<table>
<thead>
<tr>
<th></th>
<th>Title</th>
<th>Authors</th>
<th>Pages</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.</td>
<td>Rosuvastatin pharmacokinetics and pharmacogenetics in white and Asian subjects residing in the same environment • ARTICLE</td>
<td>Edmund Lee, Stephen Ryan, Bruce Birmingham, Julie Zalikowski, Ruth March, Helen Ambrose, Rachael Moore, Caroline Lee, Yusong Chen and Dennis Schneck</td>
<td>330-341</td>
</tr>
<tr>
<td>11.</td>
<td>The impact of uridine diphosphate–glucuronosyltransferase 1A9 (UGT1A9) gene promoter region single-nucleotide polymorphisms T−275A and C−2152T on early mycophenolic acid dose-interval exposure in de novo renal allograft recipients • ARTICLE</td>
<td>Dirk R.J. Kuypers, Maarten Naesens, Severine Vermeire and Yves Vanrenterghem</td>
<td>351-361</td>
</tr>
<tr>
<td>12.</td>
<td>Influence of CYP2C9 genotypes on the pharmacokinetics and pharmacodynamics of piroxicam • ARTICLE</td>
<td>Jamila A. Perini, Rosane Vianna-Jorge, Ariane R. Brogliato and Guilherme Suarez-Kurtz</td>
<td>362-369</td>
</tr>
<tr>
<td>13.</td>
<td>CYP2C9, but not CYP2C19, polymorphisms affect the pharmacokinetics and pharmacodynamics of glyburide in Chinese subjects • ARTICLE</td>
<td>Ophelia Q.P. Yin, Brian Tomlinson and Moses S.S. Chow</td>
<td>370-377</td>
</tr>
<tr>
<td>14.</td>
<td>Impact of CYP2D6 genotype on adverse effects during treatment with metoprolol: A prospective clinical study • ARTICLE</td>
<td>Richard Fux, Klaus Mörike, Anne M.T. Pröhmer, Ursula Delabar, Matthias Schwab, Elke Schaeffeler, Gernot Lorenz, Christoph H. Gleiter, Michel Eichelbaum and Kari T. Kivistö</td>
<td>378-387</td>
</tr>
</tbody>
</table>
15. Cyclosporine markedly raises the plasma concentrations of repaglinide • ARTICLE
   Pages 388-399
   Lauri I. Kajosaari, Mikko Niemi, Mikko Neuvonen, Jouko Laitila, Pertti J. Neuvonen and Janne T. Backman

PHARMACOKINETICS AND DRUG DISPOSITION

16. Oral contraceptives containing ethinyl estradiol and gestodene markedly increase plasma concentrations and effects of tizanidine by inhibiting cytochrome P450 1A2 • ARTICLE
   Pages 400-411
   Marika T. Granfors, Janne T. Backman, Jouko Laitila and Pertti J. Neuvonen

PHARMACODYNAMICS AND DRUG ACTION

17. Safety, pharmacodynamics, and pharmacokinetics of single doses of BAY 59-7939, an oral, direct factor Xa inhibitor • ARTICLE
   Pages 412-421
   Dagmar Kubitza, Michael Becka, Barbara Voith, Michael Zuehlsdorf and Georg Wensing

18. Glutathione peroxidase, thioredoxin, and membrane protein changes in erythrocytes predict ribavirin-induced anemia • ARTICLE
   Pages 422-432
   Ignazio Grattagliano, Stefan Russmann, Vincenzo O. Palmieri, Piero Portincasa, Giuseppe Palasciano and Bernhard H. Lauterburg

CLINICAL PHARMACOLOGY GRAND ROUNDS

19. Imatinib induces hypothyroidism in patients receiving levothyroxine • ARTICLE
   Pages 433-438
   Jan Willem B. de Groot, Bernard A. Zonnenberg, John T.M. Plukker, Winette T.A. van Der Graaf and Thera P. Links

LETTERS TO THE EDITOR

20. Informed consent or acknowledgment of disclosure • CORRESPONDENCE
   Pages 439-440
   Marcus M. Reidenberg
| 21. | Additional discussions regarding the altered metabolism and transport of omeprazole after long-term use of St John’s wort • CORRESPONDENCE  
*Pages 440-441*  
Hong-Guang Xie |
|---|---|
| 22. | The CYP2C9 genotype does not influence sildenafil pharmacokinetics in healthy volunteers • CORRESPONDENCE  
*Pages 441-443*  
Alexander Jetter, Andreas Lazar, Edgar Schömig, Uwe Fuhr, Martina Kinzig-Schippers and Fritz Sörgel |

**NOTES OF THE AMERICAN SOCIETY FOR CLINICAL PHARMACOLOGY AND THERAPEUTICS**

| 23. | A message from the ASCPT membership committee chairperson • ANNOUNCEMENT  
*Page 444*  
John T. Sullivan |
COMMENTARIES

311 Ethnic differences in statin disposition
Rommel G. Tirona, PhD, Nashville, Tenn, Division of Clinical Pharmacology, Vanderbilt University Medical Center

