a mandatory increase in urinary glucose and loss of calories. Dapagliflozin is the first in a new class of selective oral SGLT2 inhibitors currently in development for the treatment of T2DM.[10,11] As demonstrated in two phase I studies,[12] dapagliflozin inhibited up to 50% of filtered glucose from being reabsorbed by the kidney, thereby leading to glucose excretion of ~60 g/day and up to 3 g/h. Close-to-maximal inhibition was evident with doses between 20 and 100 mg, and this served as the rationale for dose selection in this phase IIa study. In this study we investigated whether (i) overall drug exposure by itself is comparable between healthy subjects and T2DM patients, (ii) dapagliflozin produces dose-dependent increases in glucosuria in T2DM patients (i.e., in subjects with increased glucose load), (iii) increases in glucosuria lead to changes in glycemic parameters (e.g., fasting serum glucose (FSG) and serum glucose following oral glucose tolerance test (OGTT)) in T2DM patients, and (iv) changes in glycemic parameters result in differences in glucosuria between days 1 and 14. Preliminary data from this study were presented at the

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67th Scientific Sessions of the American Diabetes Association, 22–26 June 2007.[13]

RESULTS

A total of 47 subjects (19 men and 28 women) with established T2DM and unimpaired renal function were randomly assigned to one of four treatment arms: 5, 25, or 100 mg dapagliflozin, or placebo. The treatment groups were balanced with respect to demographic and baseline characteristics. The ages and body weights of the subjects ranged from 55 to 60 years and from 84 to 91 kg, respectively. The subjects were of various races, including white (Hispanic and non-Hispanic/Latino), black/African American, Asian, and Filipino. The median estimated glomerular filtration rate (eGFR) of the subjects was 89 ml/min/1.73 m^2, with a range of 60–136 ml/min/1.73 m^2. One subject withdrew consent and discontinued partway through the study. Eighteen subjects who had been taking a stable dose of metformin for at least 4 weeks before entry into the study continued taking their maintenance dose during the study (six in the 5-mg group, five in the 25-mg group, and seven in the 100-mg group).

Safety and tolerability

In this study, multiple oral doses of 5, 25, and 100 mg dapagliflozin over a 14-day period, either alone or with metformin, were found to be safe and well tolerated. There were no deaths, serious adverse events, or discontinuations due to adverse events (AEs). AEs did not appear to be dose-related with respect to either dapagliflozin or metformin. The most frequent treatment-emergent AEs were typically gastrointestinal in nature (**Table 1**) and were more frequent in subjects who were taking metformin concomitantly.

Two episodes of hypoglycemia were reported as AEs, and these resolved spontaneously. One subject who was on a daily dose of 5 mg dapagliflozin plus metformin had a blood glucose level nadir (by glucometer) of 39 mg/dl on day 13. Another subject who was on a daily dose of 25 mg dapagliflozin plus metformin had a blood glucose level nadir (by glucometer) of 75 mg/dl on day 14. In addition, two subjects (one receiving daily dose of 100 mg of dapagliflozin plus metformin and one receiving the 25-mg daily dose of dapagliflozin alone) had vulvovaginal mycotic infections on day 11. The conditions were mild in both subjects and were resolved with miconazole. Dizziness was reported in one subject receiving the 5-mg daily dose of dapagliflozin and in two subjects receiving the 100-mg dose, and asthenia was reported in two subjects receiving the 5-mg daily dose and in one subject receiving the 100-mg dose. All three subjects who reported dizziness and two of three subjects who reported asthenia were taking metformin concomitantly.

Multiple oral doses of 5, 25, and 100 mg dapagliflozin produced no changes that were apparent during physical examination nor any changes in eGFR; renal tubular markers, including N-acetyl-β-D-glucosaminidase and β2-microglubulin; plasma electrolyte levels; vital signs; or electrocardiograms (data not shown). Although microalbuminuria was not specifically listed as an exclusion criterion, only 7 of 47 subjects had abnormal urinary albumin levels ≥30 mg/24 h on one or more occasions.

Table 1 Treatment-emergent adverse events (AEs) reported in >5% of subjects receiving dapagliflozin

	Dapagliflozin dose			
Adverse event	5 mg ($n = 11$)	25 mg ($n = 12$)	100 mg ($n = 16$)	Placebo ($n = 8$)
Total subjects with an AE, n *(%)*	8 (72.7)	4 (33.3)	8 (50)	7 (87.5)
Asthenia	2 (18.2)	0	1 (6.3)	0
Blepharospasm	0	0	1 (6.3)	0
Chest pain	0	0	1 (6.3)	0
Constipation	1 (9.1)	1 (8.3)	2 (12.5)	3 (37.5)
Diarrhea	1 (9.1)	0	2 (12.5)	1 (12.5)
Dizziness	1 (9.1)	0	2 (12.5)	0
Dyspepsia	1 (9.1)	1 (8.3)	0	0
Epigastric discomfort	2 (18.2)	0	0	0
Flank pain	1 (9.1)	0	0	0
Headache	0	1 (8.3)	2 (12.5)	1 (12.5)
Hot flush	0	0	1 (6.3)	0
Hypoglycemia	1 (9.1)	1 (8.3)	0	0
Nausea	2 (18.2)	0	2 (12.5)	1 (12.5)
Paraesthesia	0	0	1 (6.3)	0
Pharyngolaryngeal pain	1 (9.1)	0	0	0
Thermal burn	0	0	1 (6.3)	0
Tremor	0	1 (8.3)	0	0
Toothache	1 (9.1)	0	0	0
Vulvovaginal mycotic infection	0	1 (8.3)	1 (6.3)	0

Across all doses of dapagliflozin, there was no apparent change in the 24-h urine excretion of electrolytes after 14 days of dosing, with the exception of an acute transient increase in sodium excretion observed on day 1. During the first 24 h after administration of the drug dose, median changes from baseline in sodium excretion were −15.1 mEq for the placebo group, and +34.7, +40.2, and +48.0 mEq for the 5-, 25-, and 100-mg dapagliflozin dose groups, respectively. By day 13, the earlier increase in sodium excretion appeared to have normalized; median changes from baseline at this time point were +16.4 mEq for placebo and +1.8, +8.9, and −5.7 mEq for the 5-, 25-, and 100-mg dapagliflozin dose groups, respectively.

Change in glycemic parameters and glucosuria

Dapagliflozin induced a dose-dependent lowering of FSG on day 13 (**Figure 1a**). On day 2, FSG significantly decreased from baseline after a single dose of 100 mg of dapagliflozin (−9.3%, $P < 0.001$). On day 13, dapagliflozin treatment was associated with dose-dependent reductions in FSG of −11.7% ($P < 0.05$), −13.3% ($P < 0.05$), and −21.8% ($P < 0.0001$), corresponding to absolute mean reductions in FSG of 18.8, 28.8, and 38.7 mg/dl in the 5-, 25-, and 100-mg dose groups, respectively. No significant reduction in FSG was observed in subjects who received the placebo.

Dapagliflozin induced a significant reduction in post-OGTT glucose excursion, as measured in terms of the area under the

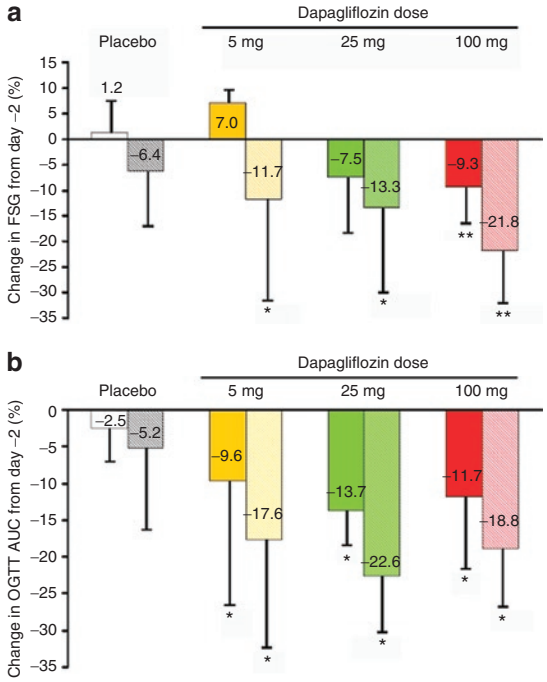

Figure 1 Effect of dapagliflozin on fasting serum glucose (FSG) and postprandial glucose. (**a**) Effect on FSG. Open bars, day 2; striped bars, day 13; *$P < 0.05$, **$P < 0.001$. (**b**) Effect on postprandial glucose. Open bars, day 2; striped bars, day 13; *$P < 0.001$. AUC, area under the glucose concentration–time curve; OGTT, oral glucose tolerance test.

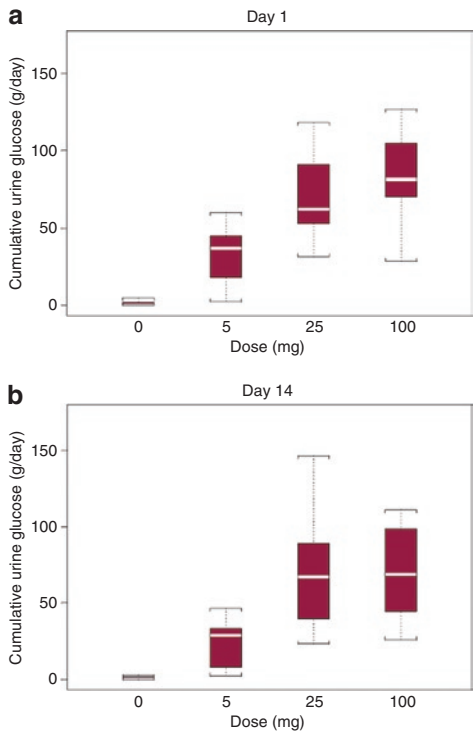

Figure 2 The total (cumulative) amount of glucose excreted in the urine over 24 h from type 2 diabetes mellitus patients was found to be dose-dependent. (**a**) Day 1 and (**b**) day 14. The midline numbers of each box are median values, and the boundaries of each box are 95% confidence intervals of the median. The median values for each are 36.9 g/day (5-mg dose, day 1) and 29.1 mg/day (5-mg dose, day 14); 62.3 g/day (25-mg dose, day 1) and 67.0 mg/day (25-mg dose, day 14); 79.7 g/day (100-mg dose, day 1) and 68.4 mg/day (100-mg dose, day 14).

plasma concentration–time curve (AUC) (0–4h), on days 2 and 13 of the study (**Figure 1b**). The overall reduction in post-OGTT glucose excursion was greater on day 13 (range: −17.6 to −22.6%; $P < 0.001$) than on day 2 (range: −9.6 to −13.7; $P < 0.001$). No significant change from baseline was seen at either time point in subjects who were given the placebo.

Daily urinary glucose excretion increased in a dose-dependent manner with dapagliflozin, and the 24-h cumulative amount of glucose excreted in the urine on days 1 and 14, respectively, is shown in **Figure 2a,b**. Although subjects remained dose-responsive to dapagliflozin at the end of the study, the amount of glucose excreted in the urine on day 14 appeared to be slightly lower than the amount excreted on day 1. After 2 weeks of once-daily treatment with the 5-, 25-, and 100-mg doses of dapagliflozin, urinary glucose excretion was 36.6, 70.1, and 69.9 g/day, respectively, as compared with 45.2, 75.3, and 81.3 g/day, respectively on day 1. Subjects receiving the placebo showed no change in cumulative 24-h urine glucose amounts or rate of glucose excretion throughout the study.

Minimal levels of glucose were present in the urine of all subjects before the study despite FSG values of up to 240 mg/ dl, thereby indicating that nearly 100% of filtered glucose was reabsorbed by the kidney. Baseline glucose clearance was negligible (i.e., <1 ml/min) in all treatment groups. Glucose clearance increased in all dapagliflozin groups 0–4 h after each dose and was 19.4, 35.2, and 35.1 ml/min for the 5-, 25-, and 100-mg doses as compared to 0.06 ml/min for placebo. On day 14, glucose clearance was 20.6, 40.8, and 42.1 ml/min for the 5-, 25-, and 100-mg dose groups, respectively, as compared to 0.97 ml/min

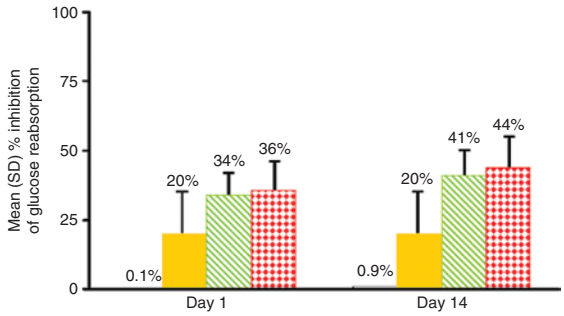

Figure 3 Percentage inhibition of glucose reabsorption by dapagliflozin on days 1 and 14. Open bars, placebo; filled bars, 5-mg dose; striped bars, 25-mg dose; and diamond bars, 100-mg dose.

for the placebo group. Effectively, the 5-, 25-, and 100-mg doses of dapagliflozin inhibited 20, 34, and 36%, respectively, of filtered glucose from being reabsorbed on day 1, and 20, 41, and 44%, respectively, as measured on day 14 (**Figure 3**). In this 14-day study, dapagliflozin had no apparent effect on serum insulin, serum fructosamine, or serum C-peptide levels, or on body weight (data not shown).

Pharmacokinetics

Mean dapagliflozin pharmacokinetic parameters of interest are summarized in **Table 2**. Dapagliflozin was rapidly absorbed

Table 2 Pharmacokinetic (PK) parameters of dapagliflozin

PK parameter	Dapagliflozin dose (mg)	Study day	
		Day 1	Day 14
C_{max} (ng/ml) Geometric mean (CV%)	5 ($n = 11$)	66 (37)	68[a] (32)
	25 ($n = 12$)	279 (19)	288 (41)
	100 ($n = 16$)	1,490 (40)	1,617[b] (46)
$AUC_{(TAU)}$ (ng·h/ml) Geometric mean (CV%)	5 ($n = 11$)	220 (32)	281[a] (28)
	25 ($n = 12$)	1,037 (25)	1,373 (31)
	100 ($n = 16$)	5,427 (30)	7,070[b] (36)
AI Geometric mean (CV%)	5 ($n = 11$)	—	1.23[a] (16)
	25 ($n = 12$)	—	1.32 (17)
	100 ($n = 16$)	—	1.33[b] (11)
% UR Mean (SD)	5 ($n = 11$)	1.44 (0.75)	2.02[a] (1.21)
	25 ($n = 12$)	0.83 (0.34)	1.28 (0.66)
	100 ($n = 16$)	1.49 (0.80)	2.41[b] (0.90)
CLR (ml/min) Mean (SD)	5 ($n = 11$)	5.88 (4.07)	6.37[a] (3.91)
	25 ($n = 12$)	3.44 (1.83)	3.95 (2.47)
	100 ($n = 16$)	4.41 (1.89)	5.50[b] (1.89)

AI, accumulation index; AUC, area under the glucose concentration–time curve; CLR, renal clearance; C_{max}, maximum observed plasma concentration; CV, coefficient of variation; UR, urinary recovery.

[a]$n = 10$. [b]$n = 14$.

after oral administration, with a median time to concentration of 1 h (range: 0.5–4 h). Dapagliflozin had a half-life of ~16 h, with modest increases in maximum observed plasma concentration C_{max} and $AUC_{(TAU)}$ of 30% after 14 days of dosing (accumulation index = 1.3). The increases in both C_{max} and $AUC_{(TAU)}$ were approximately proportional to the increase in dose. Across all dose groups, overall renal clearance of dapagliflozin was low, ranging from 3.4 to 6.4 ml/min. The percentage of dapagliflozin excreted intact into the urine over 24 h was <2.5%. In this study, there were no apparent interactions of dapagliflozin with metformin (data not shown).

DISCUSSION

Dapagliflozin, a selective SGLT2 inhibitor, has previously been shown to induce dose-dependent glucosuria in healthy subjects,[12] and we now observe a dose-dependent effect in T2DM patients. This is the first clinical study demonstrating that dapagliflozin produces not only dose-dependent increases in glucosuria but also induces insulin-independent improvements in glycemic parameters in T2DM patients (e.g., significant changes in FSG and serum glucose after OGTT). These findings are consistent with data from animal models that predicted that this insulin-independent mechanism could improve glycemic control.[11]

Once-daily administration of dapagliflozin resulted in a rapid, dose-dependent appearance of glucose in the urine and a sustained rate of glucosuria over the 24-h dosing intervals through to day 14, which was linked to decreased FSG and serum glucose following OGTT. Equally rapid and significant reductions were observed in FSG (by day 2 with 100 mg dapagliflozin and by day 13 with the 5- and 25-mg doses) and serum glucose following OGTT (all doses on days 1 and 14).

In this study, the amount of glucosuria induced by dapagliflozin was less than that reported in subjects with familial renal glucosuria, in whom renal glucose reabsorption can be completely absent. Familial renal glucosuria cases have been associated with glucose excretion of 30–144 g/day, often without associated hypoglycemia or renal dysfunction,[14–16] although a few subjects with genetic variations of familial renal glucosuria may experience renal sodium wasting and/or mild volume depletion.[17]

Dapagliflozin inhibited up to 40% of filtered glucose from being reabsorbed by the kidney, resulting in glucose excretion of up to 70 g/day or 3 g/h by day 14. The absence of complete inhibition may be the result of the action of additional SGLT2 or other tubular transport mechanisms such as SGLT1. Alternatively, the increased tubular concentration of glucose in response to dapagliflozin may compete with dapagliflozin for binding to SGLT2, thereby self-limiting the extent of inhibition of glucose reabsorption.

Given that dapagliflozin works independently of the action of insulin and results in incomplete inhibition of glucose reabsorption, hypoglycemia is not an expected side effect of treatment.[18,19] Hypoglycemia, of mild and moderate intensity, was reported in two patients during this study. Given the small sample size, the incidence of hypoglycemia requires further study and has been planned in larger trials with dapagliflozin.

Glucose is reabsorbed in the proximal tubule via SGLTs. In this study, we observed an acute transient increase in urine sodium as a result of SGLT2 inhibition. Changes in urine sodium were marginal after 2 weeks of once-daily treatment, indicating that urinary sodium excretion had normalized.

Symptomatic vulvovaginal Candida infections have been shown to be more prevalent in patients with diabetes than in the general population.[20,21] In this study, 2 of the 24 women who received dapagliflozin were diagnosed with mild vulvovaginal mycotic infections that resolved in 4 days with treatment. Although it is known that T2DM patients are prone to recurrent vaginal candidiasis,[22] it is not known whether the two women in this study had histories of similar infections. The incidence of genitourinary infection will be further assessed in longer-term clinical trials with larger populations.

The pharmacokinetics of dapagliflozin observed here in T2DM patients was comparable to that in healthy subjects[12] and has been adequately described in terms of a 2-compartment model with first-order absorption.[23] The levels of inhibition produced by dapagliflozin in T2DM patients and in healthy subjects (20–44% vs. 16–50%, respectively) were also comparable, resulting in higher amounts of glucosuria in T2DM subjects because of higher initial serum glucose levels[12] (**Figure 4**). These T2DM patients also appeared to demonstrate a greater variability in daily glucose excretion, a finding that may be caused by greater variability in filtered glucose load (i.e., the product of eGFR and serum glucose concentrations). In this study, the T2DM patients who had the highest FSG values had the greatest cumulative amount of glucose excreted in the urine on day 14; correspondingly, high serum glucose AUC values following OGTT were

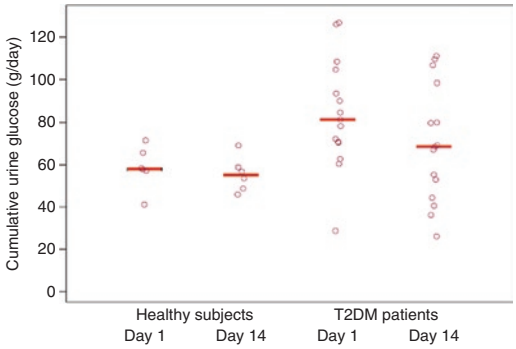

Figure 4 Total (cumulative) amount of glucose excreted in the urine on days 1 and 14 by healthy subjects and by type 2 diabetes mellitus (T2DM) patients on 100 mg dapagliflozin. Bars, median values; open circles, observed values. Data for healthy subjects from ref. 12.

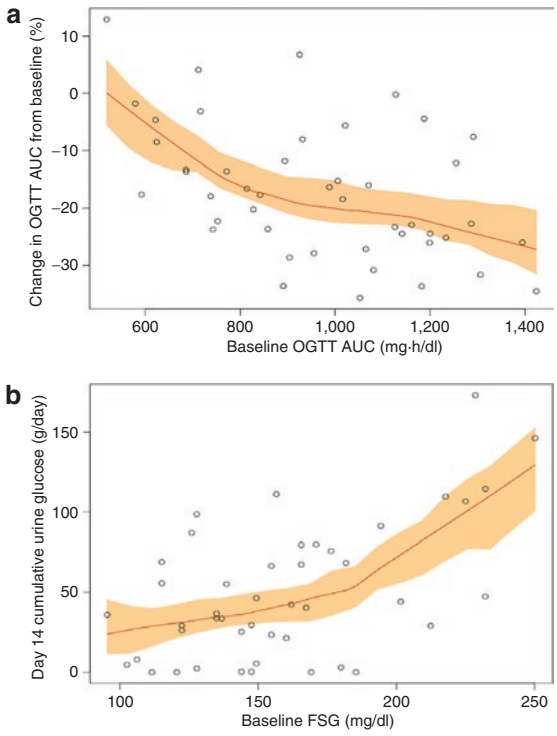

Figure 5 Baseline effect of glucose load parameters on serum and urine glucose on day 14. Higher baseline values are indicative of greater response to dapagliflozin. (**a**) Change in oral glucose tolerance test (OGTT) on day 14 relative to baseline OGTT status and (**b**) cumulative amount of glucose in the urine on day 14 relative to fasting serum glucose status. Lines: smooth line, shaded areas: 90% confidence intervals of the smoothed response from bootstrap analysis ($n = 500$).

associated with greater measurable responses to dapagliflozin treatment on day 14 (Figure 5a,b). Interestingly, a similar level of SGLT2 inhibition produced a lower amount of glucosuria in T2DM patients on day 14 than on day 1 (Figure 4). These findings suggest that SGLT2 activity is not upregulated in these T2DM patients over the course of 2 weeks and indicate that increases in glucose excretion lead to reduced filtered glucose loads in hyperglycemic subjects following improved short-term glycemic control, which in turn can result in lower amounts of glucosuria over time.

Inhibition of renal glucose reabsorption represents a potential therapeutic mechanism that utilizes the excretory capacity of the kidney to improve hyperglycemia through controlled, pharmacologically induced glucosuria. Additional studies are under way to determine whether the significant reductions in FSG and improvements in glucose tolerance observed in this study result in long-term glycemic control in patients with T2DM.

METHODS

Subjects. Men and women 18–70 years of age, with an established diagnosis of T2DM and receiving treatment with either a stable dose of metformin or diet alone (drug-naive), were eligible for participation in the study. Additional inclusion criteria were BMI <42 kg/m^2, FSG ≤240 mg/dl, glycosylated hemoglobin HbA_{1c} in the range of 6.0–10.0%, and unimpaired real function (serum creatinine ≤1.4 mg/dl for women and 1.5 mg/dl for men). Subjects were excluded if they had a history of renal, hepatic, cardiovascular, neurological, or gastrointestinal disease, or if they had undergone recent surgery, donated blood within 4 weeks prior to enrollment, had a history of polyuria and/or polydipsia within the past 2 months, or had a history of any of the following: diabetic ketoacidosis, hyperosmolar nonketotic syndrome, incontinence, or nocturia. Subjects were also excluded if they had prior exposure to any investigational drug or insulin, inducers or inhibitors of CYP1A1, prescription or over-the-counter medications, or herbal preparations within 1 week prior to the start of the study.