317 Genetic and nongenetic determinants of between-patient variability in the pharmacokinetics of mycophenolic acid
Dennis A. Hesselink, MD, and Teun van Gelder, MD, PhD, Rotterdam, The Netherlands, Department of Internal Medicine, Renal Transplant Unit, and Hospital Pharmacy, Clinical Pharmacology Unit, Erasmus MC

322 Confounding factors for sex differences in pharmacokinetics and pharmacodynamics: Focus on dosing regimen, dosage form, and formulation
Mei-Ling Chen, PhD, Rockville, Md, Office of Pharmaceutical Science, Center for Drug Evaluation and Research, Food and Drug Administration

Continued on page 2A
PHARMACOGENETICS AND GENOMICS

330 Rosuvastatin pharmacokinetics and pharmacogenetics in white and Asian subjects residing in the same environment
Edmund Lee, PhD, Stephen Ryan, MD, Bruce Birmingham, PhD, Julie Zalikowski, MS, Ruth March, PhD, Helen Ambrose, PhD, Rachael Moore, BSc, Caroline Lee, PhD, Yusong Chen, PhD, and Dennis Schneck, MD, PhD, Singapore, Singapore, Wilmington, Del, and Macclesfield, United Kingdom, National University Hospital and Changi General Hospital, Singapore; AstraZeneca, Wilmington; AstraZeneca, Alderley Park, Macclesfield

342 Effect of OATP1B1 (SLCO1B1) variant alleles on the pharmacokinetics of pitavastatin in healthy volunteers
Jae-Yong Chung, MD, Joo-Youn Cho, PhD, Kyung-Sang Yu, MD, PhD, Jung-Ryul Kim, MD, Dal-Seok Oh, OMD, Hye-Ryung Jung, MS, Kyoung-Soo Lim, MD, Ki-Ho Moon, Sang-Goo Shin, MD, PhD, and In-Jin Jang, MD, PhD, Seoul, Korea, Department of Pharmacology and Clinical Pharmacology Unit, Seoul National University College of Medicine and Hospital, and Choongwae Pharma

351 The impact of uridine diphosphate–glucuronosyltransferase 1A9 (UGT1A9) gene promoter region single-nucleotide polymorphisms T–275A and C–2152T on early mycophenolic acid dose-interval exposure in de novo renal allograft recipients
Dirk R. J. Kuypers, MD, PhD, Maarten Naesens, MD, Severine Vermeire, PhD, and Yves Vanrenterghem, MD, PhD, Leuven, Belgium, Department of Nephrology and Renal Transplantation and Department of Gastroenterology, Laboratory of Absorption and Digestion, University Hospitals Leuven

362 Influence of CYP2C9 genotypes on the pharmacokinetics and pharmacodynamics of piroxicam
Jamila A. Perini, PharmD, Rosane Vianna-Jorge, PharmD, PhD, Ariane R. Brogliato, and Guilherme Suáres-Kurtz, MD, Doct Med, Rio de Janeiro, Brazil, Divisão de Farmacologia, Instituto Nacional de Câncer, and Departamento de Farmacologia Básica e Clínica, Universidade Federal do Rio de Janeiro

370 CYP2C9, but not CYP2C19, polymorphisms affect the pharmacokinetics and pharmacodynamics of glyburide in Chinese subjects
Ophelia Q. P. Yin, PhD, Brian Tomlinson, MD, and Moses S. S. Chow, PharmD, Shatin, Hong Kong, School of Pharmacy and Department of Medicine and Therapeutics, Faculty of Medicine, The Chinese University of Hong Kong

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Impact of CYP2D6 genotype on adverse effects during treatment with metoprolol: A prospective clinical study

Richard Fux, MD, Klaus Mörike, MD, Anne M. T. Pröhmer, MD, Ursula Delabar, PhD, Matthias Schwab, MD, Elke Schaeflfer, PhD, Gernot Lorenz, MD, Christoph H. Gleiter, MD, Michel Eichelbaum, MD, and Kari T. Kivistö, MD, Tübingen and Stuttgart, Germany, Abteilung Klinische Pharmakologie, Lehrbereich Allgemeinmedizin der Medizinischen Fakultät, and Koordinierungszentrum Klinische Studien, Universitätsklinikum Tübingen, Tübingen; Dr Margarete Fischer-Bosch-Institut für Klinische Pharmakologie, Stuttgart

Cyclosporine markedly raises the plasma concentrations of repaglinide

Lauri I. Kajosaari, MB, Mikko Niemi, MD, Mikko Neuvonen, MSc, Jouko Laitila, Pertti J. Neuvonen, MD, and Janne T. Backman, MD, Helsinki, Finland, Department of Clinical Pharmacology, University of Helsinki, and Helsinki University Central Hospital

Oral contraceptives containing ethinyl estradiol and gestodene markedly increase plasma concentrations and effects of tizanidine by inhibiting cytochrome P450 1A2

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Safety, pharmacodynamics, and pharmacokinetics of single doses of BAY 59-7939, an oral, direct factor Xa inhibitor

Dagmar Kubitza, MD, Michael Becka, PhD, Barbara Voith, PhD, Michael Zuehlsdorff, PhD, and Georg Wensing, MD, Wuppertal, Germany, Bayer HealthCare AG