Before enrollment, signed informed consent, approved by institutional review boards and an independent ethics committee, was obtained from each subject. The study was conducted in accordance with the principles of the Declaration of Helsinki (as amended in 2000) and Good Clinical Practice, as defined by the International Conference on Harmonisation and ethical principles of the European Union Directive 2001/20/EC.

Study design. This double-blind, placebo-controlled, randomized, parallel-group, multiple-dose phase IIa study was conducted at five sites in the United States: Orlando Clinical Research Center, Orlando, FL; Diabetes and Glandular Disease Assoc., PA., San Antonio, TX; New Orleans Center for Clinical Research, New Orleans, LA; Elite Research Institute, Miami, FL; and Comprehensive Neuroscience, Fort Lauderdale, FL (Bristol-Myers Squibb study identifier MB102003; ClinicalTrials.gov identifier NCT00162305). Subjects were screened within 21 days of enrollment and randomly assigned in the ratio 1:1:1:2 to treatment groups of placebo, 5-, 25-, and 100-mg doses of dapagliflozin, respectively (BMS-512148; Bristol-Myers Squibb, Lawrenceville, NJ). By subsequent protocol amendment, eight additional subjects were to be enrolled and randomized 1:1 to the 5- and 25-mg dapagliflozin groups. Eventually, 8, 11, 12, and 16 subjects were randomized to the placebo, 5-, 25-, and 100-mg groups, respectively. Those who had been taking metformin for at least 4 weeks prior to the commencement of the study continued to receive their maintenance dose throughout the study. The use of metformin was not a criterion for stratifying patients for randomization. By coincidence, none of the subjects taking metformin was randomized to placebo. On day 1, subjects received either dapagliflozin or placebo at time point 0 h in a double-blind fashion, once daily, for 14 days. Metformin administration for each subject was according to the individual's dosing regimen established before the study.

The subjects were put on a fixed diet of 2,200 calories daily (60–70% from carbohydrate and monounsaturated fat, 15–20% from protein, 1.0 g phosphorus, 1.3 g calcium, and 6.0 g sodium chloride), and had free access to drinking water throughout the study. Meals were identical on days of pharmacokinetic and/or pharmacodynamic measurements (days −2, −1, 1, 2, 8, 13, and 14). Changes in body weight were not evaluated as a study end point. The subjects were discharged from the research unit on day 15 and were required to return on day 21 for follow-up.

Safety. Measurements performed at baseline, at various time points during the 14-day dosing period, and poststudy included vital signs, 12-lead electrocardiographic and physical examinations, and safety laboratory analyses involving routine hematology, serum chemistry, and urinalysis. AEs were assessed throughout the study. Investigators evaluated all clinical AEs and characterized them with respect to intensity, severity, duration, relationship to study drug, and outcome. Hypoglycemia was defined as the presence of symptoms (e.g., sweating, shaking, increased heart rate, confusion, dizziness, lightheadedness, or hunger) and/or documented glucose value ≤50 mg/dl on multiple occasions. In addition, 24-h urine volume, creatinine clearance, osmolality, electrolytes, and renal tubular markers (calcium, magnesium, sodium, potassium, phosphate, chloride, uric acid, oxalate, citrate, protein, albumin, N-acetyl-D-glucosaminidase, and β-2 microglobulin) were assessed at baseline, at various times during dosing, and after the study.

Pharmacodynamic measurements. Three end points were measured to determine glycemic efficacy in subjects receiving dapagliflozin: urinary glucose excretion and the glycemic parameters FSG and postprandial glucose measured by OGTT.

Blood samples were collected to assess FSG and 2-h postprandial glucose, insulin, and C-peptide concentrations at baseline and on various days during dosing. To measure serum glucose and serum insulin concentrations, blood samples were obtained at 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, and 4 h on days −2, 2, and 13. The area under the glucose concentration–time curve from time point 0 h to time point 4 h (AUC(0–4)) was calculated using log- and linear-trapezoidal summations. A baseline 75-g OGTT was administrated predose (day −2) and on days 2 and 13 (after a 10-h fast). The OGTT began at 0 h, with subjects being administered a 75 g Glucola oral solution (Ames Laboratories, Elkhart, IN).

The eGFR (ml/min/1.73 m^2) of the subjects was calculated as 186.3 × serum creatinine$^{-1.154}$ × age$^{-0.203}$ × 1.212 (for African-American subjects) × 0.742 (for female subjects), based on the auxiliary table for GFR estimation in accordance with the Modification of Diet in Renal Disease equation.[24] The filtered glucose load (FL) was calculated as the product of eGFR and mean serum glucose concentrations, and the renal (tubular) glucose reabsorption rate (TG) was calculated as TG = FL − ER. Urine samples were collected over a 24-h period on days −1, 1, and 14 to assess the rate and total amount of urinary glucose excreted (measured at 4-h intervals). The subjects were required to fast 8 h before and 8 h after dapagliflozin administration on those days. For each subject, the amount of renally filtered glucose was estimated on days −1, 1, and 14 from the eGFR (calculated using serum creatinine values) on days −2, 2, and 13, respectively. This value was multiplied by mean serum glucose concentrations over the 0–4, 4–8, and 8–12 h postdose intervals on days −1, 1, and 14, and the percentage inhibition of renal glucose reabsorption for each collection interval was calculated.

Pharmacokinetic measurements. Intensive plasma and urine sampling for dapagliflozin and metformin was carried out on days 1 and 14, and trough plasma samples were taken on days 2, 4, 6, 14, and 15. Plasma concentrations were used to derive maximum observed plasma concentration (C_{max}), time to C_{max} (t_{max}) half-life, and $AUC_{(TAU)}$ using standard noncompartmental methods. The accumulation index was calculated as the ratio of $AUC_{(TAU)}$ on day 14 to $AUC_{(TAU)}$ after the first dose. For each urine collection interval, the amount of dapagliflozin excreted was divided by the dose and multiplied by 100 to obtain (i) the percentage of dose excreted during each collection interval and (ii) the total percentage urinary recovery (% UR). The total amount of dapagliflozin excreted was divided by $AUC_{(TAU)}$ to obtain the renal clearance.

Liquid chromatography with tandem mass spectrometry detection measurement of dapagliflozin and metformin. Assays for dapagliflozin plasma and urine concentrations were performed using liquid chromatography with tandem mass spectrometry detection. For samples in human EDTA plasma, the between-run variability and within-run variability for the analytical quality controls of dapagliflozin were ≤10.9 and ≤10.9% coefficient of variation (CV), respectively. For samples in human urine, the between-run variability and within-run variability for the analytical quality controls of dapagliflozin were ≤5.0 and ≤6.1% CV, respectively (Bristol-Myers Squibb Department of Bioanalytical Sciences, Lawrenceville, NJ).

The assay for metformin plasma concentrations was also performed using liquid chromatography with tandem mass spectrometry detection. For samples in human EDTA plasma, the between-run variability and within-run variability for the analytical quality controls of metformin were ≤3.19 and ≤6.67% CV, respectively. Reference and internal standards for metformin hydrochloride (HCL) and metformin HCL-d_6 were obtained from USP and PPD Discovery (PPD, Richmond, VA).

Statistical analyses. The administration of dapagliflozin to 8 subjects per group or 16 subjects per group provided 80% probability of observing at least one AE in a group with 18 or 10% incidence, respectively. Summary statistics were calculated on days 1 and 14 for data relating to dapagliflozin maximum observed plasma concentration, $AUC_{(TAU)}$, accumulation index, time to maximal concentration, percentage urinary recovery, and renal clearance in plasma and urine. Summary statistics and corresponding changes from baseline were tabulated for total amount of glucose excreted in urine over 24 h, rate of glucose excretion over each collection interval, and percentage inhibition of renal glucose reabsorption. Similar analyses were carried out for serum insulin and serum C-peptide. For all safety markers, summary statistics were tabulated for the total amount excreted in urine over 24 h for serum concentration values and for corresponding changes from baseline relative to treatment group and study day.

To determine the effect of dapagliflozin on FSG concentrations, analysis of variance was performed. The fixed effect was the study day and the random effect was the subject. Point estimates and 90% confidence intervals for study day differences (day 2 to day −2, and day 13 to day −2) were provided. Analysis of variance was also carried out on serum glucose AUC(0–4) after OGTT. The fixed effect was study day and the random effect was the subject. *A priori*, the variable AUC(0–4) was log transformed. Point estimates and 90% confidence intervals for study day differences on the log scale were exponentiated to obtain point estimates and 90% confidence intervals for the day 2 to day −2 and day 13 to day −2 ratios of the geometric mean values of AUC(0–4) after OGTT. All statistical analyses were performed using SAS/STAT version 8.2 (SAS Institute, Cary, NC).

A nonparametric bootstrapping with 500 replications was applied to assess the uncertainty of tested (or characterized) relationships between glycemic parameters (i.e., baseline OGTT AUC vs. change in OGTT AUC and baseline FSG vs. day 14 cumulative urine glucose) (**Figure 5**), using the S-Plus 6.2 software package (TIBCO Software, Palo Alto, CA). Bootstrapping is a resampling methodology that provides a nonparametric assessment of the variances and confidence intervals without requiring asymptotic assumption on the distribution of parameters. Fifth, 50th, and 95th percentiles of the smoothed responses were obtained by means of this bootstrap analysis.

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CONFLICT OF INTEREST

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Dapagliflozin, a Novel SGLT2 Inhibitor, Induces Dose-Dependent Glucosuria in Healthy Subjects

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Dapagliflozin selectively inhibits renal glucose reabsorption by inhibiting sodium–glucose cotransporter-2 (SGLT2). It was developed as an insulin-independent treatment approach for type 2 diabetes mellitus (T2DM). The safety, tolerability, pharmacokinetics, and pharmacodynamics of the drug were evaluated in single-ascending-dose (SAD; 2.5–500 mg) and multiple-ascending-dose (MAD; 2.5–100 mg daily for 14 days) studies in healthy subjects. Dapagliflozin exhibited dose-proportional plasma concentrations with a half-life of ~17 h. The amount of glucosuria was also dose-dependent. Cumulative amounts of glucose excreted on day 1, relating to doses from 2.5–100 mg (MAD), ranged from 18 to 62 g; day 14 values were comparable to day 1 values, with no apparent changes in glycemic parameters. Doses of ~20–50 mg provided close-to-maximal SGLT2 inhibition for at least 24 h. Dapagliflozin demonstrates pharmacokinetic (PK) characteristics and dose-dependent glucosuria that are sustained over 24 h, which indicates that it is suitable for administration in once-daily doses and suggests that further investigation of its efficacy in T2DM patients is warranted.

Type 2 diabetes (T2DM) is a chronic disease that presents a growing worldwide problem.[1,2] Currently there are an estimated 246 million people with diabetes, and this number is expected to increase to 380 million by the year 2025.[3] T2DM is associated with serious complications and comorbidity and is quickly becoming one of the leading causes of death and disability in the world.[1,2,4] Complications of diabetes arise from chronic hyperglycemia, which can cause damage to large and small blood vessels and peripheral nerves, potentially leading to heart attack, stroke, blindness, the need for limb amputation, and kidney failure.[5,6] Current therapies act to improve metabolism by increasing insulin secretion, improving insulin sensitivity, or replacing insulin altogether.[7] Most of these agents lose their glycemic efficacy over time.[1,8] For example, in a prospective study of insulin, sulfonylurea, and metformin monotherapy, 50% of patients were unable to maintain glycemic goals after 3 years.[9] Moreover, after 9 years, only 25% of patients were able to maintain glycemic control.[9] Therefore, additional agents, especially those that work independently of insulin, are needed for the successful management of T2DM.

The kidneys contribute to maintaining normal blood glucose levels by reabsorbing ~180 g of glucose each day.[10] In the context of diabetes, blocking the reabsorption of glucose has become an intriguing therapeutic strategy, one based on the inhibition of sodium–glucose cotransporter-2 (SGLT2). SGLT2 is localized to the brush border in the S1 segments of the proximal tubule in the renal cortex and is purportedly the major transporter involved in glucose reabsorption, as shown in expression and loss-of-function studies (**Figure 1a**).[11] Glucose is transported against a concentration gradient in proximal tubules by a secondary active transport system involving the co-transport of glucose and sodium ions.[12] Increased renal glucose transporter expression and activity have been associated with T2DM in a human cellular model.[13] Mutations in the gene that codes for SGLT2 (*SLCA5*) cause renal glucosuria, a predominantly benign condition in which patients have normal kidney function, are not hypoglycemic, and generally have no significant clinical manifestations,[11] although a few subjects with mutational variations may experience renal sodium wasting and/or mild volume depletion.[14] The idea that pharmacologic inhibition of SGLT2 may provide a noninsulin-dependent option toward glycemic control for T2DM patients is promising.[15] Preclinical studies indicate that inhibitors of SGLT2 can induce renal glucose excretion and consequently lower plasma glucose levels (**Figure 1b**).[16–20]

Dapagliflozin is a potent and highly selective SGLT2 inhibitor with a distinct chemical structure containing a C-glucoside[21] (**Figure 2**), which preclinical studies predict will provide a longer half-life because of increased metabolic stability, thereby allowing once-daily dosing. Dapagliflozin is unlikely to significantly affect the pharmacokinetics of concurrently administered medications that are cytochrome P450 (CYP) or P-glycoprotein

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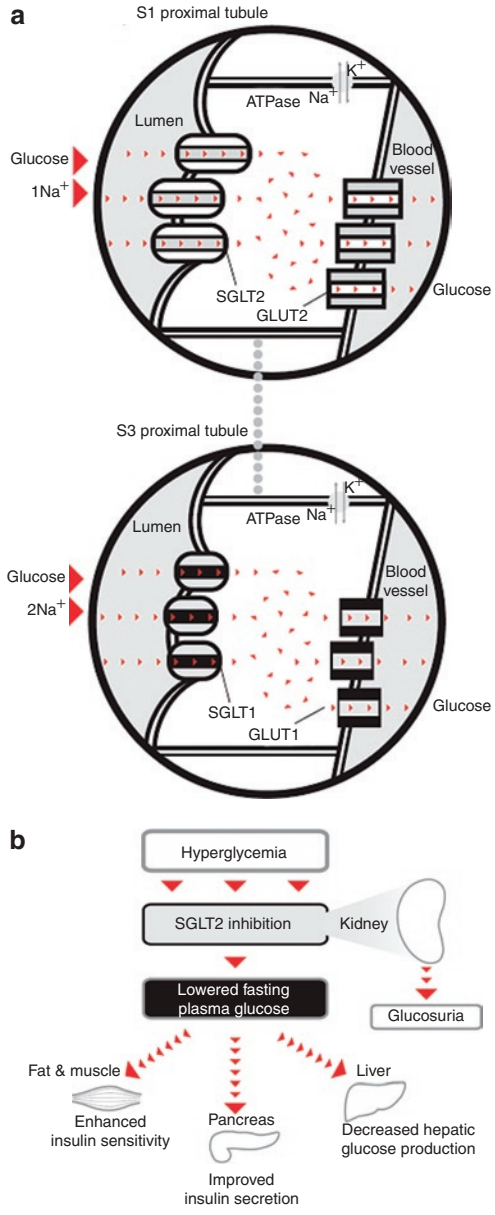

Figure 1 Sodium–glucose cotransporter-2 (SGLT2) reabsorbs glucose in the proximal tubule. (**a**) Renal glucose reabsorption by SGLT1 and SGLT2 occurs in the S3 and S1 segments of the proximal tubule, respectively. (**b**) The physiologic outcome of SGLT2 inhibition is increased renal glucose excretion.

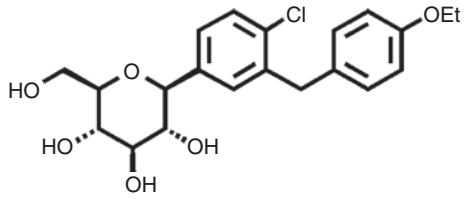

Figure 2 Chemical structure of dapagliflozin.

substrates. *In vitro* studies with recombinant CYP isoforms indicate that the metabolism of dapagliflozin may be catalyzed by multiple CYP enzymes, including CYP1A1, CYP1A2, CYP2A6, CYP2C9, CYP2D6, and CYP3A4, although turnover was low in these experiments. *In vitro* metabolic profiling experiments identified an o-de-ethylated metabolite, BMS-511926, as being an active metabolite with SGLT2 IC50 values similar to those of the parent dapagliflozin; this metabolite was quantitated in the single- and multiple-ascending-dose (SAD and MAD) studies reported here. Data from subsequent *in vitro* studies indicated that dapagliflozin is predominantly metabolized via UGT1A9, a member of the phase II enzyme UGT family, to another (inactive) metabolite, which will be measured in future studies.

The SAD and MAD studies with dapagliflozin were designed to confirm that it has a pharmacokinetic (PK) profile consistent with once-daily dosing and produces a dose-dependent increase in glucosuria in humans. We describe the first two clinical studies that have assessed the initial safety and the PK and pharmacodynamic (PD) parameters of both single and multiple doses of dapagliflozin in healthy subjects.

RESULTS

Subjects

A total of 64 subjects enrolled and completed the SAD study, and 40 subjects enrolled and completed the MAD study conducted at the Bristol-Myers Squibb Clinical Research Center, Hamilton, NJ. The subject demographics were comparable between groups. In both studies, the mean age of subjects ranged from 28 to 37 years, and the mean body weight ranged from 74 to 84 kg. The subjects were men of various races, including white (Hispanic and non-Hispanic/Latino), black/African American, Asian, and American Indian/Alaskan.

Safety outcomes

Single and multiple doses of dapagliflozin appeared to be well tolerated. Adverse events (AEs) were reported in 10 (21%) and 11 (37%) subjects who received single and multiple doses of dapagliflozin, respectively, and in 9 (35%) subjects who received placebo. Incidents of AEs did not appear to be dose-related. In both studies, treatment-emergent AEs included upper abdominal pain, contact dermatitis, dizziness, ecchymosis, erythema, fatigue, a feeling of abnormality, flank pain, headache, hyperhidrosis, hypotension, pallor, pruritic rash, other rash, stress symptoms, and swelling of the face. Two events of hypoglycemia that were mild and asymptomatic (one in placebo) were reported in the SAD study. There were no deaths or discontinuations because of AEs during these studies.

Forty-nine and 54 marked laboratory abnormalities were reported in the SAD and MAD studies, respectively. The most frequent abnormality in both studies was low absolute neutrophils + bands (five total incidents in the SAD study measured as lowest level $\times 10^3$ cells/μl: 1.43 (placebo), 1.49 (2.5 mg), 1.50 (10 mg), 1.46 (50 mg), and 1.43 (100 mg); and six in the MAD study: 1.04 (placebo), 1.32 (placebo), 1.41 (2.5 mg), 1.31 (10 mg), 1.38 (20 mg), and 1.08 (100 mg). None of these was considered clinically significant. In the two studies, a total of 17 serum chemistry abnormalities occurred. The highest reported values for individual subjects included total bilirubin: 1.4 mg/dl (10 mg), 2.7 mg/dl (20 mg), and 1.5 mg/dl (500 mg); direct bilirubin: 0.40 mg/dl (20 mg); aspartate aminotransferase (AST): 57 U/l (10 mg); alanine aminotransferase (ALT): 93 U/l

(placebo), 71 U/l (2.5 mg), and 65 U/l (50 mg); blood urea nitrogen (BUN): 24 mg/dl (10 mg), 26 mg/dl (10 mg), and 25 mg/dl (20 mg); potassium: 5.8 mEq/l (placebo), 5.8 mEq/l (2.5 mg), 5.8 mEq/l (10 mg), and 6.3 mEq/l (20 mg); and creatinine: 1.1 mg/dl (100 mg). After receiving a 20-mg dose of dapagliflozin in the SAD study, one subject had an elevated total bilirubin (2.7 mg/dl at study discharge on day 3 and 2.1 mg/dl after the 20-day follow-up) before returning to the normal range (0.1–1.2 mg/dl) at day 36. The investigator did not consider this abnormality to be clinically significant. There were no clinically relevant changes in vital signs, electrocardiograms, or physical examination findings in either study. Overall, there was no apparent relationship between the frequency of any laboratory abnormality and the dose of dapagliflozin in either study.

In the MAD study, 24-h urinary excretion of sodium was measured at baseline and on days 8 and 13. There were no notable increases in excreted sodium relative to baseline values on days 8 and 13 and no apparent differences between the placebo group and the dapagliflozin dose groups. The SAD study did not assess this parameter. Neither study evaluated plasma renin, serum aldosterone, or changes in body weight.

There were no treatment-related serious AEs in these studies. One unrelated serious AE occurred in a subject in the 20-mg dapagliflozin group who was hospitalized for severe stress symptoms. This subject later revealed a history of anxiety episodes.

Pharmacokinetics

SAD study. Dapagliflozin was rapidly absorbed after oral administration, and maximum plasma concentrations (C_{max}) were observed within 2 h of administration. Key PK parameters are reported in **Table 1**. Total exposure of dapagliflozin, as measured in terms of the area under the plasma concentration–time curve (AUC), increased dose-proportionally, up to the 100-mg dose. Between the 100- and 500-mg doses AUC increase slightly greater than dose-propotionally. C_{max} values increased slightly less than dose-propotionally. After a high-fat meal, the median T_{max} was delayed by 2.5 h (**Figure 3** and **Table 1**), C_{max} was reduced by 39%, and $AUC_{(INF)}$ was reduced by 7% as compared to values during fasting. The active metabolite of dapagliflozin was observed only with doses of >50 mg because of its substantially lower AUC values compared with those of the parent

Table 1 Summary of dapagliflozin PK parameters for a dose of 250 mg administered to fasted and fed subjects (PK population)

Pharmacokinetic parameter	n[a]	Fasted	Fed
Geometric mean C_{max}, ng/ml (CV%)	5	2,510 (31)	1,532 (24)
Geometric mean $AUC_{(INF)}$, ng·h/ml (CV%)	5	13,337 (28)	12,455 (31)
Median T_{max}, hours (min, max)	5	1.50 (1.00, 2.00)	4.00 (4.00, 4.00)
Mean $T_{1/2}$, hours (SD)	5	17.33 (19.75)	18.25 (15.94)
Mean urinary recovery, % (SD)	6	1.54 (0.40)	1.90 (0.60)
Mean CLR, ml/min (SD)	6	4.99 (0.91)	6.33 (1.19)

$AUC_{(INF)}$, area under concentration–time curve; CLR, renal clearance; C_{max}, maximum observed concentration; CV, coefficient of variation; PK, pharmacokinetic; $T_{1/2}$, half-life; T_{max}, time to C_{max}.

[a]In one subject, the 1-h dapagliflozin plasma concentration in the fasted state was greater than the upper limit of quantitation for the assay, with insufficient sample remaining to reanalyze. Because this data point was likely to contribute significantly to the systemic PK parameters, the values from this subject were excluded from the summary statistics for both the fed and fasted states.