Glutathione peroxidase, thioredoxin, and membrane protein changes in erythrocytes predict ribavirin-induced anemia

Ignazio Grattagliano, MD, Stefan Russmann, MD, Vincenzo O. Palmieri, MD, Piero Portincasa, MD, PhD, Giuseppe Palasciano, MD, and Bernhard H. Lauterburg, MD, Bari, Italy, Lexington, Mass, and Bern, Switzerland, Section of Internal Medicine, Department of Internal Medicine and Public Medicine, University of Bari, Bari; Boston Collaborative Drug Surveillance Program, Boston University, Lexington; Department of Clinical Pharmacology, University of Bern, Bern

Continued on page 4A
CLINICAL PHARMACOLOGY GRAND ROUNDS

433 Imatinib induces hypothyroidism in patients receiving levothyroxine
Jan Willem B. de Groot, MD, Bernard A. Zonnenberg, MD, John T. M. Plukker, MD, PhD, Winette T. A. van Der Graaf, MD, PhD, and Thera P. Links, MD, PhD, Groningen and Utrecht, The Netherlands, Departments of Endocrinology, Surgical Oncology, and Medical Oncology, University Medical Centre Groningen, Groningen; Department of Medical Oncology, University Medical Centre Utrecht, Utrecht

LETTERS TO THE EDITOR

439 Informed consent or acknowledgment of disclosure
Marcus M. Reidenberg, MD, New York, NY

440 Additional discussions regarding the altered metabolism and transport of omeprazole after long-term use of St John’s wort
Hong-Guang Xie, MD, PhD, Nashville, Tenn

441 The CYP2C9 genotype does not influence sildenafil pharmacokinetics in healthy volunteers
Alexander Jetter, MD, Andreas Lazar, MD, Edgar Schömig, MD, Uwe Fuhr, MD, Martina Kinzig-Schippers, PhD, and Fritz Sörgel, PhD, Köln and Nürnberg-Heroldsberg, Germany

NOTES OF THE AMERICAN SOCIETY FOR CLINICAL PHARMACOLOGY AND THERAPEUTICS

444 A Message from the ASCPT Membership Committee Chairperson
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In this issue of the Journal, Lee et al\textsuperscript{1} prospectively examined the pharmacokinetics of the 3-hydroxy-3-methylglutaryl–coenzyme A reductase inhibitor (statin) rosuvastatin in white and Asian individuals living in Singapore. This study demonstrates striking and intriguing population differences in rosuvastatin plasma exposure between white subjects and Asians of Chinese, Malay, and Indian descent. Importantly, the 1.6- to 2.3-fold greater rosuvastatin exposures in Asians in comparison with white subjects did not appear to be accounted for by differences in macroenvironment, weight, and gross dietary intake among subjects. Such convincing and overt population differences between Asians and white subjects in the disposition of drugs have not been previously described\textsuperscript{2} until now. The current findings are in accordance with other unpublished data that indicate similar disparities in rosuvastatin exposure among white subjects and Asians but not Hispanic, black, or Afro-Caribbean groups living in the United States.\textsuperscript{3} Collectively, these results strongly suggest a genetic basis for interindividual variability in rosuvastatin pharmacokinetics for which relevant mechanisms have not been determined. Moreover, whether the ethnic differences in rosuvastatin disposition also have an impact on lipid-lowering effects or susceptibility to known adverse effects of statins remains to be seen.

DETERMINANTS OF ROSUVASTATIN DISPOSITION

To better understand and evaluate the implications of the study by Lee et al,\textsuperscript{1} the determinants of rosuvastatin exposure require additional elaboration. Previous pharmacokinetic analyses of rosuvastatin disposition indicate that the drug has an oral bioavailability ranging from 20\% to 29\%.\textsuperscript{1} The fraction of the oral dose absorbed by the intestines has been estimated to be 50\% whereas the hepatic extraction ratio (fraction of incoming drug removed) is moderate, at 0.63,\textsuperscript{4} indicating that both first-pass organs contribute significantly to the bioavailability and clearance of rosuvastatin. Metabolism is not considered to be a major mechanism for rosuvastatin elimination\textsuperscript{3,5} in which, presumably, cytochrome P450 (CYP) 2C9- mediated \textit{N}-demethylation\textsuperscript{5} and uridine diphosphate–glucuronosyltransferase (UGT) 1A1– and UGT1A3-mediated acyl-glucuronidation,\textsuperscript{6} followed by spontaneous lactonization,\textsuperscript{6} are minor pathways. Hence, because rosuvastatin is a hydrophilic hydroxy acid (octanol/water partition coefficient of
Fig 1. Schematic diagram of known and unknown processes in enterocytes and hepatocytes that determine rosuvastatin (RVA) disposition. The site(s) of metabolism are not clearly established but potentially include the small intestine and liver. ATP, Adenosine triphosphate; BCRP, breast cancer resistance protein; UGT, uridine diphosphate–glucuronosyltransferase; Gluc, glucuronide; HMG-CoA, 3-hydroxy-3-methylglutaryl–coenzyme A; OATP, organic anion transporting polypeptide.

Table I. Statin transporters

<table>
<thead>
<tr>
<th>Transporter</th>
<th>Transport direction</th>
<th>Tissue localization</th>
<th>Statin substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-gp</td>
<td>Efflux</td>
<td>Intestine, liver, kidney, brain</td>
<td>Atorvastatin&lt;sup&gt;33&lt;/sup&gt; Cerivastatin&lt;sup&gt;34&lt;/sup&gt; Simvastatin&lt;sup&gt;35&lt;/sup&gt;</td>
</tr>
<tr>
<td>MRP2</td>
<td>Efflux</td>
<td>Intestine, liver, kidney, brain</td>
<td>Pravastatin&lt;sup&gt;36&lt;/sup&gt; Cerivastatin&lt;sup&gt;4&lt;/sup&gt; Fluvastatin&lt;sup&gt;8&lt;/sup&gt; Pitavastatin&lt;sup&gt;8&lt;/sup&gt; Pravastatin&lt;sup&gt;8&lt;/sup&gt; Rosuvastatin&lt;sup&gt;7&lt;/sup&gt;</td>
</tr>
<tr>
<td>BCRP</td>
<td>Efflux</td>
<td>Intestine, liver, brain</td>
<td>Pravastatin&lt;sup&gt;7&lt;/sup&gt;</td>
</tr>
<tr>
<td>BSEP</td>
<td>Efflux</td>
<td>Liver</td>
<td>Atorvastatin&lt;sup&gt;17&lt;/sup&gt; Cerivastatin&lt;sup&gt;38&lt;/sup&gt; Pitavastatin&lt;sup&gt;39&lt;/sup&gt; Pravastatin&lt;sup&gt;40&lt;/sup&gt; Rosuvastatin&lt;sup&gt;7&lt;/sup&gt;</td>
</tr>
<tr>
<td>OATP1B1</td>
<td>Uptake</td>
<td>Liver</td>
<td>Pravastatin&lt;sup&gt;41&lt;/sup&gt;</td>
</tr>
<tr>
<td>OATP1B3</td>
<td>Uptake</td>
<td>Liver</td>
<td>Atorvastatin&lt;sup&gt;43&lt;/sup&gt;</td>
</tr>
<tr>
<td>OATP2B1</td>
<td>Uptake</td>
<td>Intestine, liver, kidney, brain</td>
<td>Pravastatin&lt;sup&gt;43&lt;/sup&gt;</td>
</tr>
<tr>
<td>OAT3</td>
<td>Uptake</td>
<td>Kidney, brain, skeletal muscle</td>
<td>Pravastatin&lt;sup&gt;44&lt;/sup&gt;</td>
</tr>
<tr>
<td>MCT1</td>
<td>Uptake</td>
<td>Ubiquitous</td>
<td>Pravastatin&lt;sup&gt;44&lt;/sup&gt;</td>
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<tr>
<td>MCT4</td>
<td>Uptake</td>
<td>Ubiquitous</td>
<td>Atorvastatin&lt;sup&gt;33&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

P-gp, P-glycoprotein; MRP, multidrug resistance–associated protein; BCRP, breast cancer resistance protein; BSEP, bile salt export pump; OATP, organic anion transporting polypeptide; OAT, organic anion transporter; MCT, monocarboxylate transporter.

*Indirect evidence.
0.13), membrane transporters have been implicated as important determinants of its intestinal absorption and hepatobiliary clearance. This idea is tenable because older statins are well-recognized substrates for a number of cellular uptake and efflux transporters (Table I). In fact, currently, we understand that the hepatic uptake transporter organic anion transporting polypeptide (OATP) 1B1 and, recently, the entercyte and hepatocyte efflux transporter breast cancer resistance protein (BCRP) mediate transmembrane passage of rosuvastatin. Another important efflux transporter, P-glycoprotein, does not appear to play a role in rosuvastatin disposition. It is notable that for several processes in rosuvastatin absorption and elimination the identities of the pertinent rosuvastatin transporters remain unknown. A scheme that depicts the molecular and cellular events involved in rosuvastatin disposition in the intestine and liver is shown in Fig 1. Conceivably, variation in the efficiencies in any one or more of the processes shown could contribute to the differences in rosuvastatin pharmacokinetics between Asians and white subjects. Hence, Lee et al focused their attention on analysis of SLCO1B1, the gene encoding OATP1B1.