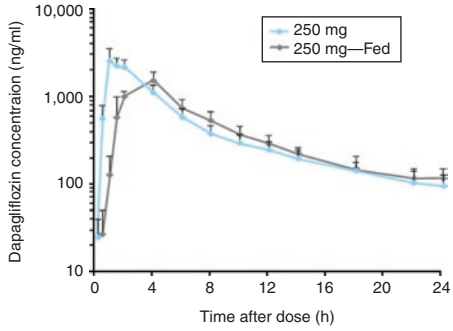

Figure 3 Single-ascending-dose study. Mean (SD) plasma concentration–time profiles for dapagliflozin on day 1 after a single 250-mg dose in fasted and fed subjects.

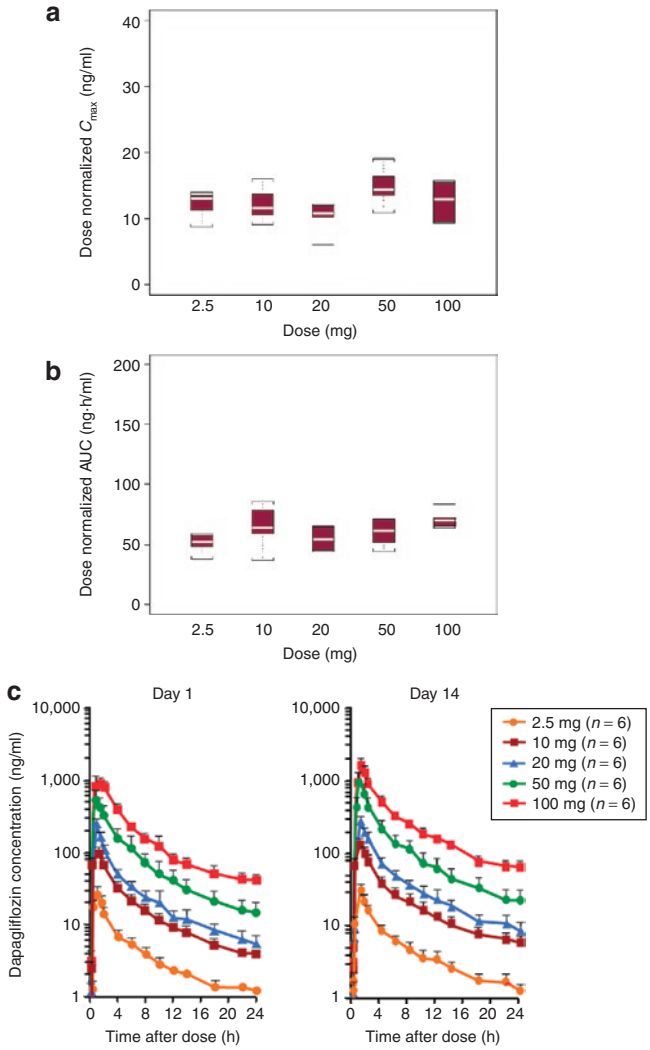

Figure 4 Multiple-ascending-dose study. Dose-normalized (**a**) C_{max} and (**b**) AUC for dapagliflozin at day 14; midlines of boxes are median values, boundaries are ~95% confidence limits for the median. (**c**) Mean (SD) plasma concentration–time profiles for dapagliflozin on days 1 and 14.

compound. Approximately 4% and 0.1% of the dose of dapagliflozin were excreted in the urine as parent compound and metabolite, respectively. The mean renal clearance ranged from 2 to 14 ml/min for the parent compound (all doses) and 30–52 ml/min for the metabolite (250- and 500-mg doses only).

MAD study. **Figure 4a,b** shows analyses of C_{max} and AUC dose-proportionality after normalization for differences in dose. The flatness of the slopes demonstrates that increases in C_{max} and AUC were proportional to increments in dosage. The mean $T_{1/2}$ after the last dose ranged from 11.2 to 16.6 h and the data were similar for the SAD study high dose (**Figure 4c**). Consistent with these half-life values, the mean day 14:day 1 AUC accumulation index for dapagliflozin in each dose panel ranged from 1.20 to 1.30; these values appeared to be independent of dose. As in the SAD study, the active metabolite had substantially lower AUC values than the parent compound. For example, after 14 days of daily dosing with 100 mg dapagliflozin, the $AUC_{(TAU)}$ (AUC over a dose interval of 24 h) of the parent compound was 5,599 ng·h/ml, whereas the $AUC_{(TAU)}$ of the metabolite was 46 ng·h/ml (0.008% of that of the parent compound). Neither the parent compound nor the metabolite was extensively excreted in the urine (<3.0 and 0.2% of the dapagliflozin dose were excreted in the urine as parent compound and metabolite, respectively). The mean renal clearances of dapagliflozin and its metabolite were comparable with the clearance observed with single doses of dapagliflozin in the SAD study.

Pharmacodynamics

SAD study. The amount of glucosuria was dose-dependent. Doses on the order of 20–50 mg maintained a close-to-maximal rate of glucose excretion of ~3 g/h for at least 24 h (**Figure 5**). The mean serum glucose AUC 0–4 h after lunch ranged from 413 to 446 mg·h/dl in subjects in the placebo group and from 393 to 405 mg·h/dl in subjects in the dapagliflozin group. Single oral doses of dapagliflozin did not alter urinary calcium excretion. Other than the expected elevations in urinary glucose observed in the dapagliflozin group, there were no apparent changes in clinical laboratory parameters that were attributable to dapagliflozin.

MAD study. The cumulative amount of glucose excreted per day was dose-dependent on days 1 and 14 (**Figure 6a,b**, respectively). Close-to-maximum glucose excretion per day was achieved with doses of 20 mg and higher. Cumulative amounts of glucose excreted over 24 h on day 1 with the 2.5-, 10-, 20-, 50-, and 100-mg doses of dapagliflozin were 17.7, 40.0, 58.0, 62.0, and 58.3 g, respectively, and were dose-dependent. This translates to ~20–30% inhibition of renal glucose reabsorption. By day 14, the 2.5-, 10-, 20-, 50-, and 100-mg doses of dapagliflozin produced 20.4, 33.6, 49.2, 53.3, and 55.4 g, respectively, of glucose excreted over 24 h. Inhibition of renal glucose reabsorption at day 14 was ~16–50%. No clear change in daily glucose excretion was observed at day 14 as compared with day 1, which suggests there were no meaningful changes in filtered glucose load in this study of healthy adult men. Consistent with this finding, serum glucose, serum insulin, and serum C-peptide concentrations were unchanged. In addition, dapagliflozin had no apparent effect on urinary calcium, magnesium, sodium, potassium, phosphate, chloride, uric acid, oxalate, citrate, total protein, albumin, osmolality, or renal tubular markers such as N-acetyl-b-D-glucosaminidase and β2-microglobulin. Furthermore, there were no observed effects on serum osteocalcin, parathyroid hormone, 25-hydroxy vitamin D, 1,25dihydroxy-vitamin D, deoxypyridinoline crosslinks, and C-telopeptide.

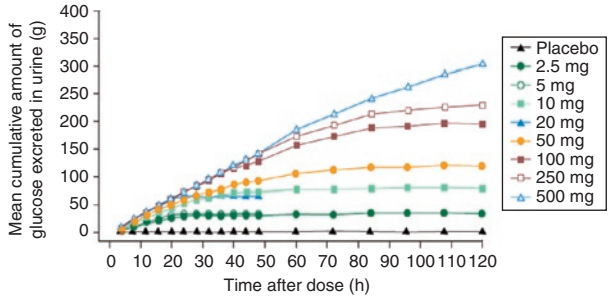

Figure 5 Single-ascending-dose study. Total mean cumulative amount of urinary glucose after a single dose of dapagliflozin. The mean cumulative amount of glucose excreted in the urine was dose-dependent. Data are shown for up to 120 h after a single dose.

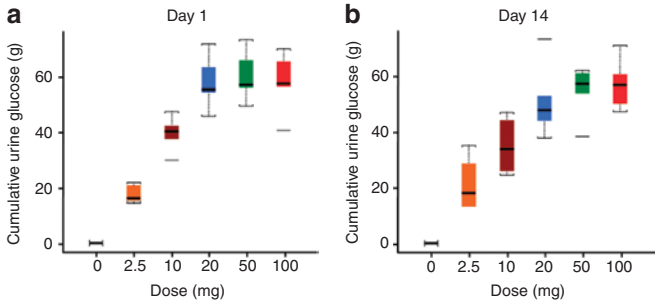

Figure 6 Multiple-ascending-dose study. The cumulative amount of glucose (g/day) in the urine 0–20 h after multiple doses of dapagliflozin at (**a**) day 1 and (**b**) day 14. The mean amount of glucose excreted in the urine at dapagliflozin doses 20–100 mg was comparable after days 1 and 14. The cumulative 24-h glucose excretion after dapagliflozin doses of 2.5 and 10 mg was ~40 and ~70%, respectively, of the glucose excreted after dapagliflozin doses of 20–100 mg.

DISCUSSION

The results of these first-in-human studies in healthy subjects support the evidence from preclinical models that SGLT2 inhibition produces dose-dependent, sustained glucosuria.[20,21] In these normoglycemic subjects, dapagliflozin doses of 20 mg and higher inhibited up to 50% of filtered glucose from being reabsorbed by the kidney, which resulted in glucose excretion of ~60 g/day (**Figure 5**) and up to 3 g/h. The dose–response curve for glucosuria may be shifted to higher doses in hyperglycemic patients. Therefore doses up to 100 mg will be tested in T2DM patients to ensure that the entire dose–response curve for glucosuria is characterized.

In humans, mutations in the SGLT2 gene, *SLC5A2*, result in familial renal glucosuria and produce varying degrees of

glucosuria depending on the type of mutation and its zygosity.[22,23] In the MAD study, doses between 20 and 100 mg produced urine glucose levels similar to those seen in some individuals with moderate renal glucosuria (between 50 and 60 g glucose/day, i.e., inhibition of only 25–50%). These glucosuria levels are approximately half of those observed in individuals with severe renal glucosuria, in whom glucose excretion can exceed 125 g glucose/day.[23,24] Generally, even severe forms of renal glucosuria do not produce other clinically relevant effects and are considered to be benign conditions. In these studies, healthy subjects who exhibited moderate glucosuria (i.e., ~60 g glucose/day), likewise demonstrated no other clinically remarkable findings.

Study volunteers did not experience reduction in serum glucose after receiving dapagliflozin. This is an expected finding, given that healthy individuals typically maintain glucose homeostasis through elaborate endocrine feedback loops and counter-regulatory pathways.[25] In contrast, T2DM patients have an excess glucose load because glycemic regulation is impaired by defects in homeostatic pathways, and preclinical studies support the hypothesis that inducing glucosuria by inhibition of SGLT2 reduces serum glucose and improves glycemic parameters in diabetic animals. An early study of the SGLT inhibitor phlorizin demonstrated that phlorizin treatment normalized insulin sensitivity in diabetic rats but had no effect on insulin action in nondiabetic controls.[26] A later study found that T-1095, another SGLT2 inhibitor, effectively suppressed postprandial hyperglycemia in diabetic rats, leading investigators to conclude that saturation of the SGLT reabsorptive mechanism increases excretion of urinary glucose more effectively under hyperglycemic than normoglycemic conditions.[27] Consistent with these reports, a significant increase in urine glucose excretion was seen within 6 h after administration of dapagliflozin in diabetic rats, whereas in normal rats on the same dose no significant increase was observed over 24 h.[20]

Over the full range of doses studied, dapagliflozin C_{max} and $AUC_{(TAU)}$ increased in proportion to the increment in dose. The PK parameters of dapagliflozin, such as its half-life of ~17 h, and its PD characteristic of maintaining maximal glucosuria over 24 h support the viability of a once-daily dosing regimen. This is in line with preclinical data attributing the enhanced glucosuric potency of dapagliflozin to the C-glucoside linkage that confers metabolic stability.[21] Renal excretion of both analytes was minimal because they are highly bound to protein in the plasma (to an extent of ~97%). Food had only a modest effect on the PK and PD parameters of a single 250-mg dose of dapagliflozin and slightly delayed and reduced C_{max}; however, the overall daily exposure was almost unchanged. Furthermore, subjects who received this dose after a high-fat meal did not have a substantially different maximal rate of urinary glucose excretion, duration of maximal rate of urinary glucose excretion, or cumulative amount of glucose excreted in the urine.

The incidence of AEs after single and multiple dapagliflozin doses did not appear to be dose-related in either study. Over the course of 2 weeks, only two mild episodes of hypoglycemia were reported, one in the placebo group and the other in the SAD 20-mg group, whereas none was reported in the MAD study. In addition, there was no increase in the occurrence of genitourinary infections in the dapagliflozin groups as compared with the placebo groups in either study; however, longer studies will be required to evaluate the effect of dapagliflozin in this context. Multiple oral doses of dapagliflozin over 2 weeks had no apparent effect on the safety and laboratory parameters measured, including renal tubular and bone turnover markers.

In summary, these first-in-human studies indicate that the SGLT2 inhibitor dapagliflozin induces sustained dose-dependent glucosuria without reducing serum glucose in healthy subjects. Further investigation in T2DM patients is needed to characterize the dose–response curve for glucosuria in hyperglycemic subjects and to demonstrate whether dose-dependent glucosuria with dapagliflozin will lead to clinically meaningful changes in glycemic parameters. A phase IIa study is also reported in this issue.[28]

METHODS

Subjects. In general, inclusion and exclusion criteria were similar for the two trials. Adult subjects (aged 18–45 years) with a body mass index in the range of 18–30 kg/m^2 were eligible for inclusion if they were deemed healthy in terms of medical history, physical examination findings, 12-lead electrocardiogram findings, and clinical laboratory evaluations. Women who were nursing, pregnant, or of childbearing age were excluded from the study. For the MAD study, subjects with urinary calcium >140 mg/g or creatinine or fasting serum glucose >110 mg/dl at screening were also excluded. Evidence of organ dysfunction or any clinically significant deviation from normal in physical examination, vital signs, electrocardiogram, or clinical laboratory determinations were criteria for exclusion. Subjects who had received calcium or vitamin D supplements within 2 weeks prior to enrollment, had undergone major surgery within 4 weeks prior to enrollment, had acute or chronic medical illness, or had a history of drug allergy or exposure to the study drug were also excluded from the trial. Studies were conducted in accordance with good clinical practice guidelines and were approved by an institutional review board. All subjects provided informed consent.

Design of the SAD study. This was a double-blind, randomized, placebo-controlled, two-period, sequential, ascending single-dose study (MB102001). Healthy subjects were randomly assigned in a 3:1 ratio to receive either a single dose of 2.5, 5, 10, 20, 50, 100, 250, or 500 mg dapagliflozin or placebo. Dapagliflozin or matching placebo was administered as an oral solution (2.5–5 mg vs. placebo) or capsule formulation (20–500 mg vs. placebo). If a dose regimen (beginning with the lowest dose) was found to be safe and well tolerated, then the succeeding panel of eight subjects received the next higher dose of dapagliflozin ($n = 6$) or placebo ($n = 2$). In period 1, subjects were given a single oral dose of dapagliflozin or placebo after a 10-h fast. Urine samples were collected for a minimum of 120 h (48 h for the 5- and 20-mg dose groups) after the dose. All subjects, excluding those in the 250-mg dapagliflozin group, were discharged from the study on day 6 (day 3 for 5- and 20-mg dose groups) if the morning urine voided was negative for glucose. If the morning urine was positive, 12-h urine samples continued to be tested for glucose and calcium excretion. In period 2, subjects who received 250 mg dapagliflozin entered a 7-day washout phase. After the washout interval, the subjects were given a high-fat breakfast and a second oral dose of 250 mg dapagliflozin or matched placebo. During the study, all subjects received diets containing fixed amounts of calcium and sodium chloride. The subjects were discharged on day 6 of period 2 if their urine was negative for glucose on the morning of that day. If the morning urine was positive, 12-h urine samples continued to be tested for glucose and

calcium excretion. The subjects were not discharged until the morning urine was negative for glucose.

Design of the MAD study. This was a double-blind, randomized, placebo-controlled, sequential, ascending multiple-dose study (MB102002). Healthy subjects were randomly assigned to one of the drug treatment groups (five sequential doses of 2.5, 10, 20, 50, or 100 mg of dapagliflozin) or to the placebo group in a ratio of 3:1. Dapagliflozin or matching placebo was administered as a capsule formulation. As in the SAD study, subjects were not enrolled at the next dose level until safety data from at least six subjects within the group had been reviewed by the sponsor in conjunction with the investigator. Beginning on day 1, subjects received a daily oral dose of dapagliflozin or matched placebo for 14 days. All subjects received diets containing fixed amounts of calcium and sodium chloride. The subjects were released from the clinical facility on day 20, and they returned on day 27 for discharge procedures.

Safety measurements. Safety assessments were similar in the two studies and were based on medical review of AE reports. This included vital signs, electrocardiograms, physical examinations, and clinical laboratory tests. AEs were recorded throughout the study period and defined as any new medical occurrence or worsening of a preexisting condition after administration of the study drug or placebo. Serious AEs were recorded for 30 days after the last dose of study medication; a serious AE was defined as an AE that resulted in death, hospitalization, or persistent or significant disability, or was life-threatening.

PK and PD assessments. Single-dose PK parameters (C_{max}, T_{max}, $AUC_{(INF)}$, $T_{1/2}$, % urinary recovery, and renal clearance) were derived from plasma concentration-vs.-time and urinary excretion data. The effect of food on dapagliflozin PK parameters was evaluated in the group receiving the 250-mg dose. PD measurements included serum glucose concentration and the amounts of glucose and calcium excreted in the urine. Samples were collected before and after lunch (within a 4-h window) on day 1 of period 1 and period 2. Urine samples were collected every 12 h for a minimum of 48 h (in the 5- and 20-mg dose groups) or 120 h (in all other groups) after the dose in period 1 and period 2 (for the 250-mg dose group). Multiple-dose PK parameters (C_{max}, T_{max}, $AUC_{(TAU)}$, accumulation index, $T_{1/2}$, % urinary recovery, and renal clearance) of dapagliflozin and its pharmacologically active metabolite were derived from plasma concentration-vs.-time data. PD parameters included urinary glucose, urinary calcium, serum glucose, serum insulin, serum C-peptide, and inhibition of renal glucose resorption. Samples of blood (0.25, 0.5, 1, 1.5, and 2 h after the dose and then every 2 h for a total of 24 h or hourly for 12 h) and urine (every 4 h for 24 h) were collected on days 1, 7, 8, 13, and 14, with the subjects in fasting condition for 8 h before collection. Urine samples were collected to calculate the excretion rate for glucose (ER). The filtered load (FL) is the product of estimated GFR and serum glucose concentrations, and the renal (tubular) glucose reabsorption rate (T_G) can be calculated as $T_G = FL - ER$.

Bioanalytical methods. Assays for plasma and urine concentrations of dapagliflozin and its metabolite, BMS-511926, were performed by Bristol-Myers Squibb using liquid chromatography atmospheric pressure ionization with tandem mass spectrometry detection in multiple-reaction monitoring mode within the period of known analyte stability. The between-run variability and within-run variability for the analytical quality controls of dapagliflozin were <7.5 and <10.1%, respectively, of the coefficient of variation, with deviations from the nominal concentrations of no more than ±3.5%. For dapagliflozin and BMS-511926, the assay range representing the lower and upper limits of quantitation in plasma and urine were 1–1,000 and 10–2,000 ng/ml, respectively.

Statistical methods. All subjects who received dapagliflozin or placebo were included in the safety and PD populations. Subjects who received dapagliflozin were included in the PK population; only subjects with data from both study periods were included in the tabulation of summary statistics for the food effect assessment. All statistical analyses were performed using SAS/STAT version 8.2 (SAS Institute, Cary, NC). Summary statistics were calculated for PK parameters by dose and period (fed, fasted), or study day, for each analyte. Scatter plots of C_{max} and $AUC_{(INF)}$ or $AUC_{(TAU)}$ relative to dose were analyzed by study day to assess the dependency on dose. Point estimates and 90% confidence intervals were calculated for the ratios of population geometric means (fed/fasted) for C_{max} and $AUC_{(INF)}$ to assess the effect of food on the PK of dapagliflozin. Geometric means and coefficients of variation were calculated for C_{max}, $AUC_{(TAU)}$, and accumulation index. Scatter plots of C_{max} and $AUC_{(INF)}$ were also provided, to further assess the effect of food on the PK of dapagliflozin. PK parameters were analyzed using noncompartmental methods. Summary statistics for PD measurements were tabulated for the total amount of glucose and calcium excreted in urine >120 h after dosing, by treatment group and period. Serum glucose concentrations were summarized by treatment group and by time elapsed since lunch for each period. Although the number of subjects included in the studies was not based on statistical power considerations, six subjects in each panel would have provided an 80% probability of observing at least one occurrence of any AE that occurred with an incidence of 24%.

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CONFLICT OF INTEREST

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Pro32Thr Polymorphism of Inosine Triphosphate Pyrophosphatase Gene Predicts Efficacy of Low-Dose Azathioprine for Patients With Systemic Lupus Erythematosus

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We evaluated the relationship between the efficacy of low-dose azathioprine (AZA) therapy and the inosine triphosphate pyrophosphatase (*ITPA*) 94C>A (Pro32Thr) polymorphism in patients with systemic lupus erythematosus (SLE). We performed a multiple regression analysis to assess the influence of various factors on the reduction in SLE disease activity index (SLEDAI) scores. The *ITPA* 94C>A polymorphism had the highest correlation with the change in SLEDAI score ($r = 0.354$, $P = 0.006$).

Azathioprine (AZA) is a thiopurine derivative widely used in organ transplantation and the management of acute lymphoblastic leukemia, inflammatory bowel disease, and several connective tissue or vasculitic disorders, including systemic lupus erythematosus (SLE).[1–3] AZA is converted via a non-enzymatic reaction to 6-mercaptopurine, which is then metabolized either by thiopurine methyltransferase (TPMT) to methylmercaptopurine or by xanthine oxidase to thiouric acid. Inosine triphosphate pyrophosphatase (ITPA) catalyzes the futile cycle to 6-thioinosine monophosphate to prevent the accumulation of 6-thioinosine triphosphate (6-TITP). Guanosine monophosphate synthetase then catalyzes 6-thioionosine monophosphate to thioguanine nucleotides (TGNs). TGNs are the clinically effective active metabolites, but they are also toxic. Mutations of the *TPMT* gene reduce TPMT enzymatic activity, which can lead to toxic serum concentrations of 6-thioprine nucleotides and severe leucopenia in patients with SLE treated with standard doses of AZA.[4–6] Recently, in addition to mutations in *TPMT*, a deficiency in the polymorphic enzyme activity of ITPA, which catalyzes the conversion of inosine triphosphate to inosine monophosphate, was identified.[7] This deficiency can cause adverse drug reactions (ADRs) in patients treated with thiopurine. The 94C>A (Pro32Thr) polymorphism of *ITPA* is of particular importance because the enzyme activity levels of homozygous and heterozygous variants have been reported to be 0 and 22.5%, respectively, of control erythrocyte ITPA activity.[7,8] In a case–control study of AZA therapy, Marinaki *et al.* reported a significant association of the *ITPA* 94C>A polymorphism with ADRs, including flu-like symptoms, rash, and pancreatitis, probably as a result of the accumulation of 6-TITP.[9] Zelinkova *et al.* showed that *ITPA* 94C>A and *TPMT* polymorphisms are associated with AZA-related leukopenia in patients with inflammatory bowel disease.[10] Little information is available on the association between the *ITPA* 94C>A polymorphism and the clinical effects of AZA in patients with SLE.

The reported frequency of the *ITPA* 94A allele polymorphism is significantly higher in Asians (11–19%) including Japanese, than in other ethnic groups such as Caucasians, Hispanics, and Africans (1–7%).[7,8,11] The purpose of this study was to investigate the contribution of *ITPA* polymorphisms to the efficacy of low-dose AZA therapy in patients with SLE and to ADRs associated with this treatment. The contributions of gene polymorphisms of *TPMT* and glutathione-S transferase (GST), which are thought to metabolize AZA, were also evaluated.

RESULTS

Patient characteristics

Characteristics of the 51 SLE patients (3 male and 48 female subjects) are shown in **Table 1**. The mean age was 35.3 ± 13.0 (range 14–70) years. The mean values of duration of SLE, SLE disease activity index (SLEDAI) score, and dose of AZA prescribed were 6.2 years, 5.2, and 0.97 mg/kg/day, respectively. At the start of AZA treatment, all patients were being treated with low to moderate doses of corticosteroids (5–30 mg/day of prednisolone in 42 patients and 1–3 mg/day of dexamethasone in 9 patients).

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Table 1 Characteristics of SLE patients before treatment

Characteristics	
Number of patients, *N*	51
Age, years, mean ± SD	35.3 ± 13.0
Sex, *n* (%)	
Male	3 (5.9)
Female	48 (94.1)
Duration of SLE, years, mean ± SD	6.2 ± 6.2
SLEDAI score, mean ± SD	5.2 ± 2.7
Urine protein/creatinine ratio,[a] mean ± SD	1.4 ± 2.0
Maintenance dose of AZA, mg/day, mean ± SD	51.5 ± 10.5
Maintenance dose of AZA, mg/kg/day, mean ± SD	0.97 ± 0.26
Receiving concomitant corticosteroid	
Prednisolone, *n* (%)	42 (82.4)
Dexamethasone, *n* (%)	9 (17.6)
Prednisolone dose, mg/day	14.7 ± 7.7
Dexamethasone dose, mg/day	1.8 ± 0.8
ITPA 94C>A mutations, *n* (%)	
C/C	30 (58.8)
C/A	19 (37.2)
A/A	2 (4.0)
A allele frequency (%)	22.5
GSTM1	
Present	26 (51.0)
Null	25 (49.0)
GSTT1	
Present	29 (56.9)
Null	22 (43.1)
*TPMT*3C* mutations, *n* (%)	
**1/*1*	49 (96.1)
**1/*3C*	2 (3.9)
**3C* allele frequency (%)	2.0

AZA, azathioprine; SLEDAI, systemic lupus erythematosus disease activity index.
[a]*n* = 24; the other patients had no record of urinary protein.

All genotype frequencies were in Hardy–Weinberg equilibrium. The allele frequencies of *ITPA* 94A, *GSTM1* null, *GSTT1* null, and *TPMT*3C* were 22.5, 49.0, 43.1, and 2.0%, respectively. No patient had *ITPA* IVS2+21C, *TPMT*2*, **3A*, or **3B* polymorphisms. The overall frequency of defective alleles recorded in this study was comparable to frequencies reported for these genes in other populations of Japanese origin.[12–14]

Relationship between *ITPA* gene variations and response to AZA

After 1 year of low-dose AZA treatment, a significant reduction in SLEDAI score was observed in patients carrying the *ITPA* 94CA or 94AA genotypes (Wilcoxon signed-rank test, $P < 0.001$, Figure 1b) but not in those carrying the *ITPA* 94CC genotype (Wilcoxon signed-rank test, $P = 0.30$, Figure 1a). The mean

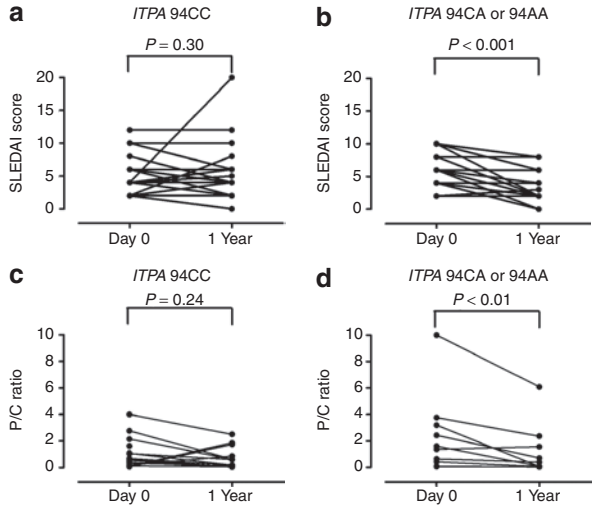

Figure 1 Changes between baseline evaluation and 1-year follow-up in (**a**,**b**) systemic lupus erythematosus disease activity index (SLEDAI) score and (**c**,**d**) urine protein/creatinine (P/C) ratio. **a**,**c**: patients carrying the *ITPA* 94CC genotype; **b**,**d**: patients carrying the *ITPA* 94CA or 94AA genotype.

change in SLEDAI score during 1 year of AZA therapy was significantly different between the two groups (−2.8 ± 3.0 in CA/AA vs. 0.4 ± 3.4 in CC, $P = 0.002$). Likewise, the urine protein/creatinine ratio significantly decreased after 1 year of AZA treatment in patients carrying the *ITPA* 94CA or 94AA genotypes (Wilcoxon signed-rank test, $P < 0.01$, Figure 1d) but not in those carrying the 94CC genotype (Wilcoxon signed-rank test, $P = 0.24$, Figure 1c), although the change in urine protein/creatinine ratio before and after AZA therapy was not significantly different between the two groups (−1.2 ± 1.4 in CA/AA vs. −0.4 ± 1.2 in CC, respectively, $P = 0.5$). Changes in anti-dsDNA and CH50 levels after AZA therapy were not significantly different between the two groups. One patient carrying the *ITPA* 94CC genotype (2%) had a flare during the year of AZA treatment. The dose of AZA was not significantly different between the two groups (0.95 ± 0.28 mg/kg/day in CA/AA vs. 0.99 ± 0.25 mg/kg/day in CC, $P = 0.77$), and the percentage decrease in steroid dose over 1 year was not significantly different between the two groups (%, 16.3 ± 39.1 in CA/AA vs. 18.8 ± 20.3 in CC, $P = 0.77$). During AZA therapy, steroid dosage decreased significantly in both groups over the course of 1 year ($P = 0.004$ in CA/AA; $P = 0.007$ in CC).

Factors contributing to the change in SLEDAI score

To evaluate the relative contributions of factors thought to be relevant to the efficacy of AZA treatment, we performed a multiple regression analysis that factored in gene polymorphisms (*ITPA*, *GSTM1*, *GSTT1*, and *TPMT*), patient age, duration of SLE, SLEDAI score at baseline, and dose of AZA. The dose of AZA and the *TPMT* genotype were calculated to have no effect on the change in SLEDAI score in a stepwise analysis (backward elimination procedure) and were eliminated as factors from the multiple regression analysis. The main results obtained from the multivariate analysis, adjusted for clinically important covariates, are shown in Table 2 (overall model $R^2 = 0.433$). The

Table 2 Partial correlation coefficients and multiple correlation coefficient in multiple regression analysis between change in SLEDAI score and various factors

Factors	Partial regression coefficient	*P* value
ITPA 94C>A polymorphism	−0.354	0.006
Age	−0.332	0.006
SLEDAI score	−0.240	0.061
GSTT1 polymorphism	0.142	0.232
Duration of SLE	0.139	0.235
GSTM1 polymorphism	0.115	0.327

Multiple correlation coefficient was 0.658.

SLEDAI, systemic lupus erythematosus disease activity index.

ITPA 94C>A polymorphism was most highly correlated with the change in SLEDAI score ($r = 0.354$, $P = 0.006$); the second-strongest factor was patient aging ($r = 0.332$, $P = 0.006$). No correlation with the change in SLEDAI score was found for the genotypes for *GSTM1* ($r = 0.115$, $P = 0.33$) or *GSTT1* ($r = 0.142$, $P = 0.23$), SLEDAI score at baseline ($r = -0.240$, $P = 0.06$), or duration of SLE ($r = 0.139$, $P = 0.24$), as shown in **Table 2**.

Relationship between genotypes and ADRs

Four patients (7.8%) discontinued AZA therapy within 1 year as a result of ADRs. One patient had bone marrow suppression (the white blood count decreased to 500/μl), and three patients had severe hepatotoxicity. Statistical analysis could not show a significant association between the gene polymorphisms studied (*ITPA* 94C>A, *GSTM1*, *GSTT1,* and *TPMT*3C*) and ADRs. Two patients heterozygous for the *TPMT*3C* gene had no ADRs during AZA therapy. Their maintenance doses of AZA were 0.56 and 1.11 mg/kg/day, respectively. Their AZA doses were not changed during the AZA therapy.

DISCUSSION

In this study, we provide evidence that the *ITPA* 94C>A polymorphism is associated with the clinical response to low-dose AZA therapy in patients with SLE. Our results show that patients carrying the *ITPA* 94CA or 94AA genotypes exhibited a significantly better response to AZA than those carrying the *ITPA* 94CC genotype. One case–control study has shown a significant association of the *ITPA* 94C>A polymorphism with ADRs to AZA therapy, probably due to the accumulation of the metabolite 6-TITP (a substrate of ITPA).[9] This metabolite might contribute to the immunosuppressive properties of AZA, and therefore the *ITPA* 94C>A polymorphism may be associated with a beneficial AZA effect, rather than toxicity, at relatively low doses (0.97 mg/kg/day).

In contrast to *ITPA* polymorphism, there was no correlation in our study between the efficacy of AZA treatment and other genotypes of major enzymes involved in AZA metabolism. Because the frequency of *TPMT*3C* mutant alleles, which exhibit low enzyme activity, is extremely low in the Japanese population (see **Table 1**), the applicability of our study to the evaluation of the contribution of the *TPMT* polymorphism is limited. The absence of a correlation between the *GST* polymorphism and the efficacy of AZA treatment contradicts a recent report to some extent.[15] AZA might be converted to 6-mercaptopurine non-enzymatically in patients receiving low doses of AZA, which may obscure the impact of the *GST* polymorphism.

The 1-year rate of relapse-free survival was almost 90% with AZA therapy (1–3 mg/kg/day) for proliferative lupus erythematosus.[15] In the case of ulcerative colitis and Crohn's disease, low-dose AZA treatment (0.6–1.2 mg/kg/day) is also effective and safe for the maintenance of remission in Japanese patients.[16] Of the patients on low doses of AZA, 88% were maintained in remission for 6 months.[16] In our study, SLE patients were maintained in remission on relatively low doses of AZA (0.97 ± 0.26 mg/kg/day, mean ± SD), whereas in many previous trials the recommended dose of AZA was 2–3 mg/kg/day for SLE[15,17]. Our results indicate that the 1-year rate of flare-free survival was 98% (50/51) for SLE patients taking a low dose of AZA, which is similar to the results of a trial with higher doses in Caucasian patients.[15] The relatively high efficacy of low-dose AZA therapy in Japanese patients may be linked to the high frequency of the *ITPA* 94A allele. The frequency of the *ITPA* 94A allele is significantly higher in Japanese (22%, in our study) than in Caucasians (6–7%).[7]

We have shown the correlation between ITPA genotype and clinical efficacy; however, the mechanism of this correlation remains unclear because we could not obtain blood concentration data. Stocco *et al.* reported that genetic polymorphism of ITPA is a significant determinant of 6-mercaptopurine metabolism and of severe febrile neutropenia after combination chemotherapy for acute lymphoblastic leukemia in which 6-mercaptopurine doses are individualized on the basis of *TPMT* genotype.[18] Hawwa *et al.* reported that carriers of the *ITPA* 94C>A variant allele had lower concentrations of TGNs, but this result did not reach statistical significance.[19] Thus, the concentration of TGNs might be limited as a predictor of clinical outcome for the carriers of the *ITPA* 94C>A variant allele. Marinaki *et al.* suggested that the concentration of 6-TITP (a substrate of ITPA) might be linked to ADRs, especially for patients carrying the *ITPA* 94CA or 94AA genotypes.[9] However, no method has been established for calculating 6-TITP concentration. Additional studies are needed to ascertain the association of *ITPA* 94C>A with 6-TITP and TGN concentrations and of 6-TITP and TGN concentrations with the clinical efficacy/toxicity of AZA treatment.

The patients carrying the *ITPA* 94CA or 94AA genotypes showed a significantly better response to low-dose AZA treatment than those with the *ITPA* 94CC genotype. Our findings suggest that the *ITPA* 94C>A polymorphism can be used to predict the response to AZA treatment in patients with SLE. We observed that the *ITPA* polymorphism, but not the *TPMT*3C*, *GSTM1,* or *GSTT1* polymorphisms, is a useful diagnostic marker to predict the response to low-dose AZA therapy in patients with SLE.

In conclusion, this is the first report to show the relationship between the effects of low-dose AZA therapy and certain genetic markers in the treatment of SLE.

METHODS

Patients. We retrospectively studied 58 unrelated Japanese patients with SLE treated with AZA in the nephrology and rheumatology units of the Gunma University Hospital. AZA prescriptions for the patients were recorded during the period January 1996 to December 2006. All patients gave written informed consent to participate in the study, which was approved by the institutional review board for clinical trials at the Gunma University Hospital. All patients were diagnosed with SLE according to the American College of Rheumatology 1982 classification criteria.[20] Criteria for excluding patients from the study included pregnancy, a history of AZA treatment before December 1995, and the use of allopurinol (a drug that interacts pharmacokinetically with AZA) at the start of AZA therapy. These criteria resulted in the exclusion of 7 of the 58 patients screened.

Patient characteristics, including age, sex, duration of SLE, SLE activity, dose of AZA, and concomitant medications, were recorded for 1 year from the start of AZA treatment. Clinical examination and laboratory tests, including blood count, liver and renal function tests, urinary protein/creatinine ratio, serum complement titer (e.g., CH50), and autoantibody level (anti-dsDNA antibody), were carried out at every consultation. The SLEDAI according to Gladman *et al.*,[21] urinary protein/creatinine ratio, and CH50 and anti-dsDNA values were determined to assess SLE activity. All clinical data were followed from the beginning of AZA treatment, and the efficacy of AZA treatment was evaluated 1 year after its start or at the time of its interruption.

Hepatotoxicity due to AZA treatment was defined as occurring when the level of serum aspartate aminotransferase, alanine aminotransferase, or alkaline phosphatase increased to more than two times the upper limit of the normal range. Myelosuppression was defined as occurring when leukocyte counts were reduced to $<2.0 \times 10^9$/l or when platelet counts were reduced to $<1.0 \times 10^{11}$/l.

DNA isolation and genotyping. Genomic DNA was extracted from whole blood using the QIAamp Blood Mini Kit (QIAGEN, Valencia, CA). Genotypes of *GSTM1, GSTT1, ITPA* 94C>A, *ITPA* IVS2+21A>C, *TPMT*2, *3A, *3B*, and **3C* were determined in all patients. *GSTM1* and *GSTT1* genotypes were determined using a multiple PCR method described by Chen *et al.*,[22] and *ITPA* 94C>A and IVS2+21A>C were determined using PCR-restriction fragment length polymorphism analyses described previously.[9] Genotyping for *TPMT*2, *3A, *3B*, and **3C* alleles was performed using the pyrosequencing method, as previously described.[23]

Statistical analysis. The correlations among the change in SLEDAI score, age, AZA dose, duration of SLE, SLEDAI score before treatment, and the polymorphisms considered in this study (*ITPA*, *GSTM1*, *GSTT1*, and *TPMT*) were evaluated using a hierarchical multiple regression analysis. A multivariate analysis method with a stepwise backward elimination procedure was used. Differences between data collected before and after treatment were examined for statistical significance using the Wilcoxon matched-pairs signed-rank test. Independent group comparisons were made using the Mann–Whitney *U*-test. Results are presented as mean ± SD unless otherwise stated. A P value of <0.05 was considered statistically significant.

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

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Small Solutions for Big Problems: The Application of Nanoparticles to Brain Tumor Diagnosis and Therapy

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Nanotechnology has been projected to have a significant impact on the future treatment of brain tumors. Specifically, nanoparticles have the potential to revolutionize brain tumor imaging as well as surgical and adjuvant treatments. The translation of current research in nanotechnology into clinical practice will rely on solving challenges relating to the pharmacology of nanoparticles.

WHY NANOPARTICLES?

One of the key reasons that nanoparticles have promise in the treatment of cancer is that they can be targeted to tumors through antigen-dependent (specific) or antigen-independent (nonspecific) mechanisms. Specific targeting relies on the interaction of antigens on the surface of nanoparticles with tumor cell receptors. A variety of molecules including peptides (arginine–glycine–aspartic acid,[1] F3,[2] and chlorotoxin[3]), cytokines (interleukin-13[4]), drugs (methotrexate[5]), antibodies (anti-epithelial growth factor antibodies[6]), and ferromagnetic agents[7] have been proposed as targeting modalities. Multiple targeting molecules can be added to the surface of nanoparticles to tailor targeting of brain tumors through a concept referred to as "surface-mediated multivalent affinity effects."[1]

Nonspecific targeting relies on the preferential extravasation of nanoparticles into the brain through vascular access provided by blood–brain barrier (BBB) breakdown, which occurs in many brain tumors. Other small molecules can also cross BBB defects. However, unlike small molecules that diffuse freely into and out of a tumor, nanoparticles accumulate within a tumor because of the enhanced permeability and retention effect. The effect accounts for the observation that nanoparticles are retained within tumor tissue after serum levels decline. The enhanced permeability and retention effect results from active angiogenesis, the expression of vascular mediators of extravasation, and altered vascular architecture.[8]

In addition to their potential for targeting, the physicochemical properties of nanoparticles make them ideal devices for the delivery of compounds to brain tumors. Molecules such as contrast agents or drugs can be loaded into the core of a nanoparticle or applied as a coating to its surface. The process of a single nanoparticle carrying a large number of drug molecules or ions is referred to as "nanoparticle amplification"[1] and explains the concept of nanoparticles as delivery devices. In addition, molecules with different functions can be incorporated into a nanoparticle to create multifunctional nanoparticles (**Figure 1**). The size and chemical composition of a nanoparticle can be altered to control the efficiency of small-molecule loading.

The performance of nanoparticles in biological systems suggests that by isolating their payload from the surrounding environment, they may reduce the systemic toxicity associated with conventional chemotherapeutic agents. Moreover, nanoparticles create a barrier to degradation of their payload by preventing contact with plasma enzymes.

DIAGNOSTIC APPLICATIONS

IO MRI contrast agents

One of the most mature applications of nanotechnology to the diagnosis of brain tumors is in magnetic resonance imaging (MRI). Various nanoparticles have been developed as MRI contrast agents. To date, nanoparticle-based contrast agents have been designed with a core of iron oxide (IO) crystals with or without a shell of organic material, such as polyethylene glycol (PEG).[9–11]

The key benefit of nanoparticle-based materials is that they may provide better information about the extent of tumor. Both gadolinium-based contrast agents and nanoparticle-based contrast agents cause enhancement of tumors by passing through areas of disrupted BBB where they alter MR signal intensity. However, unlike gadolinium, nanoparticle-based contrast agents,

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such as ultrasmall superparamagnetic IO (SPIO) nanoparticles, are taken up by the phagocytes found at tumor margins.[12] Therefore, areas of tumor not seen with gadolinium-enhanced MRI can be detected using IO-based nanoparticles.[11] Moreover, unlike freely diffusing gadolinium chelates, IO-based nanoparticles tend to persist longer within the tumor and more accurately delineate tumor margins.[12] Another advantage of nanoparticles is their capacity for highly selective molecular tumor targeting. It is possible that nanoparticles could be engineered to image certain subpopulations of cells, such as stem cells or endothelial cells. Various IO nanoparticles under development show promise as tumor-specific contrast agents.[2,13,14]

Intraoperative brain tumor delineation

The central challenge of brain tumor surgery is achieving a complete resection without damaging normal structures near the tumor. Achieving maximal resection currently relies on the neurosurgeon's ability to judge the presence of residual tumor during surgery. The use of fluorescent and visible dyes has been proposed as a means of visualizing tumor margins intraoperatively. However, investigators have been hampered by three main difficulties: (i) achieving tumor specificity, (ii) achieving adequate visual contrast, and (iii) identifying a dye useful for a wide range of tumors. Dye-loaded nanoparticles have been reported to meet each of these challenges.

IO-based nanoparticles loaded with the near-infrared fluorescing molecule Cy5.5 for intraoperative tumor delineation have been created and characterized.[13,14] These nanoparticles can be visualized in experimental models with fluorescence imaging and MRI. Under the appropriate lighting conditions, Cy5.5-loaded nanoparticles delineate margins of implanted tumors (**Figure 2**).[15]

Optical semiconductor nanocrystals, called quantum dots, have also been evaluated as a method to visualize brain tumors and have been shown to induce fluorescent staining of implanted C6 brain tumors.[16] However, because of their heavy metal content, quantum dots are potentially toxic toward normal tissues. Moreover, like Cy5.5-loaded nanoparticles, the quantum dots characterized to date require a darkened operative field for visualization. Still, it is possible that the composition of the quantum dots could be modified to emit light in the visible spectrum.[16]

We have developed and characterized a nontoxic dye–loaded polyacrylamide nanoparticle that shows promise for delineating neoplastic tissue under normal lighting conditions. Coomassie-blue loaded nanoparticles have been shown to visibly stain 9L gliosarcoma cells, and *in vivo* data are currently being collected.

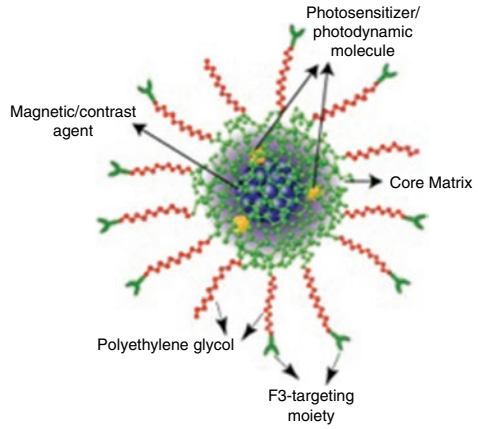

Figure 1 Schematic diagram of multifunctional polyacrylamide nanoparticle. While this nanoparticle contains contrast agents, photosensitizers, and F3-targeting peptides, all of these components can be interchanged for other small molecules with various functions. The size of the nanoparticle shown is ~20 nm. Generally larger than circulating proteins (hemoglobin tetramer (6 nm) and albumin (8 nm)), nanoparticles range in size from 1 to 100 nm. Adapted with permission from Reddy *et al*. Vascular targeted nanoparticles for imaging and treatment of brain tumors. *Clin Cancer Res* **12**: 6677–6686 (2006). Figure 1.

THERAPEUTIC APPLICATIONS

Chemotherapeutics

Nanoparticles are in a unique position to enable the development of novel chemotherapeutics by facilitating passage of these compounds across the BBB or across the blood–tumor barrier and delivering drugs to brain tumors at levels that would not otherwise be possible. Nanoparticles may also meet the challenge of efficiently delivering hydrophobic drugs to tumor cells and overcoming drug resistance.