**SLCO1B1 GENETIC VARIATION AND ROSUVASTATIN PHARMACOKINETICS**

Facilitative hepatic uptake from the portal circulation by OATP1B1 is thought to contribute to tissue selectivity and therapeutic response to statins including rosuvastatin. Moreover, OATP1B1 is a component of the vectorial transport pathway of statins from blood to bile, and, therefore, the possibility arises that relatively lower OATP1B1 activities in Asians may have been responsible for the observed higher drug exposures when compared with white subjects. This contention is not without merit, given that several studies have demonstrated an association between SLCO1B1 genotype and pravastatin drug levels. Specifically, individuals carrying the SLCO1B1 521C (V174A) variant, as found in SLCO1B1*5 and *15 haplotypes, are associated with higher pravastatin exposures, a result consistent with decreased in vitro transport function. Whereas Lee et al found an association between the homozygous SLCO1B1 521CC genotype and increased rosuvastatin levels in white subjects, heterozygosity at this allele did not confer differences in drug concentrations within each ethnic group. Notably, the genetic polymorphism in SLCO1B1 at the 521 position did not account for the clear population differences in rosuvastatin exposures among white subjects and the Asian groups. These results may not be surprising, given the lack of profound differences in the allelic frequencies of the 521C variant among white (0.222), Chinese (0.086), Malay (0.129), and Asian-Indian (0.071) subjects, where “all-or-none” genotypes would be expected for observed ethnic population effects. Because, to date, no other known SLCO1B1 polymorphism is more predominant in Asians and white subjects, it appears that the pharmacogenetics of other rosuvastatin disposition pathways may better explain the ethnic differences in pharmacokinetics.

**GENETIC INVOLVEMENT OF OTHER RELEVANT ROSUVASTATIN DISPOSITION PATHWAYS?**

On the basis of the lack of association with SLCO1B1 genotype, other pathways involved in rosuvastatin disposition should now be examined for polymorphisms associated with ethnically determined variability in exposures. Metabolic enzymes are unlikely to contribute to the population differences in rosuvastatin disposition because polymorphisms in CYP2C9 or UGT1A1 that are associated with decreased activity are not found in greater prevalence in Asians than in white subjects. Among transporters, genetic variation in BCRP could also play a role. However, the lack of a striking difference in the allelic frequencies between ethnic groups for known BCRP nonsynonymous polymorphisms suggests that other rosuvastatin disposition pathways should also be investigated. Therefore other unknown disposition pathways, probably involving transport proteins, will need to be examined to clearly establish the mechanisms involved in ethnic variations in rosuvastatin exposure. Of particular interest would be those transporters involved in the absorptive transport of rosuvastatin in enterocytes, in addition to other hepatic transporters (Fig 1).

**ETHNIC DIFFERENCES IN DRUG DISPOSITION WITH OTHER STATINS?**

The observations by Lee et al beg the question of whether other statins may also exhibit ethnic differences in systemic exposures. There are, however, differences in the physicochemical characteristics and metabolic/transporter susceptibilities among the available statins that often argue against this extrapolation. For instance, many statins are efficiently metabolized by CYP3A (atorvastatin, cerivastatin, fluvastatin, lovastatin, simvastatin), CYP2C9 (fluvastatin), or CYP2C8 (cerivastatin) and are also substrates for numerous transporters (Table I). The multiplicity in metabolic and transport pathways often found for statins precludes ethnic population variation in one pathway causing a significant impact on the overall pharmacokinetics.
This may be the case for cerivastatin, for which ethnic differences among white, Japanese, black, and Hispanic subjects in drug exposures were not observed in a retrospective study, despite the fact that ethnically determined genetic variations in major disposition pathways (CYP2C8, multidrug resistance 1 gene, and SLCO1B1) exist. Although there may be significant interindividual differences in the exposures of statins that are cleared predominantly by polymorphic enzymes such as fluvastatin with CYP2C9, relevant variants do not remarkably segregate among ethnicities in a manner that would manifest as a population phenomenon.

INTERINDIVIDUAL VARIATION IN ROSUVASTATIN DISPOSITION AND PHARMACODYNAMIC EFFECTS

The wide variation among individuals in the response to statins is thought to be partly determined by genetics. Polymorphisms in cholesterol homeostasis genes have been the major focus of statin therapy pharmacogenetics research, but there has also been considerable attention directed toward the impact of variation in drug disposition genes on pharmacologic effects. It is worth noting that for all statins, including rosuvastatin, the factors that affect their clearance from the systemic circulation are, importantly, also those that may determine therapeutic response because the liver is both the site of the drug target and the major eliminating organ. Hence, interindividual differences in the expression and function of hepatocyte drug transporters that introduce (ie, basolateral uptake transporters) or take away (ie, biliary or basolateral efflux transporters) rosuvastatin to and from its intracellular target, 3-hydroxy-3-methylglutaryl-coenzyme A reductase, would be expected to translate into variability in hepatocellular drug levels and the resulting antihyperlipidemic effects. In support of this notion, a small retrospective study by Tachibana-Iimori et al indicated that individuals with SLCO1B1 521C alleles had attenuated lipid-lowering effects in comparison with those homozygous for 521T, consistent with in vitro evidence for decreased hepatocyte uptake transport function of this variant. Because individuals with SLCO1B1 521C alleles have associated higher drug levels of pravastatin than those with 521T, the concentration-response relationship is likely to differ among individuals depending on SLCO1B1 genotype. Whether polymorphisms in SLCO1B1 or other hepatic rosuvastatin transporters influence the lipid-lowering effect of rosuvastatin will require additional investigation. Furthermore, studies determining the pharmacokinetic-pharmacodynamic response relationship for rosuvastatin in Asians and white subjects will be of clinical interest.