Solid lipid nanoparticles (SLNs) are among the best characterized, nontoxic, nanoscale devices for brain tumor drug delivery. Although the exact mechanism by which SLNs cross the BBB and blood–tumor barrier is unknown, binding, endocytosis, and phagocytosis by endothelial cells are central components.[17] The lipid matrix of SLN provides a means of loading drugs such as doxorubicin and paclitaxel in a microenvironment that protects them from degradation and improves their therapeutic window by maximizing release within tumor tissue. Drug-loaded SLNs have been shown to enhance tumor concentrations and decrease plasma concentrations of doxorubicin and paclitaxel as compared to equivalent drug doses, even without the need for toxic surfactants.[17] The unloading of drugs within target tumor tissues can also be controlled through modification of the SLN surface and constituent lipids.[18]

Similar in composition to SLNs, nanoparticle formulations of low-density lipoproteins have also been proposed as novel drug delivery devices. Tumor cells preferentially take up low-density lipoprotein nanoparticles via corresponding receptors, which are

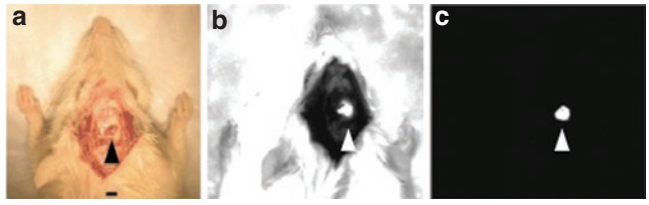

Figure 2 Fluorescent staining of implanted green fluorescent protein (GFP)-expressing 9L gliosarcoma by nanoparticles containing Cy5.5 (near-infrared dye). The tumor is visualized under (**a**) normal lighting conditions, (**b**) GFP channel, and (**c**) Cy5.5 channel. The tumor is above the black or white triangle. Adapted with permission from Kircher MF, Mahmood U, King RS, Weissleder R, Josephson L. A multimodal nanoparticle for preoperative magnetic resonance imaging and intraoperative optical brain tumor delineation. *Cancer Res* **63**: 8122–8125.

upregulated in these tissues. The utility of low-density lipoprotein nanoparticles as drug delivery devices has been suggested by *in vitro* studies that demonstrate their rapid internalization by glioma cell lines.[19]

Like lipid-based nanoparticles, non-lipid-based nanoparticles consisting of matrices of synthetic biocompatible polymers also isolate their payload from the environment. The clinical use of doxorubicin[20] and paclitaxel[21] in gliomas has been limited by the inability of these drugs to cross the BBB because of the p-glycoprotein drug efflux system in endothelial cells. The feasibility of employing polymeric doxorubicin-loaded nanoparticles for the treatment of brain tumors has been demonstrated in a rat glioma model.[20] Similarly, paclitaxel-loaded poly(D,L-lactide-*co*-glycolide) nanoparticles were more cytotoxic to C6 glioma cells than was free paclitaxel *in vitro*, probably due to internalization and intracellular unloading of the drug from the nanoparticles.[22] Moreover, when paclitaxel is used, poly(D,L-lactide-*co*-glycolide) nanoparticles eliminate the need for coadministration of surfactant. Data from clinical trials of Abraxane, a nanoparticle formulation of paclitaxel, in cancer patients will help to determine how nanoparticle-based drugs may be applied in treating glioma patients.

In addition to enabling the entry of chemotherapeutics into tumor cells, nanoparticles can be engineered to potentiate the activity of chemotherapeutics by inhibiting the p-glycoprotein drug efflux system, which confers resistance to therapy to some tumor cells. Specifically, polycyanoacrylate, cetyl alcohol/polysorbate, lipid polymer–based, and surfactant polymer–based nanoparticles have been shown to potentiate the effects of chemotherapeutic agents by inhibiting p-glycoprotein activity in glioma cell lines.[23] The activity of chemotherapeutic compounds in glioma cells has also been potentiated when they are co-incorporated with nanoparticles containing p-glycoprotein inhibitors.[24]

Nonconventional therapeutics

In addition to the delivery of conventional anticancer agents, nanoparticles may be capable of delivering gene therapy plasmids. Nanoparticles are gaining favor as vectors for gene therapy because they cross the BBB more efficiently, can be dosed more precisely, and are less immunogenic than traditional vectors. Recently, a plasmid encoding proapoptotic Apo2 ligand/tumor necrosis factor–related apoptosis–inducing ligand was incorporated into a cationic albumin-conjugated PEGylated nanoparticle and found to have potent antitumor activity against the C6 glioma model *in vitro* and *in vivo*.[25]

Attempts at thermotherapy for the treatment of brain tumors using focused ultrasound have been hampered by the challenges of the electromagnetic properties of the skull and of achieving even temperature distribution throughout a lesion.[26] An alternative strategy was developed in which SPIO nanoparticles were injected intratumorally and heated via a magnetic field. SPIO nanoparticles are well suited for this application since they are retained within tumor tissue for the duration of therapy. Injected SPIO nanoparticles were first tested in a rat model of glioma and shown to be effective in improving survival.[27] A recent phase I clinical trial suggests that SPIO can be safely administered to patients with glioblastoma.[26]

Photodynamic therapy (PDT) is an experimental adjuvant therapy for brain tumors that carries little local or systemic treatment associated morbidity. PDT was initially applied clinically to cutaneous and bladder malignancies, which can easily be exposed to light. While brain tumors cannot be exposed to light as easily, even the deepest brain tumors become exposed during surgery. Thus far, the efficacy of PDT for treating brain tumors has been limited in clinical trials, probably because of the difficulty of creating tumor-specific, sufficient accumulation of photosensitizer within neoplastic cells.[28] Polymeric nanoparticles offer a solution to this problem by allowing the delivery of a large quantity of photosensitizers to tumor cells via tumor-specific ligands. Reddy *et al.* have induced long-term remission of implanted 9L gliomas through PDT mediated by F3-targeted, Photofrin-loaded magnetic nanoparticles.[2] The ability of nanoparticles to mediate PDT is an exciting possibility that merits further investigation.

FUTURE CHALLENGES

Challenges related to nanoparticle clearance and toxicity must be overcome before nanoparticles can be used clinically. While the addition of PEG to the nanoparticle surface can prevent opsonization and delay clearance,[29] the clearance of PEGylated nanoparticles through the liver is relatively slow, thereby increasing the risk of toxicity. Because of conflicting evidence in various animal models, lack of details about the mechanism by which PEG prevents opsonization, and a limited understanding of the biochemical properties of PEG polymers for coating nanoparticles, significant experimental work remains to be done before PEGylated nanoparticles can be used clinically.[30]

Beyond preliminary evidence that suggests that nanoparticles may have toxicity toward astrocytes and neurons in culture, there are few *in vitro* data on the toxicity of nanoparticles toward the central nervous system. Nonetheless, nanoparticles have been used in experimental studies in humans as imaging and drug delivery agents without significant adverse consequences. An understanding of the relationship between toxicity and particle size, geometry, pharmacokinetics, and surface coating is required before nanoparticles can be used in clinical practice.

SUMMARY

Nanoparticles have the potential to advance the diagnosis, operative management, and adjuvant therapy of brain tumors. Nanoparticle-based MR contrast agents have the potential to visualize portions of tumor, especially along the tumor–brain interface, that would have been unclear with conventional MRI. In addition, nanoparticles may ultimately improve the completeness of brain tumor resection. Finally, delivery of chemotherapy and nontraditional therapies to brain tumors will likely be improved by nanoparticle-based drug delivery devices. As our knowledge of their pharmacology expands, nanoparticles are likely to play a central role in the future management of brain tumor patients.

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

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Predictive Biomarkers in the Development of Oncology Drugs: A Therapeutic Industry Perspective

BM Fine[1] and L Amler[1]

The codevelopment of predictive biomarkers along with therapeutic agents, particularly in oncology, is becoming increasingly important. This approach presents both substantial opportunities and challenges. We illustrate these by reviewing some specific recent examples relating to the development of anticancer drugs targeting the HER family of cell surface receptors. We argue that ultimate success may require significant changes to the current approach to drug development.

Over the past several decades, remarkable advances in clinical medicine have been made through the use of data from large randomized studies to determine which therapies will benefit large populations of patients with relatively common diseases. However, it has long been recognized that, although these therapies may benefit patients on average, many individuals within these patient populations may not benefit. It would clearly be preferable to tailor individual therapy more precisely to individual patients. This treatment approach—described as "personalized medicine" in which, for example, biomarkers (see **Table 1** for definition) are used to guide the choice of treatment—is increasingly becoming expected by patients and the general public.

This expectation of receiving personalized medical treatment is fueled, in part, by numerous highly publicized advances in human biology. Examples of these advances include the completion of the draft sequence of the human genome and the development of high-throughput profiling technologies that allow the simultaneous assessment of large numbers of expressed genes, proteins, and single-nucleotide polymorphisms (e.g., refs. 1–3). Frequently, high-profile publications describing these technologies and their use in evaluating human disease are accompanied by enthusiastic descriptions of how these advances will lead to "personalized medicine."

Discussions about personalized medicine and the use of biomarkers are frequently confusing because the word "biomarker" is such a broad term. Under the National Institutes of Health Biomarker Definitions Working Group's broad definition of "biomarker" (see **Table 1**) fall a wide variety of different types of biomarkers, including (i) markers for disease screening, early detection, or disease susceptibility; (ii) pharmacodynamic biomarkers (which reflect the biological activity of a therapy); (iii) prognostic biomarkers (which reflect the "natural" history of an individual's disease); (iv) surrogate end-point biomarkers (which are associated with clinically meaningful end points such as survival); and (v) predictive biomarkers (pretreatment markers that predict who will/will not benefit from a particular therapy).

A discussion of all these different types of biomarkers and their roles in drug development and personalized medicine is beyond the scope of this article. Instead, we focus specifically on predictive biomarkers and their codevelopment with therapeutic agents. A sense of the significant progress that has already been made with predictive biomarkers can be gleaned from a list issued by the US Food and Drug Administration, "Valid Genomic Biomarkers in the Context of Approved Drug Labels" (http://www.fda.gov/cder/genomics/genomic_biomarkers_table.htm). At last count, this list included 28 biomarkers associated with 40 drugs for all kinds of diseases. Some selected examples are listed in **Table 2**.

To illustrate our perspective on both the opportunities and the challenges associated with developing predictive biomarkers along with new therapeutics, we describe a few specific examples targeting cancer. The use of predictive biomarkers in oncology is particularly important and promising because (i) cancer is a disease caused by molecular alterations resulting in huge molecular heterogeneity; (ii) effective cancer therapy still constitutes a major unmet need; and (iii) cancer patients exhibit substantial heterogeneity of benefit from therapies. With the aid of illustrative examples, we argue that, although a more personalized approach to drug development is increasingly important, it is accompanied by significant challenges. Achieving success in this effort may require a dramatic change in the current approach to drug development.

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Table 1 Glossary of key terms

Term	Definition
Biomarker	The NIH Biomarker Definitions Working Group's definition: "A characteristic that is objectively measured and evaluated as an indicator of normal biologic or pathogenic processes or pharmacological responses to a therapeutic intervention."[23]
IHC	Immunohistochemical staining: staining of tissue with polyclonal or monoclonal antibodies coupled to dyes, enabling the qualitative or semi-quantitative evaluation of presence of a particular antigen (e.g., HER2) in a tissue sample by examination under a microscope.[24]
FISH	Fluorescent *in situ* hybridization: staining of tissue with specific DNA probe sequences coupled to a fluorescent dye to enable the assessment of the presence of specific DNA or messenger RNA sequences in a tissue sample.[24]
KRAS	Gene encoding a key intracellular protein that is involved in the signaling of activated HER family members to affect cellular proliferation and survival (see, e.g., ref. 4), suggesting the hypothesis that activating mutations in this gene may be associated with lack of benefit from anti-EGFR therapy. Activating mutations are known to occur in a number of different tumor types, including non–small cell lung cancer and colorectal cancer.[25]

NIH, National Institutes of Health.

Table 2 Examples of predictive biomarkers

Disease/ therapeutic area	Drug	Predictive biomarker
Breast cancer	Hormonal therapy	Estrogen and progesterone receptor expression
	Trastuzumab	HER2/neu
Chronic myelogenous leukemia	Imatinib, dasatinib, nilotinib	BCR-ABL
Gastrointestinal stromal tumor	Imatinib	c-Kit
Colorectal cancer	Cetuximab, panitumumab	*KRAS* mutations
	Irinotecan	*UGT1A1* polymorphisms
Myelodysplastic syndrome	Lenalinomide	5q-Chromosomal abnormality
Leukemia	6-Mercaptopurine	*TPMT* polymorphisms
Anticoagulation	Warfarin	*VKORC1* polymorphism

The specific examples we focus on are anticancer drugs targeting the HER family of cell surface receptor tyrosine kinases. The HER family has four known members: HER1 (also known as epidermal growth factor receptor, EGFR), HER2, HER3, and HER4. These molecules are shown schematically in **Figure 1**. Signaling through these receptors drives cellular proliferation and survival. There is considerable evidence that members of this family are important in the development of a variety of cancers. For example, EGFR and HER2 have been shown to be activated by mutation (EGFR) or amplification (EGFR, HER2).

Trastuzumab (Herceptin), the first successful anticancer therapeutic targeting the HER family, is a humanized monoclonal antibody that has high affinity and specificity for human HER2. It was first approved by the Food and Drug Administration in 1998 for the treatment of HER2-positive metastatic breast cancer. For a detailed review of trastuzumab, see ref. 4.

Prior to the initiation of clinical studies with trastuzumab, it was known that

1. HER2 amplification and overexpression occur in 20–25% of breast cancers.[5]
2. HER2 expression is associated with a higher grade of malignancy, hormone receptor negativity, a greater likelihood of metastasis, and poor prognosis for survival.[5]
3. Preclinical data suggested that gene amplification and overexpression of HER2 were potential predictive biomarkers for trastuzumab. For example, experiments with breast cancer cell lines showed that growth inhibition was greatest in cell lines with high HER2 expression and that there was little if any growth inhibition in breast cancer cell lines with low HER2 expression.[6]

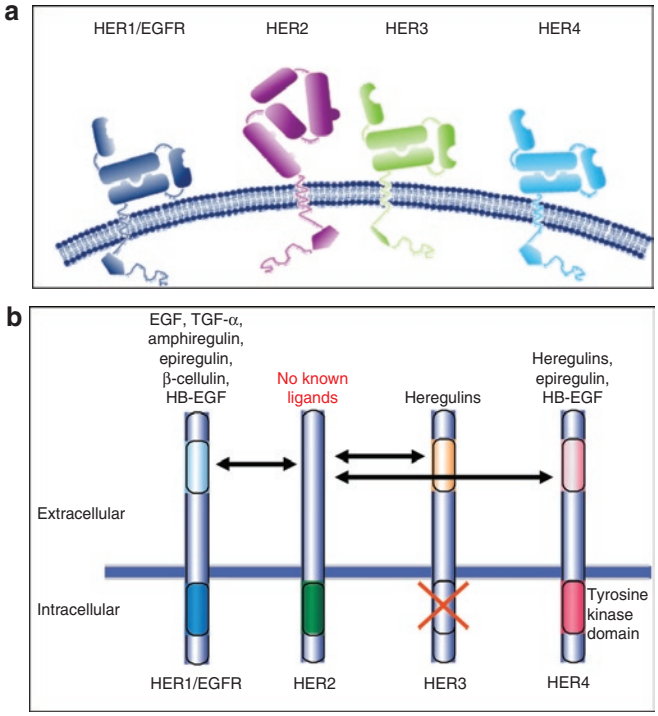

Figure 1 HER family of cell surface receptors. (**a**) Schematic representation of receptor structure. (**b**) Schematic representation of interactions between HER family members, ligands, and kinase activity. As shown schematically, HER2 has no known ligands and HER3 has no tyrosine kinase activity. EGF, epidermal growth factor; HB-EGF, heparin-binding EGF-like growth factor; TGF-α, transforming growth factor–α.

As a result of this prior knowledge, the clinical development of this molecule was largely confined to studies in patients with HER2-positive tumors,[4] initially defined as high expression of HER2 by immunohistochemical (IHC) testing (see **Table 1** for definition). The pivotal study of chemotherapy with or without trastuzumab in 648 patients with HER2 IHC–positive first-line metastatic breast cancer[7] showed a

significant increase in overall survival in the trastuzumab treatment arm as compared with the control arm and led to the approval of trastuzumab for use in this indication. Subsequent studies in HER2-positive adjuvant breast cancer[8] also showed a significant increase in progression-free and overall survival in the trastuzumab treatment arm as compared with the control arm.

On the basis of the trastuzumab experience, many researchers have concluded that incorporating predictive biomarkers into clinical drug development results in faster, smaller, cheaper, and ultimately more successful clinical development. However, for many drugs it is not quite so simple, as examples of other therapies targeting the EGFR family illustrate.

EGFR is expressed in many tumors, including colorectal and non–small cell lung cancer. At least four drugs have been developed that target EGFR for the treatment of cancer: the monoclonal antibodies cetuximab (Erbitux) and panitumumab (Vectibix) and the small-molecule tyrosine kinase inhibitors erlotinib (Tarceva) and gefitinib (Iressa).

The development of cetuximab, a chimeric anti-EGFR antibody, is particularly illustrative. Analogous to trastuzumab, cetuximab's clinical development was primarily confined to patients with EGFR IHC–positive tumors. Its development included two large randomized phase III studies in EGFR IHC–positive metastatic colorectal cancer, both of which were positive. One study in the third-line setting compared cetuximab with best supportive care[9] and showed an improvement in overall survival. The other study, known as CRYSTAL, in the first-line metastatic setting compared cetuximab plus chemotherapy with chemotherapy alone and showed an improvement in progression-free survival.[10]

Although these results appeared to support the use of EGFR IHC as a predictive biomarker for cetuximab, additional data emerged that called this into question. Even before the results of these pivotal studies were released, there was a report that the benefit of cetuximab may not be confined to EGFR IHC–positive tumors.[11] In addition, data began to emerge that suggested other molecular markers that might be predictive of benefits of cetuximab treatment. For example, Khambata-Ford *et al.*[12] described the results of a single-arm, single-agent cetuximab study of 110 patients with metastatic colorectal cancer in which pretreatment biopsies were obtained from all patients prior to initiation of cetuximab treatment. Among other findings, this study reported that patients whose tumors had wild-type *KRAS* showed a higher rate of disease control than patients whose tumors had mutated *KRAS*.

These observations led investigators to evaluate *KRAS* mutations in large randomized studies in which tumor samples had been banked. To date, all cetuximab studies in colorectal cancer in which testing has been reported have consistently shown an association between *KRAS* wild-type tumors and increased benefit from cetuximab treatment and have demonstrated that *KRAS* mutations are not prognostic.[13,14] Similar findings for the relationship between *KRAS* mutational status and therapeutic benefit have also been described for the anti-EGFR antibody panitumumab (e.g., ref. 15).

In contrast to EGFR antibodies, the development of small-molecule EGFR inhibitors was not restricted to EGFR-positive tumors. However, as with EGFR antibodies, data supporting potential predictive biomarkers for erlotinib and gefitinib have emerged relatively late in their clinical development. For example, after completion of the pivotal studies of erlotinib and gefitinib in advanced non–small cell lung cancer, a number of investigators reported that patients with EGFR mutations had high rates of tumor shrinkage following treatment with erlotinib or gefitinib.[16,17] However, a determination of whether these mutations are also associated with improvement in progression-free survival or overall survival has been limited by (i) the relatively low number of tumor samples available from the large pivotal studies to test this hypothesis[18,19] and (ii) the finding that EGFR mutation is a good prognostic marker in non–small cell lung cancer.[20] Also, from retrospective analyses of relatively small numbers of patients, high EGFR gene copy number, as measured by fluorescent *in situ* hybridization (see **Table 1** for definition), has been found to be associated with improved overall survival.[19] The role of other potential predictive biomarkers, including EGFR expression by IHC, *KRAS* mutations, and markers for epithelial–mesenchymal transition, continue to be explored through retrospective studies. However, the small number of available patient samples, particularly in non–small cell lung cancer, is limiting progress.

A common feature of all the predictive biomarker results for EGFR-targeted therapies is that they arose from analyses done retrospectively on relatively small sets of patient samples. Although the conclusions from these analyses are sometimes limited and many questions remain unanswered, these examples show that retrospective biomarker analyses on clinical trial samples are likely to be critical to making progress in the development of predictive biomarkers.

More recently, even with respect to trastuzumab, our understanding of biomarkers is evolving. For example, although HER2 by IHC was the initial predictive marker, more recently HER2 by fluorescent *in situ* hybridization has emerged as a predictive marker as well. In addition, it is becoming increasingly recognized that the implementation of HER2 testing in clinical laboratories requires careful attention to testing guidelines to ensure reliable results.[21] Finally, somewhat reminiscent of the findings with cetuximab, a recent report at the American Society of Clinical Oncology 2007 annual meeting suggested that some breast cancer patients whose tumors were HER2-negative may benefit from trastuzumab in the adjuvant setting.[22] This is yet another example of a result obtained through retrospective analysis of clinical trial samples. Although these results are very preliminary—and it is too early to know whether this finding will be confirmed in other studies—it highlights the fact that the understanding of predictive biomarkers for a particular drug is likely to evolve for many years after initial regulatory approval is given for the drug.

We would argue—based on experience in codeveloping predictive biomarkers for therapeutic agents, particularly in oncology—that there are two points that should be emphasized. First, codeveloping predictive biomarkers with therapeutic

agents is not as easy as the trastuzumab example (initially) made it look. Second, in codeveloping predictive biomarkers with therapeutics, we should expect our understanding to evolve over time; in many cases, highly predictive biomarkers may not be identified and confirmed until after completion of pivotal clinical studies. Therefore, the key to successful development of informative predictive markers may be to bank high-quality pretreatment samples from as many patients as possible during clinical development to enable the testing of novel hypotheses that may arise after trial completion.

The banking of tumor samples in clinical trials without a prespecified hypothesis is often criticized as being a "fishing expedition." However, we believe that the examples described here illustrate the critical value of banked samples. If samples had not been banked in the large randomized studies of EGFR inhibitors described earlier, the only way to assess whether, for example, *KRAS* mutations are predictive of lack of benefit from anti-EGFR antibody therapy would be through the initiation of large, expensive, randomized trials, the results of which would not become available for years.

Although we have highlighted some of the challenges of codeveloping predictive biomarkers with therapeutic agents, we do not think this should lead to the conclusion that such development should not be pursued. In fact, the more we learn about the biology of human disease, the more unlikely it seems that any drug will be equally effective in all patients, and the greater will be the demand for predictive biomarkers. The challenge now is to find a successful strategy for codeveloping predictive biomarkers and therapeutic agents that recognizes and overcomes these hurdles.

We would argue that a successful strategy must take a broader view of drug development; i.e., it must focus on diseases and biological processes rather than on individual drugs. By doing this, we more fully embrace the iterative nature of therapeutic and predictive biomarker development, recognizing that our understanding of diseases and drugs will continue to evolve over time, particularly after a drug's launch. An important consequence of embracing this iterative approach is that it raises the importance of banking adequate numbers of relevant samples during clinical trials to enable retrospective testing of biomarkers as our understanding of disease biology evolves. A challenge posed by this approach is that it requires a long-term commitment to develop drugs and biomarkers for a particular disease or biological process; this marks a significant departure from the approach that many therapeutics developers currently take.

ACKNOWLEDGMENT

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CONFLICT OF INTEREST

Both authors are employees and stockholders of Genentech, Inc.

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Ophthalmic Drug Delivery: Development and Regulatory Considerations

GD Novack[1]

This review presents an update on the development of ophthalmic drug delivery systems (Table 1), as well as regulatory aspects of product development. This review is based on full papers and published patents as of November 2008.