SUMMARY

The investigation performed by Lee et al addressed important questions regarding the appropriateness of extrapolating apparent population differences in rosuvastatin disposition inferred from separate pharmacokinetic studies in Japan, England, and the United States, through a formal prospective study in white and Asian groups living in the same city (Singapore). Undoubtedly, their dramatic findings on ethnically determined drug disposition directly impacted product labeling. How this information will affect the safety and efficacy of rosuvastatin therapy will be viewed with certain interest. In the meantime, the mechanisms underlying ethnic differences in rosuvastatin disposition will have to be unearthed. We can expect that, through targeted clinical and basic research, better prediction of interindividual variability in drug response will improve clinical outcomes and optimize drug development.

The author has no conflict of interest.

References

Ethnic differences in statin disposition


Genetic and nongenetic determinants of between-patient variability in the pharmacokinetics of mycophenolic acid

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Mycophenolate mofetil (MMF) (CellCept; Hoffmann-La Roche, Basel, Switzerland) and mycophenolate sodium (Myfortic; Novartis Pharmaceuticals Corp, Basel, Switzerland) are prodrugs of the immunosuppressive compound mycophenolic acid (MPA). MMF was approved by the US Food and Drug Administration in 1995, after the publication of the results of 3 phase III, randomized, controlled, double-blind, multicenter trials performed in de novo kidney transplant recipients.1-3 These so-called pivotal trials demonstrated that combination therapy consisting of MMF, cyclosporine (INN, ciclosporin), and corticosteroids, with or without antithymocyte globulin induction treatment, significantly reduced the incidence of acute rejection compared with the combination of cyclosporine, corticosteroids, and either placebo or azathioprine.1-3 Since then, MMF has also proved effective in combination with other drugs, such as tacrolimus and sirolimus, as well as after other forms of solid organ transplantation.4 As a result, there has been a rise in the use of MMF during the past decade. In 2002, 79% of kidney transplant recipients received MMF before hospital discharge.5 At present, MMF has largely replaced azathioprine as the antimetabolite of choice after renal transplantation.5 A similar trend has occurred for heart and liver transplantation.5 In addition, MMF is being increasingly used in the prevention of graft-versus-host disease after hematopoietic stem cell transplantation6 and is currently under investigation in several autoimmune disorders, including systemic lupus erythematosus, psoriasis, and myasthenia gravis.7,8

The success of MMF in transplantation medicine and in autoimmune disease has been achieved by use of fixed doses of the drug. The official recommendation is to take 1 g twice daily, and therapeutic drug monitoring (TDM) of MMF is not routinely performed. In a recent review, it was stated that “MMF is simple to use without monitoring.”9 However, in recent years it has been recognized that there exists a large interindividual variability in both the pharmacokinetics and pharmacodynamics of this drug. As a result, there has been a heightened interest in individualization of MMF treatment as a means to further improve the good clinical results that have already been obtained with this drug.4 This goal could be achieved by using TDM, considering various patient characteristics when dosing MMF, and possibly using pharmacogenetics. In this month’s issue of the Journal, Kuypers et al10 report that single-nucleotide polymorphisms (SNPs) in the MPA-metabolizing enzyme uridine diphosphate–glucuronosyltransferase (UGT) 1A9 are related to MPA exposure in renal transplant recipients. For the first time, these findings provide evidence for the feasibility of a pharmacogenetic approach to individualized MPA treatment.

PHARMACOKINETICS OF MMF

After oral administration, MMF is rapidly and almost completely absorbed from the intestine and undergoes presystemic de-esterification to form the immunologically active MPA.4,11 MPA is extensively bound (>97%) to human serum albumin and is thought to undergo restrictive clearance by the UGT enzyme family into the pharmacologically inactive 7-hydroxyglucuronide MPA (MPAG). In addition, 2 minor metabolites are formed—the inactive phenolic glucoside and the acyl-glucuronide (AcMPAG), which has immunosuppressive properties in vitro.4,11 UGT1A9 appears to be the most important UGT isoform for the glucuronidation of MPA, accounting for more than
50% of MPAG production in the liver, kidney, and intestinal mucosa. In addition, MPAG is formed by UGT1A7, UGT1A8, and UGT1A10, which are expressed in the kidney and gastrointestinal tract. UGT2B7 is responsible for the formation of AcMPAG.12-14 MPAG is excreted into bile through multidrug resistance protein (MRP) 2–mediated transport.15,16 In the gut, bacterial deconjugation transforms MPAG back into MPA, which is absorbed from the colon. Because of this enterohepatic circulation (EHC), the initial MPA plasma concentration peak at 1 hour is followed by a second increase in the MPA plasma concentration, occurring 6 to 12 hours after oral administration. In humans the contribution of EHC to the MPA area under the concentration versus time curve ranges between 10% and 61%. Finally, the majority of the absorbed MMF is eliminated by the kidneys as MPAG.4,11