PERSPECTIVE

Local ocular delivery provides unique therapeutic opportunities and challenges. Drugs can be delivered directly to the anterior portions of the eye in relatively simple formulations, providing, *de facto*, an enhanced therapeutic index. The eye is an immune-privileged site,[1] and can be visualized noninvasively. Given the low levels of most biotransformation enzymes, drug–drug interactions are typically not a major issue.[2] However, a recent article suggests a potentially important role for certain drug transports[3] that might be involved in drug–drug interactions. On the other hand, the posterior segment has numerous barriers to drug delivery. The eye is contained in the semi-rigid sclera, changes in eye volume result in potentially dangerous changes in intraocular pressure. Multiple sampling of target tissues is difficult, and therefore pharmacokinetic–pharmacodynamic interactions are rarely possible to understand.[2]

There are many potential routes for local delivery of therapeutic molecules to the eye (as recently reviewed by Weiner,[4] **Figure 1**). The intravitreal route is now approved for two anti–vascular endothelial growth factor treatments for choroidal neovascularization caused by "wet" age-related macular degeneration (pegaptanib and ranibizumab) and also for corticosteroid treatments for intraocular inflammation (triamcinolone acetonide as Triescence and Trivaris). Sub-Tenon injections are under clinical investigation for delivering an ocular hypotensive agent (anecortave acetate).[5]

In a landmark review a quarter century ago, Shell applied to ophthalmology the principles involved in improving pharmacotherapy: minimizing pulsatile drug concentrations and keeping tissue concentrations of the drugs at just above minimally effective levels but below toxic levels.[6] This approach resulted in the pilocarpine ocular therapeutic system (Ocusert, **Figure 2a**). In the same era, Kass used electronic monitors to determine the levels of compliance of patients with respect to glaucoma medications. He reported that patients missed 13–25% of prescribed doses, made timing errors, and had trouble instilling eyedrops.[7–9]

There is a renewed interest in ophthalmic drug delivery systems due to (i) the availability of technical capacity to execute many of the early-stage ideas presented by Shell,[6] (ii) regulatory approval of three pharmacotherapies for the treatment of wet age-related macular degeneration, (iii) expanded understanding obtained through electronic monitoring studies showing that patient compliance is an issue,[10] (iv) renewed acknowledgment based that proper eyedrop instillation is challenging,[11,12] and (v) an increasing geriatric population at risk for degenerative eye disorders.

OPHTHALMIC DRUG DELIVERY

The use of eyedrops is a relatively inefficient delivery system because eyedrops range from 20 to 50 µl in volume, whereas the precorneal space in healthy volunteers is only ~7 µl.[13] The excess volume may roll down the cheek or exit through the nasolacrimal duct, where it is exposed to the vascular nasopharynx, allowing systemic absorption. In general, only ~1–5% of an applied drug is absorbed into the eye, and most of that, typically, is absorbed into the anterior segment.[14] Due to the rapid turnover of tears (precorneal) and aqueous humor (anterior chamber), the ocular residence time for most drugs is relatively short. Adequate therapy with eyedrops requires either the provision of a sufficient peak magnitude so that the effect extends for a useful period of time or more frequent applications of a lower dose. The formulation of topical ocular agents is very important from the point of view of comfort, safety, and ocular bioavailability, requiring optimization of pH, osmolarity, solubility, stability, and, for most multidose formulations, preservative effectiveness.[15–17] Ocular drug delivery systems are designed to overcome these limitations of eyedrops in various ways, including extended residence time, decreased pulsatile delivery, controlled delivery, and more local delivery to the posterior segment.[6]

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Table 1 Ophthalmic drug delivery systems

Product	Development status	Brand name (generic name, firm)
Contact lenses	Clinical	—
Collagen shields	Preclinical	—
Gellan gum	Marketed	Timoptic-XE (timolol maleate, Merck)
Polycarbophil	Marketed	Azasite (azithromycin, InSite/Inspire)
Cationic exchange resin	Marketed	Betoptic-S (betaxolol, Alcon)
Prodrug	Marketed	Betagan (levobunolol, Allergan)
		Lumigan (bimatoprost, Allergan)
		Propine (dipivefrin, Allergan)
		Travatan (travoprost, Alcon)
		Xalatan (latanoprost, Pfizer)
Soft drug	Marketed	Lotemax, Alrex (loteprednol etabonate, Bausch & Lomb)
Lyophylized Teflon strips	Clinical	—
Plastic rods	Clinical	Allergan
Non-erodible implants	Marketed	Vitrasert (ganciclovir, Bausch & Lomb); Retisert (fluocinolone acetonide, Bausch & Lomb)
	Marketed	Ocusert (pilocarpine, Alza)
	Clinical	Iluvien (fluocinolone acetonide, Alimera)
	Clinical	iVation (triamcinolone acetonide, SurModics)
	Preclinical	Subretinal implant, SurModics
	Preclinical	Subretinal injector, SurModics
Cell based	Clinical	Encapsulated cell technology (human ciliary neurotrophic factor, Neurotech); human umbilical tissue–derived cells (Centocor)
Erodible implants	Approved	Surodex (dexamethasone, Allergan)
	Clinical	Posurdex (dexamethasone, Allergan)
Iontophoresis	Preclinical and clinical	IOMED (now ReAble Empi), Aciont, EyeGate

Information on development status was obtained from published papers and the firms' websites.

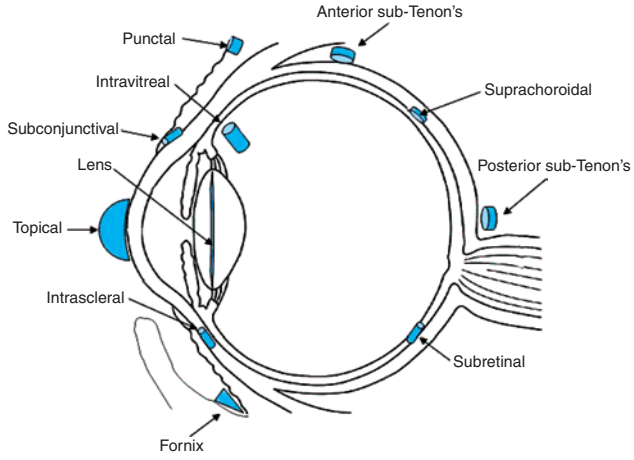

Figure 1 Schematic diagram of various routes of ocular drug administration. Reprinted with permission from ref. 4.

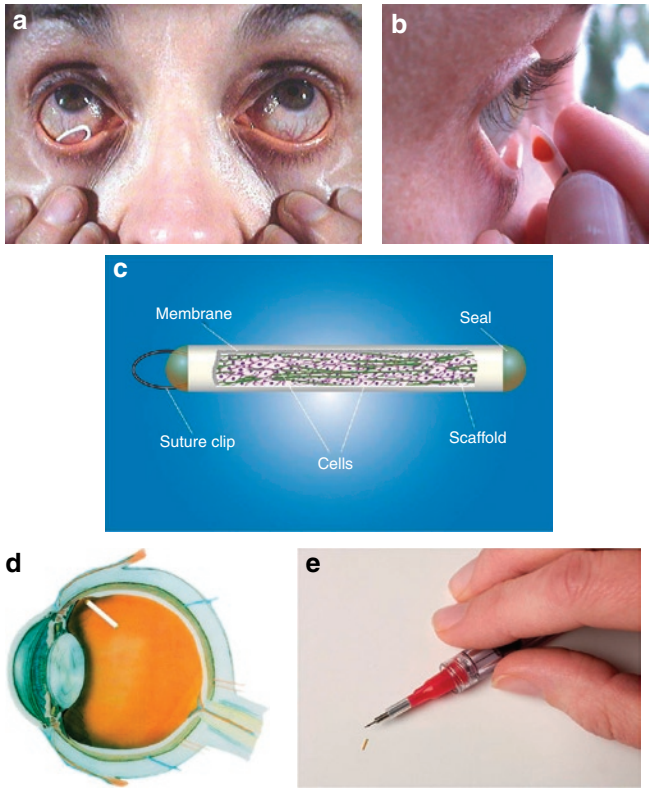

Figure 2 Examples of ophthalmic delivery systems. (**a**) Photograph of patient with Ocusert (pilocarpine) in place in lower cul-de-sac of right eye. (**b**) Photograph of use of fluorescein lyophilized on Teflon strips. Photograph courtesy of Prof. Michael Diestelhorst. (**c**) Encapsulated cell technology: schematic diagram of implant. (**d**) Encapsulated cell technology: schematic diagram of implant placed in vitreous cavity of human eye. Photograph courtesy of Neurotech, Inc. (**e**) Iluvien fluocinolone acetonide implant. Photograph courtesy of Alimera Sciences.

CONTACT LENSES AND COLLAGEN SHIELDS

The use of hydrophilic contact lenses to deliver drugs was first evaluated more than three decades ago. Although extended duration of effect was observed with various classes of drugs, no product in this category has yet been approved or commercialized.[18–20] However, recent patent activity suggests continued research interest in this area.[21,22] Collagen shields, used as therapeutic "corneal bandages," have also been evaluated as drug delivery systems. In one preclinical study using this system, enhanced intraocular delivery of gentamicin was observed in rabbits.[23] In another study there was no difference in antibiotic efficacy of gatifloxacin whether delivered through collagen shields or in standard eyedrops.[24]

IMPROVED EYEDROPS

Researchers have evaluated the benefits of thickening the consistency of eyedrops to improve contact time and thus potentially increase the duration of the minimally effective concentration. In the case of one formulation of pilocarpine (typically administered q.i.d.), this did not sufficiently prolong the duration of action to allow for twice-daily dosing.[25] A gel-forming solution of timolol that included gellan gum was slightly more effective than timolol maleate ophthalmic solution

(1 mm Hg).[26] A once-daily application of the gel-forming solution was equivalent to the standard solution (Timoptic-XE) applied twice daily.[27] A proprietary polycarbophil formulation (DuraSite) has been evaluated in relation to several drugs, including fluorometholone, and was found to be equivalent to standard fluorometholone suspension in a clinical model of allergy for up to 6 h after a single dose.[28] DuraSite is used in an approved formulation of azithromycin (AzaSite).[29] Other proprietary formulations have been developed, including the 0.25% Betoptic-S formulation of betaxolol (cationic exchange resin, Amberlite IRP-69),[30] which was found to be equal in effect to the 0.5% solution and better with respect to ocular tolerability.[31] A cationic emulsion of cyclosporine is being developed for the treatment of dry eye.[32]

Scientists have developed prodrugs for greater corneal penetration. One of the first ophthalmic prodrugs to be marketed was dipivalyl epinephrine, which is metabolized to active epinephrine by the cornea and aqueous humor.[33] Another prodrug in the market is levobunolol, which itself is active, as is its ocular metabolite, dihydrolevobunolol.[14,34,35] Several prostaglandins in the market are metabolized by ocular esterases to their active free acids; one of these, bimatoprost, may itself be active. Loteprednol etabonate, an active "soft drug" (a drug specifically designed to produce targeted local activity, with prompt metabolic inactivation and consequently limited systemic exposure), is metabolized by ocular esterases to the inactive PJ-90 and PJ-91; it is currently marketed for the treatment of ocular inflammation and allergy.[36]

Several approaches involve topical applications, created to avoid some of the problems associated with eyedrops, e.g., the use of preservatives and the issue of solubility. Diestelhorst and colleagues demonstrated the deposition of drugs by lyophilization on Teflon strips that are "painted" onto the conjunctiva. In clinical studies, they found a greater fluorescein concentration in the anterior chamber with the use of the lyophilisate than with the use of eyedrops (**Figure 2b**).[37] Another technology, which is currently in clinical trials, involves coating a plastic rod with the drug (e.g., fluorescein or clonidine).[38,39]

In the continuum between drops and implants, several agents have been formulated in erodible inserts designed to be placed in the lower cul-de-sac of the eye. Some of these agents have been evaluated clinically (ocular hypotensive agents and antibiotics).[40–44]

NON-ERODIBLE IMPLANTS

Ashton developed a non-erodible intravitreal implant to treat cytomegalovirus retinitis over a period of 8 months. In this approach, a tablet of ganciclovir is coated with polyvinyl alcohol and ethylene vinyl acetate polymers (Vitrasert) and implanted intravitreally. Clinically, this product was found to be more effective than intravenous ganciclovir and free of the risk of systemic toxicity associated with intravenous delivery of ganciclovir.[45] A similar technology was used to develop an implant consisting of a tablet of fluocinolone acetonide contained in a silicone elastomer cup with a release orifice and a polyvinyl alcohol membrane between the tablet and the release orifice (Retisert). This product was found to be effective in the treatment of recurrent posterior uveitis.[46] Both products are approved for marketing in the United States. A third-generation product that can be inserted through an intravitreal injection with a 25-gauge needle, rather than by surgical implantation, is under clinical evaluation for delivery of fluocinolone acetonide in the treatment of diabetic macular edema (Iluvien, **Figure 2c**).[47,48]

A helical device, designed for maximal surface area with minimum implantation size and intended for transconjunctival implantation, is in phase II evaluation with triamcinolone acetonide for the treatment of diabetic macular edema (iVation).[49] The same research group has also developed a subretinal injection device intended for gene delivery (RetinaJect)[50] and a polycaprolactone-based subretinal implant.[51,52] Both devices are in preclinical development.

Currently under development is an encapsulated-cell technology in which cells transfected with human growth factor genes are surgically implanted into the vitreous cavity (**Figure 2d/e**). In a phase I trial using human ciliary neurotrophic factor in patients with retinitis pigmentosa, the implants were productive for 6 months and there were no substantive safety issues.[53] Centocor is currently evaluating subretinal delivery of human umbilical tissue–derived cells in the treatment of retinitis pigmentosa.[54,55]

ERODIBLE IMPLANTS

Scientists at Oculex, now part of Allergan, have developed an erodible-implant technology that has the advantage of not requiring surgical excision after its contents are exhausted. A dexamethasone delivery system formulated for implantation into the anterior chamber of the eye (Surodex) showed results similar to those of eyedrops in the treatment of postsurgical inflammation.[56] In patients with persistent macular edema, the group that received treatment through a dexamethasone delivery system formulated for intravitreal implantation (Posurdex) showed better results than a group that received no treatment.[57] Surodex is approved in Singapore, and Posurdex is in late-stage development.

IONTOPHORESIS

The use of an electrical current to enhance the delivery of drugs through the sclera is an approach that has been known for several decades[58] and that has recently been evaluated by several firms (IOMED, now ReAble Empi;[59,60] Aciont;[61] and EyeGate) to deliver a variety of drugs, some of which are in clinical development.[62]

REGULATORY CONSIDERATIONS

A key regulatory issue is whether the ophthalmic drug delivery product is a drug or a combination product. If the delivery system is merely a platform for delivery, then the product would most likely be considered a drug for regulatory purposes. However, if the delivery system is itself a device with its own indication, then it would most likely be a combination product. Thus, Vitrasert (new drug application (NDA) 20569) and Retisert (NDA 21737), both non-erodible implants, were reviewed and approved as

drugs. Visudyne (verteporfin injection, NDA 21-119) requires the use of lasers of a specified light frequency. Each of the lasers was reviewed and approved as a device (Premarket Approval nos. P990048 and P990049, Zeiss VISULAS 690s and Coherent Opal Photoactivator, respectively), and Visudyne is considered a combination product. This is consistent with US regulation 21 CFR 3.2(e), fda.gov/oc/combination).[63] To my knowledge, there are currently no other approved ophthalmic combination products.

Although contact lenses are not currently an approved product for delivery of pharmaceutical agents, one could consider their use for this purpose.[18–20] If the contact lens was used only as a platform for the drug, then it would most likely be considered a drug. However, if the product involved both drug delivery and refractive correction, it would most likely be considered a combination product.

One approach to drug delivery is to take already approved (and off-patent) molecules and develop an ophthalmic drug delivery platform. This approach, which does not involve a new molecular entity, may employ the "505(b)(2)" section, legislated as part of the Drug Price Competition and Patent Term Restoration Act of 1984 (Waxman-Hatch).[64] These are applications in which the sponsor relies on the US Food and Drug Administration's previous finding of safety and effectiveness for a reference listed drug (21 CFR 314.54). Typically, these are reformulations with a different dosing regimen but similar indications, e.g., Timoptic-XE (timolol gel–forming suspension, NDA 20-330) and Istalol (timolol maleate ophthalmic solution, NDA 21-516, which relied on Timoptic timolol maleate ophthalmic solution, NDA 18-086). Assuming that the product is off-patent at the time of NDA approval, the sponsor could typically reference the preclinical and clinical work with respect to the innovator product. Appropriate additional preclinical work would be required on the local safety, efficacy, and pharmacokinetics of the new product. A clinical study would also be necessary to show that the new product is equivalent to the innovator product in safety and efficacy. This "505(b)(2)" approach may save literally millions of dollars and years of work and also decrease the risks of development. Of course, complete development of the pharmaceutics and manufacturing of the new product must be conducted consistent with good manufacturing practices.[65]

For both approaches, the ocular safety of the excipients used (e.g., the delivery system) must be assessed. If there is substantial systemic absorption, the issue of systemic safety may also need to be addressed. The differences in the development paths may also provide differential exclusivity from the Food and Drug Administration.

SUMMARY

Twenty-five years ago, the ophthalmic community had a few approved drug delivery systems (dipivefrin, Ocusert) and numerous ideas for future development. Today, there are numerous approved products (prodrugs, novel eyedrop formulations, non-erodible implants), and many more are in preclinical and clinical development. There is great hope that these current and future products will result in enhanced ocular therapeutics for a wide range of ophthalmic diseases.

CONFLICT OF INTEREST

Dr Novack serves as a consultant to numerous ophthalmic pharmaceutical and medical device firms, including some of those discussed in this article. He owns stock in Inspire Pharmaceuticals, Inc.

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The Next Generation of Drug-Delivery Microdevices

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Advances in microelectromechanical systems (MEMS) and miniaturization technologies have enabled the creation of biomedical microdevices intended for use in the treatment of various chronic and acute illnesses. The ability to create very precisely defined micrometer and nanometer features allows for the production of a new generation of biomedical devices that can be implanted using minimally invasive procedures to provide controlled therapeutic drug delivery. We believe that there is a wide variety of pharmacological therapies in which these novel drug-delivery systems could be implemented, including treatments for cancer and trauma.

INTRODUCTION

Advances in microelectromechanical systems (MEMS) and miniaturization technologies have enabled the creation of biomedical microdevices designed to aid in the diagnosis, monitoring, and treatment of various chronic and nonchronic illnesses. The ability to create very precisely defined micrometer and nanometer features allows for the production of a new generation of biomedical devices that can be implanted using minimally invasive procedures. Our work is focused on the translational research and development of a wide variety of implantable drug-delivery devices for the effective treatment of chronic and acute illnesses. The biomedical microdevices described here are based on our research and can be classified in two categories: passive devices and active devices.

Passive-delivery microdevices are based on the delivery of a drug from single- or multiple-reservoir implant architectures. The activation and timing of release are controlled by a polymeric membrane that is designed to hermetically seal the reservoirs from the surrounding delivery site. Membrane degradation is controlled by a chemical or biochemical reaction between the membrane material and the environment at the specific site of implantation. Passive-delivery devices depend mainly on diffusion or osmotic pressure to deliver the drug payload, making them adequate for slow, long-term drug release. Therefore, the application of these types of devices is limited mainly to continuous, long-term treatment of chronic illnesses in which there is no dose dependence and the drug delivery does not require active feedback control or telemetric activation.

Active-delivery devices use MEMS technology to release the drug from single or multiple reservoirs upon activation. The activation and timing of release are controlled electronically to trigger an actuation mechanism that allows rapid degradation of a sealing reservoir membrane. An optional and separate actuation mechanism can increase the rate of drug release. Active-delivery devices can be programmed to deliver drugs with specific pharmacokinetic profiles. Activation can be performed telemetrically or even autonomously with the use of feedback sensors to determine the required doses for treatment. Drug delivery from active devices can be customized to treat illnesses that require continuous or pulsatile delivery in dose-dependent treatments. The drug-release mechanism for active devices can also be coupled with customized MEMS actuators to rapidly pump the drug out of the reservoirs in addition to utilizing the diffusion and/or osmotic pressure mechanisms for delivery. The versatility of active-delivery devices allows for a wide range of therapeutic options. The current limitation, however, involves the extent to which the supporting electronics for powering the MEMS actuators can be miniaturized so as to be integrated within the implantable biomedical microdevices.

Active- and passive-delivery devices represent the next generation of drug-delivery modalities that can incorporate and store drugs in multiple reservoirs and locally release these drugs at the area of interest without the risk of potentially toxic systemic delivery. The devices can deliver multiple drugs in precisely defined doses at precisely dictated times and have the ability to store liquid- and solid-phase drug formulations. The choice of the type of device depends on the nature of the illness as well as the specific treatments and environmental requirements, including implantation site, device dimensions, duration of treatment, biocompatibility, delivery time constants, desired pharmacokinetics profiles, dose, and payload. Because unwanted reactions between therapeutic compounds and devices have been

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observed, the risk of such reactions must be an integral part of the design considerations. The selection of materials and their chemical and biological compatibilities are crucial for successful performance of the device. Delivery devices designed for long-term therapy must last as long as possible in order to minimize the number of implantations or revision surgeries. The design should incorporate biodegradable materials so as to avoid the need for extraction surgery. Delivery devices for short-term therapy must be designed either for easy extraction after implantation or with biodegradable and bioresorbable materials.

In the following sections, we review a new generation of state-of-the-art passive- and active-delivery devices and the advantages of utilizing these specific delivery modalities as compared with conventional pharmacological therapies.

PASSIVE-DELIVERY DEVICES

Biodegradable polymers are currently used as passive-delivery systems in clinical applications. Gliadel polymer wafers have been used for local delivery of the chemotherapeutic drug 1,3-bis(2-chloroethyl)-1-nitroso-urea (BCNU) (carmustine) over a period of 3 weeks to treat remaining tumor tissues after brain tumor resection.[1] These delivery devices release the drugs in their reservoirs slowly within the body, as the biodegradable polymer dissolves through hydrolysis.

Another type of passive-delivery device, developed by Grayson *et al.*,[2] is a passive resorbable millimeter-sized device made of compression-molded poly(L-lactic) acid (PLLA) reservoirs with poly(lactide-*co*-glycolide) membranes. Once implanted, the multiple-reservoir membranes degrade, releasing the drug over time. PLLA-based devices degrade slowly over a timescale of months, allowing drugs and other biological compounds, such as heparin, human growth hormone, and dextran, to be released through the membrane before the entire device degrades and is fully absorbed at the implant site. Release through the poly(lactide-*co*-glycolide) membranes takes place as water is absorbed into the polymer, and swelling and hydrolysis occur. Higher-molecular-weight polymers typically take longer to degrade fully, thereby making it possible to achieve pulsatile and timely compound release by incorporating a range of molecular weights for the membrane polymers.

Both the Gliadel wafer and the device designed by Grayson *et al.* offer first-generation modalities for polymer-based passive drug delivery from multiple reservoirs. The Gliadel wafers are limited by their low drug concentration capabilities and relatively large device dimensions, which render their use in intracranial implantation more invasive and even impractical for some medical applications. PLLA-based devices are also limited by their relatively large size. Drug depot reservoirs in these devices are unsatisfactory for long-term release of high-dose compounds, and fabrication of the device takes a long time (on the order of days) and is extremely labor intensive. Human error during fabrication can easily introduce a wide range of mechanical and chemical variations among devices, negatively affecting the performance of the device, both *in vivo* and *in vitro*. Unsatisfactory chemical reactions between BCNU and PLLA have resulted in a need to re-evaluate the choice of materials for use in polymer-based passive-delivery devices. Both kinds of devices are capable of releasing drugs in only one physical form, either a solid powder or a liquid solution.