The pharmacokinetics of MPA has been investigated extensively in adult and pediatric organ transplant recipients (reviewed in references 4 and 11) and has demonstrated considerable interpatient variability in drug exposure. Within-patient variability in MPA disposition appears to be modest and is not likely to hinder the clinical application of TDM for MPA.17 In addition, in renal transplant recipients, there is a structural increase of MPA exposure with time, despite a reduction of the MMF dose.18,19 The importance of the variability in MPA pharmacokinetics is demonstrated by the relationship between MPA pharmacokinetics and clinical outcomes after transplantation. Several studies have shown that the risk of acute rejection is significantly related to MPA exposure, and target levels for the MPA area under the concentration versus time curve from 0 to 12 hours have been proposed.18-20 MMF-related leukopenia and the occurrence of infections have been associated with the MMF dose and with high free, but not total, MPA exposure.18,20,22 Recently, it was suggested that high levels of AcMPAG are implicated in the gastrointestinal side effects of MMF.23,24

DETERMINANTS OF MPA PHARMACOKINETIC VARIABILITY

The recognition of the interindividual differences in MPA exposure and its relationship with clinical outcome has led to the investigation of the causes of this variability and the identification of several explanatory factors. First, MPA pharmacokinetics is influenced by a number of drug interactions. Most importantly, coadministration of MMF with cyclosporine leads to a decreased exposure to MPA (on average, 35%) and to an increase in the plasma levels of MPAG as compared with patients treated with MMF plus tacrolimus or corticosteroids.25-27 Recently, it was demonstrated that the interaction between cyclosporine and MPA is caused by inhibition of the MRP2 transporter by cyclosporine, leading to an impaired excretion of MPAG in bile, resulting in less EHC of MPA.15

The interaction between cyclosporine and MPA is clinically relevant because discontinuation of cyclosporine treatment will lead to an increased exposure to MPA and may lead to the occurrence of new MMF-related side effects. In addition, the optimal MMF dose in cyclosporine-treated patients is likely to be different from that in patients receiving tacrolimus- or sirolimus-based immunosuppression.28 Finally, (possible) differences in MPA exposure must be considered when immunosuppressive drugs are compared as part of MMF-containing regimens in clinical trials. In addition to cyclosporine, several other drugs have been shown to alter the disposition of MMF by either interfering with its absorption (antacids and iron ion preparations) or inhibiting the EHC of MPA (cholestyramine [INN, colestiramine]).11,29,30

Binding of MPA to serum albumin is a second variable influencing total exposure to MPA.17,31 Under physiologic conditions, MPA is extensively and avidly albumin-bound. Because MPA is believed to be a restrictively cleared compound, clinical conditions leading to hypoalbuminemia may cause a rise in the free fraction of MPA and, consequently, an increased MPA clearance. Free MPA may also increase as a result of competition for albumin binding. In an in vitro study, high concentrations of MPAG and salicylate were effective in displacing MPA from its albumin-binding sites.31 With MMF being increasingly used in the treatment of various renal diseases characterized by a nephrotic syndrome, it is important to recognize the possible effects of a decreased albumin concentration on MPA pharmacokinetics.

Renal function is a third important determinant of between-patient variability of MPA pharmacokinetics. Renal failure causes a decreased elimination of MPAG and an accelerated MPA clearance.17-20 The mechanism by which uremia affects MPA clearance is incompletely understood at present, but it may involve displacement of MPA from its albumin-binding sites by high levels of MPAG and, possibly, acidosis. MMF dosing in patients with delayed graft function after kidney transplantation may thus be different from that in patients with a primary functioning graft. Moreover, a more gradual improvement of kidney function is often observed during the first year after transplantation, sometimes resulting from withdrawal of the nephro-
potent cytokine or tacrolimus, and this could explain the increased exposure to MPA that several investigators have observed, despite a reduction in the MMF dose.\textsuperscript{18,19}

**ROLE OF UGT IN MPA PHARMACOKINETICS**

The possibility that, in addition to variation in absorption, distribution, and elimination, the clinical pharmacokinetics of MPA is also influenced by interindividual differences in UGT activity has been considered. The enzymatic activity of the UGT family is known to increase during early childhood, and marked interindividual differences in UGT expression and glucuronidation activity have been found in adults.\textsuperscript{20,32} In a recent study Girard et al\textsuperscript{33} found a 17-fold variation in the amount of UGT1A9 protein in adult human livers. MPA glucuronidation activity in hepatic microsomes differed by more than 9.5-fold and was significantly correlated with UGT1A9 protein levels.\textsuperscript{33}

The causes of the variable UGT expression are incompletely understood. Several drugs are known to induce or inhibit UGT.\textsuperscript{32} However, to date, there has been only 1 study that has reported a possibly clinically relevant drug interaction with MPA occurring at the UGT level.\textsuperscript{34} Evidence for a genetic basis of the variable UGT expression was provided recently with the identification of several SNPs in the \textit{UGT1A1}, \textit{UGT1A7}, and \textit{UGT2B7} genes. Some of these SNPs result in the complete or partial loss of glucuronidation activity.\textsuperscript{20,32,35,36} In addition, SNPs have been discovered in the coding and promoter region of the \textit{UGT1A9} gene, which is considered to be the most important UGT isozyme for MPA glucuronidation.\textsuperscript{33,35,37} Of all \textit{UGT1A9} promoter SNPs investigated, the −2152C>T and −275T>A SNPs were found to have the strongest association with hepatic \textit{UGT1A9} protein content.\textsuperscript{33} Carriers of these closely linked SNPs had roughly 2-fold higher UGT1A9 protein levels compared with carriers of the wild-type promoter and with noncarriers of the −2152C>T/−275T>A SNPs. Importantly, in vitro MPA glucuronidation activity was 2.1-fold higher in −2152C>T/−275T>A carriers.\textsuperscript{33}