A new generation of passive-delivery devices using liquid crystal polymer and PLLA has been designed to address these limitations. Unlike PLLA, liquid crystal polymer is not biodegradable, but it has been shown to be mechanically highly robust and chemically inert in harsh environments. We believe that use of this material will solve many of the problems encountered with polymer–drug chemical interactions. Liquid crystal polymer is also biocompatible and therefore does not adversely affect the body. In liquid crystal polymer–based devices, the drug of interest is slowly released through an ~150–180 μm opening in the cap, which is covered on the inside with a poly(lactide-*co*-glycolide) membrane. Diffusion-driven drug delivery can be controlled temporally through changes in the molecular weight of the membrane and the diameter of the hole in the device. The inner volume of the reservoir that serves as the drug depot measures ~15 μl; this allows for greater drug loading as compared with previously available passive-delivery devices. The greater drug-loading capability facilitates the local delivery of large drug payloads to tumors, which is critical for ensuring the efficacy of chemotherapeutic treatments. The millimetric dimensions of this new device also make it an ideal candidate for intracranial implantation in the rat. One key innovation is the use of the injection molding process for high-precision mass production, producing reliable and consistent results, i.e., very small tolerances in payload volume. **Figure 1** shows a schematic drawing of this passive polymer device. It is currently implemented with a single-reservoir format, but work is ongoing to produce a multiple-reservoir version with the capability of releasing a range of therapeutic compounds in solid or liquid formulations.

The need for aggressive treatment of malignant gliomas is one of the driving forces behind the development of this new generation of passive-delivery devices, which have also demonstrated the superior chemotherapeutic performance of temozolomide as compared with BCNU (carmustine). Chemotherapeutic agents, including temozolomide, O^6-benzylguanine, and STAT3 inhibitor III, given their high efficacy in treating glioblastomas, have the potential to be used with implantable drug-delivery devices. Future research focusing on passive-delivery devices will involve the use of these candidates as well as investigate methods of

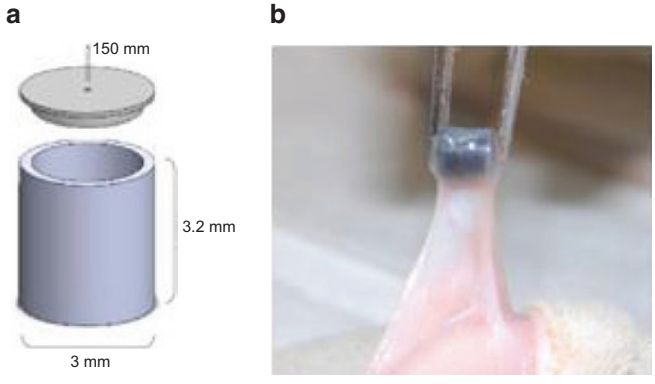

Figure 1 Passive-delivery devices. (**a**) Schematic of the liquid polymer–based passive drug-delivery device. (**b**) Actual device implanted in a rat.

delivering different drugs simultaneously from multiple reservoirs so as to improve and customize treatment efficacy in several chronic illnesses that require minimally invasive and localized delivery of more than one drug.

The key advantages of using this type of passive-delivery device are small size, biocompatibility, large payload capacity, lack of the need for supporting electronics, simplicity of operation, and the ability to load a range of drug formulations.

ACTIVE-DELIVERY DEVICES

Pioneering work relating to active-delivery devices based on MEMS technology was originally conceived for applications that require controlled release.[3] The delivery mechanism in these early devices involved the electrochemical dissolution of gold or polymeric membranes, allowing the drug to diffuse to the site of action.[4] Selective membrane activation allowed for pulsatile and controlled release of drugs from multiple reservoirs.[5] Santini *et al.* demonstrated that devices based on MEMS technology were capable of precise pulsatile release of multiple agents from a single device.[6,7] Using this device, Li *et al.* subsequently demonstrated *in vivo* delivery of the chemotherapeutic drug BCNU in rats.[8]

A subsequent version of active-delivery devices was designed to increase payload capacity and improve reliability of release. The capacity was first improved by the addition of Pyrex reservoirs to increase the payload of the device while reducing its dimensions compared with previous iterations. The size of the reservoirs can be customized by changing the thickness of the Pyrex layer. These improvements allowed Li *et al.* to deliver efficacious doses of BCNU to a tumor model in rats.[9]

The next version of the active devices was based on the modification of the mechanism for opening the membrane; instead of electrochemical dissolution of the gold membrane, the membrane is melted by resistive heating. The new actuation mechanism is based on the application of an electrical pulse to produce localized melting of gold membranes, releasing the drugs contained in the reservoirs. This modification was necessitated by the fact that the electrochemical reaction was dependent on environmental conditions outside the device and therefore could not ensure a 100% reliable opening of the membrane at certain implantation sites. The effectiveness of the modified actuation mechanism has been demonstrated both *in vitro* and *in vivo*.[10,11] **Figure 2** shows a schematic diagram as well as a photograph of the device. Ongoing research on resistive heating mechanisms aims to reduce power consumption by using silicon nitride as a membrane material and a smaller gold resistor to burst the membrane by thermal shock. *In vivo* studies are under way in a rat flank model to release the chemotherapeutic drug temozolomide for minimally invasive treatment of brain tumors.

A new generation of drug-delivery devices has been designed specifically for trauma care, for situations in which rapid intervention is critical to patient survival.[12] The implantable rapid-drug-delivery device (IRD^3) was intended for use in the immediate treatment of hemorrhagic shock, using vasopressin as a model drug. The device was also designed as a preventive subcutaneous implant for high-risk patients, e.g., soldiers on the battlefield.[13] The design involves a triggering algorithm that incorporates blood pressure and heart rate values from standard electrocardiogram and blood pressure sensors. **Figure 3** shows the architecture of the IRD^3 components and a snapshot of *in vitro* release of methylene blue for visualization purposes. The device architecture consists of a reservoir, a thermal actuator, and a sealing membrane, fabricated using MEMS technology. The actuation mechanism involves resistive heating on the bottom layer, leading to the nucleation of vapor bubbles from the liquid drug contained in the reservoir. Film boiling minimizes heat transfer to the drug, thereby preventing drug degradation. The increase in the pressure within the internal chamber bursts the sealing membranes. Further nucleation of bubbles results in greater volume displacement, rapidly forcing the rest of the drug solution out of the reservoir. The IRD^3 is capable of *in vivo* delivery by virtue of its modular design, allowing volumes of 0.02–0.10 ml of drug solution to be delivered in this manner. *In vitro* testing of the device reveals that the IRD^3 is an effective method for rapid drug delivery without significant degradation. Experimental results show that ~85% of the drug is released and is stable within 45 s. *In vivo* tests in a rabbit model have shown effective release of vasopressin. The IRD^3 also has potential applications in health care for civilians, e.g., in cardiac devices that require very rapid delivery. Future work includes the integration of a wireless platform for telemetric activation.

The rapid actuation mechanism of the IRD^3 has led to the development of rapid reconstitution devices based on MEMS technology. The advantages of storing drugs in lyophilized form are increased shelf life and improved drug stability even in harsh environmental conditions. These biomedical microdevices were

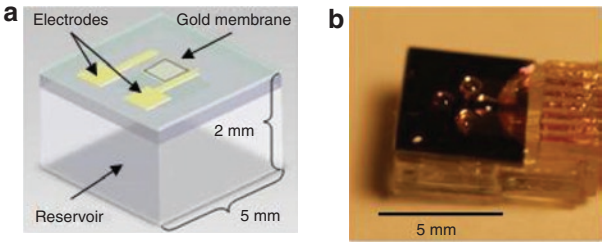

Figure 2 Active-delivery devices for slow, controlled delivery. (**a**) Schematic device. (**b**) Actual silicon-based active device.

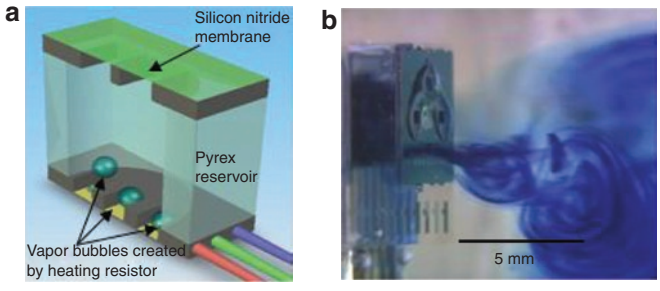

Figure 3 Active-delivery devices for rapid, controlled delivery. (**a**) Schematic of device showing actuation mechanism. (**b**) *In vitro* release of methylene blue from an implantable rapid-drug-delivery device.

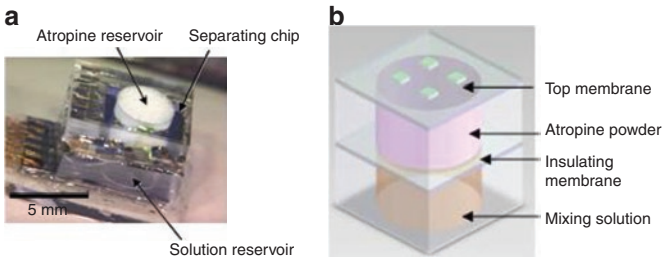

Figure 4 Active-delivery devices for rapid, controlled reconstitution and delivery. (**a**) Photograph of a reconstitution device containing water and lyophilized drug. (**b**) Modular schematic of reconstitution device.

named rapid reconstitution packages. Figure 4 shows a photograph as well as a schematic diagram of the device. It consists of the IRD[3] device filled with a mixing solution, to which another reservoir containing lyophilized atropine is appended. The reservoirs are separated by a silicon chip with hermetically sealed silicon nitride membranes, and the top reservoir is also capped with a membrane chip. Activation of the device results in the ejection of mixing solution into the lyophilized drug, reconstituting it and then forcing it out of the device, as shown in Figure 4. Preliminary results recorded for characterization of the reconstitution of atropine support the potential usefulness of this new type of device in extending the shelf life of drugs. Deployment of the rapid reconstitution packages would allow reconstitution of drugs and injection on demand, thereby enabling a wider availability of drugs for medical personnel in the field or in evacuation units that lack drug refrigeration equipment. A longer shelf life would also reduce the logistical burden of periodically replenishing stocks of a large number of drugs, such as atropine, epinephrine, factor VII, and antibiotics. Potential delivery modalities of the rapid reconstitution packages include implantable devices, transdermal patches, pen-style autoinjectors, and autoinjector cartridges for infusion bags. Deployment of rapid reconstitution packages would result in better treatment during transport to proper care facilities and improved survival rates in trauma victims.

Before clinical trials are performed, further failure analysis and reliability studies are needed to guarantee safe operation of the device for sustained periods of implantation. Total failure analysis should include a comparison with current delivery modalities such as depots and a determination of the maximum allowed dosage in case of device failure and unwanted partial or full payload release. For purposes of assessing long-term biocompatibility, investigation of the body's response to the presence of the device and the subsequent development of fibrous capsules will also need to be carried out. The fibrous capsule introduces an additional transport barrier that may affect the drug-release profile, in both passive and active devices. Current studies aim to determine the autoimmune response as well as the variability in device performance caused by the presence of fibrous capsules. Active-delivery devices that rely on successive electrical stimuli for their operation may induce further adverse immune responses, and this possibility requires special attention. Further investigation of long-term biocompatibility will therefore be required for successful implementation of the drug-delivery microdevices in clinical settings.

CONCLUSIONS

MEMS and miniaturization technologies have been used for producing novel implantable biomedical microdevices for drug-delivery applications that require minimal invasiveness of the medical procedure. Passive and active devices can deliver precise quantities of therapeutic compounds over a predetermined period of time.

The biomedical microdevices discussed in this article have the potential to advance traditional therapeutic modalities for treating acute and chronic illnesses. Their precise designs and capacity for tailoring specific pharmacokinetic profiles provide a unique platform for effective personalized therapeutic treatments while minimizing invasiveness of procedures.

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

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Delivery of Biologics in Cardiovascular Regenerative Medicine

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Stem cell–based cardiovascular regenerative medicine offers the next frontier of therapy through delivery of progenitor cells to achieve structural and functional repair of the myocardium. Initial clinical experience indicates that such therapy is feasible and safe, yet structural and functional benefits remain rather modest. A common pitfall in all studies is the very low rate of cell retention by the recipient myocardial tissue. This limitation warrants lead optimization so as to improve the outcome of stem cell delivery.

As cardiovascular regenerative medicine using stem cell–based biologics approaches the end of its first decade of clinical development, a review of the data accumulated to date is warranted to guide the next stages of development. Ongoing clinical efforts aim to salvage myocardium following acute infarction complementing state-of-the-art reperfusion therapy or to repair dysfunctional myocardium in the chronic setting beyond current therapies that are largely palliative. Initial clinical experience, based on meta-analysis of more than 1,000 patients worldwide with ischemic heart disease who have thus far received adult stem cells, indicates that the approach is feasible and safe.[1,2] However, structural and functional benefits—albeit statistically significant across studies—remain rather modest in magnitude.[1,2] This suggests that further efforts need to be applied to the development and optimization of current techniques before stem cell–based therapy can be considered for wider clinical application. Experience in the course of clinical trials has identified several factors that may influence the outcome of therapy.[3,4] These include patient-specific variables, such as functionality of autologous cells, or patient-independent parameters, such as stem cell processing.[3,4] These findings will guide the design of future hypothesis-driven clinical trials, as well as larger outcome-driven clinical trials.[4] Nevertheless, a common feature has been the apparently low cell retention rates. Accordingly, at present, a major focus of the research community is on the development of methods to improve cell retention and thereby to enhance cell survival.

DELIVERY OF BIOLOGICS AND HURDLES IN CLINICAL TRANSLATION

Clinical studies specifically addressing the delivery and homing of cell biologics (**Table 1**) have identified several issues involved in the translation of cellular therapeutics to practice.[5–10] Using current delivery techniques, the delivery of stem cell–based biologics demonstrates variable retention rates, typically not exceeding 5–10% of the injected dose regardless of the specific method of administration.[5–10] Biodistribution is also variable, depending in part on the cell type,[5] with potentially large numbers of cells finding their way to remote organs such as the lungs, liver, or spleen. The consequences of extracardiac appearance of stem cells are not known, and biovigilance monitoring has been incorporated into the development of stem cells as has been done earlier in drug development. In addition, studies indicate a progressive decrease in myocardial signals after the delivery of labeled stem cells,[7,9,10] a finding consistent with rapid cell death or washout, within hours of administration. Although this limitation does not invalidate the efficacy of stem cells, it suggests that reparative mechanisms involve additional paracrine or immunomodulatory processes that may not require the preservation of intact cells.

The mechanisms that underlie the early loss of cells from the heart are multifactorial and interrelated. With respect to methods of stem cell delivery, specific features of each technique impose an inherent "background level" of acute cell loss. For instance, systemic intravenous delivery leads to a low cell concentration with first myocardial passage as a result of immediate circulatory dilution and, depending on cell size and surface characteristics, sequestration by the pulmonary vasculature. In the case of intramyocardial delivery, the egress of cells through the injection channel, by washout via the lymphatic system, or by accidental injection into the microcirculation are all expected concerns. As described below, the outcome of cell retention is very much a function of the mode of delivery and the composition of the injectate, including its active ingredients and vehicles. However, a third and very important determinant

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Table 1 Clinical studies assessing biodistribution of biologics for cardiac repair

						Biodistribution		
						Heart (%)		
Reference	Patients	Tracer	Imaging	Cells	Delivery	Early (<2 h)	Late (>12 h)	Remote organs
Hofmann *et al.*[5]	STEMI ($n=9$)	^{18}F-FDG	3D PET	BMNC ($n=3$) BMNC ($n=3$) Enriched CD34$^+$ ($n=3$)	IC IV + IC (sequential) IC	1.3–2.6 0 IV (1.8–5.3) 14–39	NA NA NA	85% Liver and spleen
Kang *et al.*[7]	STEMI ($n=11$)	^{18}F-FDG	PET/CT	G-CSF mobilized peripheral stem cells	IC ($n=8$) IV ($n=3$)	0.5–3.3 0	<1.5* NA	24–45% Liver and spleen 19% Lung, 22% liver, and 15% spleen
	Chronic MI ($n=5$)				IC ($n=5$)	0.2–2.4	NA	8% Liver, 15.7% spleen, 5.8% brain, and 6% bladder ($n=1$)
Blocklet *et al.*[6]	STEMI ($n=6$)	^{111}In-oxine	3D PET	G-CSF mobilized CD34$^+$	IC	5.5 ± 2.3		48 ± 35% Liver, 29 ± 19% spleen
Penicka *et al.*[9]	STEMI ($n=5$) Chronic MI ($n=5$)	^{99m}Tc-HMPAO	SPECT	BMNC	IC	1.3–5.1 1.3–3.0	1.1–1.3 0	No quantitative assessment, mostly liver, spleen and lungs, not in brain
Schots *et al.*[8]	Chronic MI ($n=5$)	^{111}In-oxine	SPECT	G-CSF mobilized CD133$^+$	IC	6.9–8.0	2.3–3.2	23–26% Liver, 24–28% spleen
Schachinger *et al.*[10]	STEMI ($n=8$) Intermediate ($n=4$) Chronic MI ($n=5$)	^{111}In-oxine	PET	Cultured EPC	IC	6.3 ± 2.9 4.5 ± 3.2 2.5 ± 1.6	~1–5 ~1–2 ~0–2	~30% Liver, ~9% spleen, and ~8% lung (details, see Figure 2 in ref. 10)

CT, computed tomography; EPC, endothelial progenitor cells; FDG, fluorodeoxyglucose; IC, intracoronary; IM, intramyocardial; PET, positron emission tomography; SPECT, single-photon emission CT; STEMI, ST elevation myocardial infarction; ^{99m}Tc-HMPAO, ^{99m}Tc-d,l-hexamethylpropylene amine oxime.

of cell retention is the diseased myocardial target tissue itself (Figure 1 and Table 2).

TARGET PRIMING FOR AUGMENTED HOMING AND CELL SURVIVAL

In cardiovascular regenerative medicine, especially as applied to myocardial recovery, two fundamental clinical scenarios need to be distinguished: the patient recovering from acute myocardial infarction vs. the patient with chronically dysfunctional myocardium. Differences in the myocardial substrate and patient-specific molecular and cellular profiles governing cell retention and survival affect the choice and applicability of the technique of delivery.[11] Reperfused infarcted myocardium is often associated with microvascular obstruction and massive hemorrhage followed by invasion of white blood cells, creating a potentially adverse microenvironment for exogenously delivered cells if the latter are delivered immediately after reperfusion.[12] In the following days, the healing process is characterized by activation of adhesion molecules, growth factors, and cytokines with pleiotropic effects that may facilitate cell retention.[12] On the other hand, chronically dysfunctional myocardium displays mixed degrees of compensatory hypertrophy, maladaptive remodeling, and areas of nonviable tissue containing patchy areas of fibrosis, leaving cardiac myocytes with variable degrees of perfusion. The mechanisms of stem cell attrition differ in these two clinical situations. Whereas during the days to weeks after acute ischemic injury chemoattractive forces are generally favorable for cells to adhere to vascular or structural myocardial compartments, it is controversial as to whether these signals are present in the setting of remodeled and dysfunctional myocardium. A better understanding of the varied signaling profiles of diseased myocardium relative to the extent of remodeling and dysfunction[13] is important in predicting the amount of cell adhesion and migration that is likely to occur after stem cell administration. Armed with this information, a basis will have been established for efforts aimed at priming the tissue for more efficacious retention, and activation

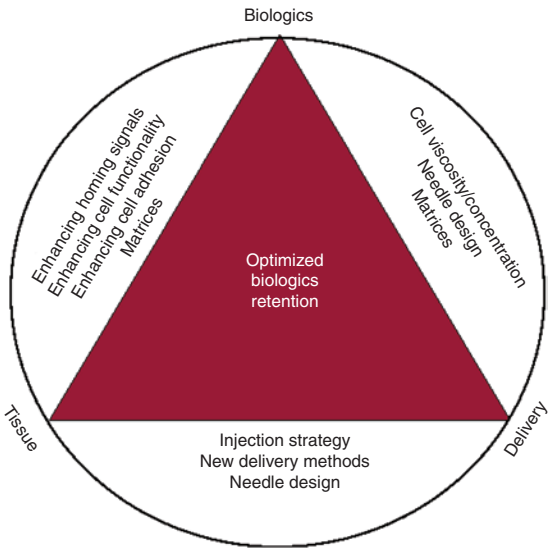

Figure 1 Factors to be considered in the future framework of optimization of biologics retention (see the text for details).

Table 2 Lead optimization for biologics retention

		Effects			
	Issue	Primary	Secondary	CR	Potential solutions
Interactions					
Device–cell product	Cell trauma 2° to:				
	Bioincompatibility	↓Viability, function	↓Functional cell dose	↓	Inert coating
	Shear				↑Core lumen size
	Cell activation	Adhesion, aggregation	Core lumen obstruction	↓	↓Viscosity, cell concentration
		Cytokine/ mediator release	↑Local, pulmonary, and systemic effects	↓	
Device–tissue	Coronary artery disease	Trauma	Acute ischemia	↓	Non-occlusive injection or low-pressure balloon
		Collateral-dependent flow	Uneven tissue distribution	↓	
	Multivenous channels	Unreliable flow patterns	Uneven tissue distribution	↓	Double-balloon catheter
			↑Cell transit to pulmonary arteries	↓	Alternative injection method (intramyocardial)
	Myocardial disease:				
	Recent infarction	Tissue friability	Myocardial perforation	↓	Alternative injection method (venous, perivascular)
		Microvascular obstruction		↓	
		Inconsistent delivery			
	Fibrosis	↓Needle penetration	Reflux via injection site	↓	↓Needle caliber
	No viability	Reduced tissue distribution	↑Systemic appearance	↓	Reconfigured needle (side-holes, multiple)
					Avoiding fibrotic, nonviable myocardium (MRI or electromechanical guidance, ultrasound)
					Controlled/programmed injections
					Imaging and spatial delineation
	Altered cardiac rhythm	Conduction trauma	Fascicular block	?	Prophylactic pacing
		Electrical simulation	Ventricular tachyarrhythmias	?	Antiarrhythmics
Tissue–cell product	Vascular:				
	Cell diameter < vessel	↑Transit to coronary venous system	↑Pulmonary and systemic effects	↓	"Stop-flow" (arterial, venous), ↑adherent cells
	Cell diameter > vessel	Microvascular obstruction	Myocardial ischemia	↑	Alternative injection (intramyocardial, perivascular)
	Cell aggregation				
	Myocardial:				
	↑Interstitial flow rates	↑Transit to coronary lymphatic/venous system	↑Pulmonary and systemic appearance	↓	Adherent carrier (fibrin, hydrogel, nanofibers)
					Engineered cells (ligand-specific antibodies)
	Inflammation	Monocyte infiltration	Cell destruction	↓	Delayed administration
	Fibrosis	Early reflux	—	↓	Alternative needle configurations
		↓Tissue vascularity	*In situ* cell death	↓	Adherent carrier (fibrin, hydrogel)

2°, secondary; CR, cell retention; MRI, magnetic resonance imaging.

of selected pathways governing cell retention and homing can be targeted. Candidate pathways are typically activated in response to ischemia. These include, among others, cytokines such as stromal-derived factor 1, interleukin 6 and 8, hypoxia-inducible factor 1, and vascular endothelial growth factor.[14,15] From the clinical perspective, activation of these pathways would be ideally achieved by nontraumatic stimuli, including low-energy shock waves.[16] Alternatively, pharmacological tissue pretreatment with growth factors or use of chemoattractive peptides and nanofibers might be considered.[15,17] In parallel, pharmacological strategies targeting matrix remodeling could contribute to changes in microenvironment favoring cell homing and survival.[18] In this regard, a concerted effort in clinical development should be deployed for optimization of delivery to dysfunctional but viable myocardium, rather than targeting nonviable tissue.

DEVELOPMENT IN BIOLOGICS

The critical path for successful clinical translation of stem cell–based biologics in regenerative cardiovascular medicine mandates further optimization of the active ingredient, its processing and functionality. From the perspective of delivery and retention, several critical leads have been identified (**Table 2**). In particular, the dose regimen relative to the injection volume is essential for maximizing safety and efficacy. Here, the bolus space in which cells are delivered may determine the immediate extent of retention. In this regard, it remains untested whether enrichment strategies for particular types of biologics may result in higher retention in parallel with superior therapeutic efficacy.[5] Likewise, to minimize loss due to washout, various injection strategies with controlled, programmed delivery or needle modification may be needed. Second, the profile of biologics may influence retention and engraftment. The propensity for attachment to structural myocardial elements (vascular, interstitial matrix, myocytes) is likely to depend on the adhesive characteristics of the given biologic. Pretreatment strategies to enhance these or other functional features open the path to the creation of more advanced cell products. These strategies utilize gene modification,[19] small protein-based conditioning,[15,20] or, alternatively, broad systematic approaches based on the increased understanding of natural, embryonic cardiomyogenesis[21] and related biology.[22] Advances in tissue engineering provide an array of multiple delivery platforms ranging from hydrogels and fibrins to self-assembling peptides or scaffolds with cell-adhesion motifs that may favorably modify retention and survival.[23–25] Further progress with a new generation of biologics may be also expected by applying a biomimetic approach[24] and by developing novel scaffolds combining the presence of growth factors or cytokines. However, such new biologics should be developed hand in hand with delivery devices and methods in order to ensure their clinical applicability.

DELIVERY METHODS AND DEVICES

There is no consensus in cardiovascular regenerative medicine regarding the most appropriate technique or device for stem cell delivery.[11] Rather, the choice depends on the assessment of the underlying pathology and the acuteness of myocardial injury or associated interventional procedure, as well as the actual cytotype to be delivered.[11] This results in several parallel leads that characterize the current stage of development (**Table 2**). One lead optimization addresses device–tissue interactions that are particular to the setting of acute myocardial infarction. The retention efficacy of the commonly used intracoronary infusion route depends on microvascular patency and function and stem cell expression of adhesion molecules. Direct intramyocardial cell delivery, associated with encouraging results with respect to retention efficacy and safety in animal models of acute myocardial infarction,[26,27] has led to the initiation of phase I studies in humans. Retention rates in both techniques are lower than desirable. Alternatively, administering a local reservoir of stem cells responsive to homing signals augments infarct tissue levels. Such a strategy, using a balloon-mounted microneedle catheter to access the periadventitial space, has been granted US Food and Drug Administration approval for early clinical testing (http://clinicaltrials.gov/ct2/show/NCT00677222?term=athersys&rank=1). As with intracoronary infusion, periadventitial delivery utilizes standard imaging techniques, but its safety profile needs to be established, especially in patients with diffuse coronary atherosclerosis in whom plaque trauma and vessel damage are a concern. Finally, novel approaches with coronary sinus delivery[28] or dual arterial-venous balloon occlusion are being explored.

In the setting of chronically ischemic myocardium, tissue heterogeneity due to fibrosis and nonviable myocardium profoundly affects cell tissue–device interaction. This complex interaction requires lead optimization in our understanding of whether delivery guidance with electromechanical mapping is superior to conventional fluoroscopic or other image-based systems. The magnetic resonance imaging–guided approach has been successfully tested in large animal models[29] and has the capacity to enable disease-specific targeting. Other strategies that employ controlled or programmed injections, or needle designs that promote cell dispersion, could also improve retention by limiting the immediate washout. New devices and refinements of earlier designs require bench testing to assess their biocompatibility and effects on cell viability. This is particularly important in the future application of second-generation biologics, where the demands of increased viscosity may require catheter or needle modifications. Complex engineered biologics may ultimately favor surgical rather than catheter delivery.

IMPLICATIONS FOR CLINICAL DEVELOPMENT AND TRANSLATION

In an attempt to optimize the effects of stem cells, novel biologic products are being developed to increase the responsiveness of cells to tissue signaling and to minimize their susceptibility to attrition. Advances in stem cell delivery systems must keep pace if progress in cardiovascular regenerative medicine is to be ensured. Development strategies should address the main aspects of delivery in a concerted manner, ensuring that integration of the three components of biologics-based tissue repair (underlying disease, cell preparation, and delivery method) is preserved. The interplay between these components is variable and dependent on their unique properties, and it is for the delivery system to ensure effective biologics administration, with minimal trauma to cells and tissue. It is important to note that preclinical and clinical development will also depend on the interventional skills of the operators. Intramyocardial injection catheters function within a framework different from that of the vascular devices, and, although a fair body of experience exists, most of these devices have seen limited use in clinical trials to date. Even though none is overly demanding in concept or mechanics, learning curves are still being charted for all of them, especially for those in which advanced myocardial disease is being targeted.

Future development should incorporate a "reverse engineering" approach, harnessing knowledge from a continuous bed–bench–bed life cycle. In this approach, growing clinical experience will provide a guide to further improvement of

components related to delivery (**Figure 1**). Ultimate clinical selection of delivery devices and methods is likely to depend on biologic characteristics and tissue properties. Parallel advances in biologics imaging for both cell tracking and functional assessment of surrogate end points will greatly facilitate progress in cardiovascular regenerative medicine.

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CONFLICT OF INTEREST

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Methods for Female Contraception: A Model for Innovation in Drug Delivery Systems

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In 2007, 1.7 billion women were in need of contraception, but only 57% of them were using modern contraceptives.[1] During a woman's 30-year reproductive life, her contraceptive needs may vary from postponing childbearing to spacing out the births of her children and, finally, to limiting family size. Modern contraceptive methods and their delivery systems reflect these changing needs as well as the challenges associated with the long-term regulation of conception, which are similar to the challenges encountered in developing therapeutics for chronic medical conditions.

CHALLENGES

Advances in contraceptive technology have been impressive in the past 50 years, beginning with the first oral contraceptive (OC) in the early 1960s, followed by long-term hormonal injectables in the late 1960s, copper T intrauterine devices (IUDs) in the 1970s and 1980s, the first progestin subdermal implant in 1983, a progestin intrauterine system (IUS) in 1990, and the first combined hormonal vaginal ring and transdermal patch in the early 2000s. Throughout the world, real-world constraints, including limited access to medical care or contraceptive supplies, high cost of contraceptives, safety concerns, cultural or religious mores, and national policies, restrict the choice and availability of contraceptives.[2]

Nonuse or incorrect use of contraception is associated with unintended pregnancy: approximately 1.5 million pregnancies, half of all unintended pregnancies in the United States annually, occur in contraceptive users, and 90% of these are related to inconsistent or incorrect method of use.[3] Dissatisfaction with the method of contraception and the need for daily attention contribute to this undesired outcome.[2] Therefore, the development of contraceptives remains challenging, given that methods must be not only safe and effective but also acceptable to the user and effective for long periods of time.

OC: A PARADIGM SHIFT

The introduction of the first OCs in the early 1960s created a paradigm shift in contraception, setting a new gold standard for contraceptive efficacy, with perfect-use pregnancy rates of <1% per year in clinical trials and typical-use pregnancy rates of 8%.[4] "Traditional" methods (withdrawal and periodic abstinence) had typical-use pregnancy rates of 25% whereas rates for barrier methods (male or female condom, the diaphragm, and the contraceptive sponge) ranged from 15 to 32%.[4] The benefits of the OC were immediately evident, but knowledge about cycle control accumulated more slowly, and this influenced adherence. There was also the issue of side effects as well as rare, but alarming, serious adverse events. These concerns led to modifications in OCs and the development of new methods.

FACTORS INFLUENCING DEVELOPMENT OF METHODS OF CONTRACEPTION

Although synthetic progestins suppress ovulation when used alone, low-dose progestin-only OCs are somewhat less effective than combined OCs in which an estrogen is added to the progestin; progestin-only methods are also associated with more irregular, unscheduled bleeding.[5]

For these reasons, combined hormonal OCs are the standard today, and most formulations include ethinyl estradiol (EE), a potent synthetic estrogen, along with a synthetic progestin. The use of combined OCs is associated with an increased risk of rare, but serious, cardiovascular events, especially venous thromboembolism (VTE), and also myocardial infarction and stroke, particularly in older women and smokers.[6] The first modification in OC formulations was the reduction of the estrogen dose from ≥50 μg per pill to 30–40 μg per pill, leading to a reduced risk of VTE.[7] EE doses of 15–20 μg per pill are used in some OC formulations today. In some studies, OCs with very low EE doses were associated with more irregular bleeding and lower continuation rates; data from comparative trials are insufficient to establish a difference in effectiveness or safety between OCs with low and very low EE doses.[8]

Tolerability and safety issues stimulated the development of new generations of synthetic progestins with fewer androgenic effects. The first progestins were designed specifically to suppress ovulation. Undesirable side effects, such as bloating or acne, were

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addressed by modifying the molecular structures of progestins,[9] but progestin–estrogen interactions led to more complications. Although OCs with nonandrogenic third-generation progestins were associated with better lipid profiles than the older progestins were,[10] VTE risk seemed to be higher.[6] Recently, the issue of VTE risk associated with OC use was addressed in a large, long-term surveillance study including 58,674 women, designed to compare VTE risk in women using OCs with drospirenone, a fourth generation progestin, with that in women using OCs with levonorgestrel (LNG) or other progestins.[11] The main Cox regression analysis showed no difference in VTE risk between women using drospirenone OCs and those using OCs with LNG or other, older progestins (hazard ratios: drospirenone vs. LNG and drospirenone vs. other progestins: 1.0 and 0.8 (upper limit of 95% confidence interval: 1.8 and 1.3). VTE risk was substantially higher in women who discontinued OC use and became pregnant (19.4/10,000 woman-years);[11] this finding highlights a serious risk associated with unintended pregnancy in former OC users.

NONORAL DELIVERY SYSTEMS FOR SHORT-TERM, USER-CONTROLLED HORMONAL CONTRACEPTIVES

Nonoral delivery systems for combined hormonal contraceptives were developed, in part, in an effort to lower VTE risk by avoiding first-pass hepatic metabolism of steroids. EE, the estrogen in most OCs, has a longer half-life than E_2 (17β-estradiol) and is not metabolized as readily, and this reduces the benefit of avoiding the first-pass effect. In a clinical study in which EE was administered alone, either vaginally or orally, the route of administration had no impact on the hepatic effects of EE.[12] In studies in which EE and a progestin were delivered by oral or vaginal routes, the hepatic impact of EE was modulated by the characteristics of progestin but not by the delivery route.[13,14]

Typically, nonoral delivery systems for user-controlled hormonal contraceptives do not require daily attention.[2] Approved short-term, user-controlled non-OC methods (**Table 1**) include two vaginal rings (a combined etonogestrel–EE ring for general use[15] and a progesterone ring for lactating women[16]) and a transdermal patch delivering norelgestromin and EE.[17] In studies comparing the patch or the etonogestrel–EE vaginal ring with an OC, the nonoral methods were equivalent in efficacy, tolerability, and cycle control to the comparator OC.[15,17]

Several recent studies have addressed the factors that influence the selection of a nonoral short-term hormonal contraceptive method and compliance with use of the method. In one study, 9,700 women beginning hormonal contraception completed a self-administered questionnaire about contraceptive selection. Approximately 46, 39, and 15% selected the vaginal ring, an OC, and the patch, respectively; 62% of those who chose the vaginal ring and 53% of those who opted for the patch chose the method because of the lower probability of inadvertent omission of use. The women who selected the OC were influenced mainly by the proven efficacy of the method.[18] In a second, cross-sectional,

Table 1 User-controlled, nonoral hormonal contraceptive delivery systems

Product	Status	Manufacturer/ developer	Active agent (average daily dose)/ technology	Duration of action	Women, % with unintended pregnancy, 1st year, typical use
Vaginal contraceptives					
NuvaRing	Approved worldwide (US approval: 2001)	Schering-Plough/ Organon	ETO/EE (120 µg/15 µg/day)/ steroid core, ethylene vinyl acetate copolymer ring; od: 54 mm; c-sd: 4 mm	1 Cycle (new ring for each cycle)	8 (4)
Progering (lactating women)	Approved in Chile, Peru	Andromaco SA	Natural P (10 mg/day)/ silicone elastomer/P mix	3 Months, continuous	0 (16)
Nestorone[a]/EE	Phase III	Population Council	NES/EE (150 µg/15 µg/day)/ two steroid cores, silicone elastomer ring body; od: 56 mm; c-sd: 8.4 mm	1 Year (ring used for 13 cycles)	NA
Ulipristal (formerly, CDB-2914)	Phase II	HRA Pharma/ Population Council	Ulipristal/proprietary	3 Months, continuous	NA
Cellulose sulfate vaginal gel	Phase II	CONRAD	Sodium cellulose sulfate	Applied prior to coitus	13.4 (95% CI: 7.5–19.4%)[b] (22)
Transdermal formulations					
Ortho Evra transdermal patch	Approved worldwide (United States: 2001)	Ortho Women's Health and Urology	NGMN/EE matrix-type patch	3 Patches per cycle (1 per week)	8 (4)
Transdermal Nestorone/E_2 gel	Phase II	Antares/Population Council	NES/E_2/proprietary	Daily	—
Transdermal spray Nestorone[a]/estrogen	Phase I	FemPharm Pty Ltd./ Population Council	NES/estrogen MDTS[c] spray	Daily	—

c-sd, cross-sectional diameter; CI, confidence interval; E_2, 17β-estradiol; EE, ethinyl estradiol; ETO, etonogestrel; MDTS, metered dose transdermal system; NA, not available; NES, Nestorone; NGMN, norelgestromin; od, outer diameter; P, progesterone.

[a]Nestorone is a registered trademark of the Population Council. [b]200 couples. [c]MDTS is a registered trademark of Acrux.

multicenter study, a self-administered questionnaire was used to assess the effects of noncompliance with contraceptive regimens. Of the 26,250 women who completed the questionnaire, 65, 23, and 12% reported using an OC, the ring, and the patch, respectively. Of the respondents, 71% of OC users reported noncompliance as compared with 32% of patch users and 21.6% of ring users ($P < 0.0001$, χ^2-test). After an episode of noncompliance, 44% of OC users, 40% of patch users, and 20% of ring users reported feeling relief from tension at the onset of their next menstrual bleed. Compliance-related problems were the most frequent cause of method change; whereas 32% of women switched from OCs or the patch to the ring, only 1 and 3% of ring users switched to an OC and the patch, respectively.[19]

A third contraceptive vaginal ring, developed by the Population Council, is currently in phase III trials. This ring combines Nestorone (NES), a 19-norprogesterone derivative with potent antiovulatory activity when administered nonorally, with EE. The NES/EE ring is designed for use for 13 cycles rather than disposal after a single cycle.[20]

Several other short-term, user-controlled contraceptives made with NES are in the early stages of development. In a phase I study, an NES-based skin-spray formulation was shown to block ovulation.[21] Spray formulations incorporating both NES and an estrogen are also being studied, as is a transdermal gel preparation combining NES with E_2. A cellulose sulfate vaginal gel, originally designed for dual protection against pregnancy and human immunodeficiency virus infection, was shown to have contraceptive efficacy similar to the spermicide nonoxynol-9,[22] but it was not effective in preventing human immunodeficiency virus infection.[23]

Finally, there is interest in developing a hormonal contraceptive system without estrogen but with acceptable cycle control. One candidate under investigation is a vaginal ring delivering ulipristal, a progesterone receptor modulator developed at the National Institutes of Health and now licensed to HRA Pharma. An oral emergency contraceptive using this agent is currently under review in Europe.

DELIVERY SYSTEMS FOR LONG-TERM REVERSIBLE CONTRACEPTIVES

The long-term reversible contraceptive methods (IUD/IUSs, subdermal implants, and injectables) described in **Table 2** are highly effective, with real-life annual failure rates of <1%.[4] The Copper T (TCu) 380A (ParaGard in the United States)

Table 2 Long-acting, reversible contraceptive methods

Product	Status	Manufacturer/ developer	Active agent (average daily dose)/technology	Duration of action	Women, % with unintended pregnancy, 1st year, typical use
Subdermal implants					
Norplant	1st Approved 1983 (US approval: 1990, not currently manufactured)	Discontinued	LNG (85 (initial) to 30 (steady state) μg/day)/ 6-capsule system	7 Years	0.7 (27)[a]
Jadelle	Approved worldwide (US approval: 1996)	Bayer HealthCare Pharmaceuticals/ Population Council	LNG (100 (1 mo) to 30 (24 mo) μg/day)/ 2-rod system	5 Years	≤1 (27)[a]
Implanon	Approved worldwide (United States: 2006)	Schering-Plough/ Organon	ETO (initial: 60–70 μg/ day; final: 25–30 μg/day)/ single rod	3 Years	0.05 (4)
Intrauterine device/system					
Copper IUDs (in United States: ParaGard T 380A intrauterine copper contraceptive)	Approved worldwide (US approval: 1984)	Many (United States: Teva Pharmaceuticals)/ Population Council	Copper (area: 380 ± 23 mm^2)/ T-shaped polyethylene stem; copper wire on vertical stem; "collars" on horizontal arms	10 Years	0.8 (4)
Mirena IUS	Approved worldwide, 1st approval: Finland 1990 (United States: 2000)	Bayer HealthCare Pharmaceuticals/ Population Council	LNG (initial: 20 μg/day)/ T-shaped polyethylene stem, steroid reservoir cylinder around stem	5 Years	0.2 (4)
Injectable contraceptives					
Depo-Provera depo-subQ provera 104	Approved worldwide (United States: 1992)	Pfizer/Pharmacia & Upjohn	DMPA (150 mg/3 months, IM, or 104 mg/3 months, SC)	3 Months	3 (4)
Combined estrogen–progestin injectables[b]	Not currently available in the United States	Many manufacturers	DHPA 75 mg + E_2EN 5 mg DHPA 150 mg + E_2EN 10 mg DMPA 25 mg + E_2C 5 mg NET-EN 50 mg + E_2V 5 mg	1 Month	—

DHPA, dihydroxyprogesterone acetophenide; DMPA, depot medroxyprogesterone acetate; E_2, estradiol; E_2C, estradiol cypionate; E_2EN, estradiol enanthate; E_2V, estradiol valerate; ETO, etonogestrel; IUDs, intrauterine devices; IUS, intrauterine system; IM, intramuscularly; LNG, levonorgestrel; NET-EN, norethisterone enanthate; SC, subcutaneously.

[a]Pregnancy rates in year 5 of a 5-year comparative study (Norplant vs. Jadelle), see ref. 27. [b]Described in ref. 29.

and TCu380S had the lowest overall pregnancy rates in randomized, controlled comparative clinical trials including other copper-releasing IUDs (TCu220, TCu200, Multiload 375 or 250, Copper 7, Copper-Safe 3000, and Nova T).[24] A recent review of published data indicated that the TCu380A and the LNG IUS were associated with roughly comparable 5-year cumulative pregnancy rates.[25]

The main side effects of the copper T IUDs are irregular menstrual bleeding and pain, especially in the first 2 years after insertion.[24] The LNG IUS is associated with reduced menstrual bleeding (leading to amenorrhea in some cases) and may have hormone-related side effects.[26]

An IUD/IUS must be inserted and removed by a health-care professional, and this takes away the user's control of fertility. These methods are suitable for women who wish to postpone childbearing for a prolonged period or who have completed their families but do not wish to undergo sterilization.

Norplant and Jadelle, the first subdermal implants, both developed by the Population Council, contain LNG. The clinical equivalence of these systems has been demonstrated for 5 years.[27] In a recent review of comparative trials of contraceptive implants, the authors concluded that Norplant, Jadelle, and Implanon were equally effective; irregular bleeding was the most common side effect; and Jadelle and Implanon were easier to remove than Norplant.[28] The subdermal implants have the same advantages and disadvantages as the IUD/IUS systems: long-term efficacy and safety without daily attention but with reduced control over fertility by the user.

Other long-term alternatives include 3-month injectable contraceptives delivering medroxyprogesterone acetate (Depo-Provera or depo-subQ provera 104, depot medroxyprogesterone acetate); these formulations have perfect-use rates equivalent to those of hormonal implants and the TCu380A IUD.[4] Concern about bone loss, especially in adolescents, led to recommendations that DMPA be used for only 2 years.[29] Combination injectable contraceptives for monthly administration are not currently available in the United States but are used elsewhere; when compared with DMPA-only injectables, combination formulations were associated with a reduced incidence of bleeding irregularities.[30]

CONCLUSION

The lessons learned during the development of modern contraceptive methods may be relevant to the development of therapeutics for chronic medical conditions requiring treatment throughout the life cycle. Despite advances in contraception, many women who wish to avoid pregnancy either choose not to use contraception, do not have access to a suitable method, or use a method incorrectly,[1,3] resulting in millions of unintended pregnancies each year accompanied by significant morbidity and mortality.[2] Clearly, improvements in methods have contributed to the increased use of contraception worldwide, but current user-controlled methods could be further improved to promote greater adherence. It would be helpful to ease restrictions on access to medical care and contraceptive supplies because such restrictions limit the use of contraceptives, particularly the long-acting, reversible methods that are associated with higher rates of compliance. Further advances in the field of contraception will require targeted development of new and improved methods combined with country-specific changes in policy and funding in order to ensure access to these methods.

CONFLICT OF INTEREST

The authors are employed by the Population Council, which develops contraceptive methods for men and women.

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CORRIGENDUM: PI-34: An Analysis of Subjects in Clinical Trials Who, After Dosing, Report Predose Adverse Events and Abnormalities in Their Medical Histories

Clin. Pharmacol. Ther. **85**, S9–S36 (2009); doi:10.1038/sj.clpt.2008.283

In this abstract, published in the 2009 *Clinical Pharmacology & Therapeutics* Abstract Supplement, the author byline is incomplete. The correct author list is:

JP Kitzmiller, DK Groen and G Apseloff; The Ohio State University, Columbus, OH

The authors regret this error.

CORRIGENDUM: Dapagliflozin, a Novel, Selective SGLT2 Inhibitor, Improved Glycemic Control Over 2 Weeks in Patients With Type 2 Diabetes Mellitus

B Komoroski, N Vachharajani, Y Feng, L Li, D Kornhauser and M Pfister

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> The median values for each are 36.9 g/day (5 mg dose, day 1), and 29.1 mg/day (5 mg dose, day 14); 62.3 g/day (25 mg dose, day 1) and 67.0 mg/day (25 mg dose, day 14); 79.7 g/day (100 mg dose, day 1) and 68.4 mg/day (100 mg dose, day 14).

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