In this month’s issue of the Journal, Kuypers et al\textsuperscript{10} report that the −2152C>T and −275T>A SNPs in \textit{UGT1A9} are associated with significantly lower MPA exposure in the early phase after renal transplantation. The less frequent \textit{UGT1A9}*3 SNP, present in less than 5% of the white population, was associated with a higher MPA exposure, which is in agreement with the previously described reduction of in vitro enzymatic activity.\textsuperscript{10,33,35} These observations demonstrate for the first time that, in vivo, interindividual variability in the pharmacokinetics of MPA can be partially explained by genetic variation. Given the high allelic frequency of the \textit{UGT1A9} −2152C>T and −275T>A SNPs (approximately 15% in white subjects), as well as the 2-fold reduction in MPA exposure in comparison with noncarriers, these findings are also likely to be clinically relevant and offer both a rationale and a means for a personalization of MMF treatment.

The recognition of the pharmacokinetic variability of MPA and its determinants, as well as the MPA concentration-effect relationship, have led to an increased popularity of TDM for MPA. Ultimately, by adjusting the dose of MMF based on plasma concentrations and considering factors such as type of calcineurin inhibitor used, albumin concentration, renal function, and possibly, UGT genotype, it may become possible to individualize MMF therapy and improve clinical outcomes. TDM is currently routinely performed for the immunosuppressants cyclosporine, tacrolimus, and sirolimus. In addition, genetic polymorphisms in the genes encoding for the drug-metabolizing enzymes cytochrome P450 3A and the drug transporter ABCB1 have recently been shown to be associated with both the pharmacokinetics of these agents and transplantation outcomes.\textsuperscript{38-43}

There are, however, still questions left unanswered. First of all, in the study of Kuypers et al,\textsuperscript{10} the reduction of MPA exposure in carriers of the \textit{UGT1A9} −2152C>T and −275T>A SNPs appears to result in part from a decreased EHC of MPA. It is possible that the investigated SNPs, in addition to an effect on hepatic UGT activity, may also increase UGT activity in the intestinal wall, thereby making deglucuronidation of MPAG (and subsequent MPA reabsorption) less effective. Second, the genetic effect on MPA exposure was only apparent in patients taking 2 g MMF, suggesting dose-dependency. A third interesting observation was the finding that the MMF-related gastrointestinal side effects occurred numerically (but not statistically significantly) less frequently in carriers of the UGT promoter SNPs. However, MPA metabolites were not determined in this study, making interpretation of this finding difficult.

At present, 3 clinical trials comparing the efficacy and cost-effectiveness of fixed-dose versus concentration-controlled MMF dosing are ongoing. One of these trials, which includes 900 de novo renal transplant recipients, has incorporated a pharmacogenetic substudy. In addition to the \textit{UGT1A9} −2152C>T and −275T>A SNPs investigated by Kuypers et al,\textsuperscript{10} SNPs in \textit{UGT2B7} and \textit{MRP2} will
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Confounding factors for sex differences in pharmacokinetics and pharmacodynamics: Focus on dosing regimen, dosage form, and formulation

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Sex differences in pharmacokinetics and pharmacodynamics have frequently been attributed to the distinction in the intrinsic factors between men and women at the molecular or physiologic level.1-8 However, often neglected by the scientists in the field are some of the extrinsic factors such as route of administration (eg, intravenous or oral), dosing regimen (eg, single or multiple dose), dosage form (eg, immediate- or extended-release tablets), and formulation (eg, composition or makeup of the dosage form) used in the pharmacokinetic (PK)/pharmacodynamic (PD) studies. It is noted that, although information on the route of drug administration and dosing regimens is generally provided in the reports, these factors (particularly the latter) are seldom taken into consideration when the study results are interpreted in relation to sex. Furthermore, several reports have failed to distinguish the dosage form or formulation used in these studies. The potential influence of these factors on the PK/PD in either men or women is likely to result from the interplay among the characteristics of sex, dosage, dosing design, duration of drug administration, and the pharmaceutic product under study. The aim of this commentary is to bring to the attention of the scientific community that disparities in the dosing regimen, dosage form, or formulation used for the PK/PD studies may in part contribute to or even play a major role in the observed sex-related differences in these studies. It is suspected that such interactions may have confounded the interpretation of some of the literature reports on the finding of a sex difference (or lack thereof) in PK/PD.

Different routes of administration (eg, intravenous versus oral dosing) give rise to distinct PK and PD profiles in humans. Similarly, different PK or PD responses in men and women can be identified as a result of a specific route of administration.1-9 In some cases a sex-related difference in PK/PD may be found after intravenous administration but may not be observed after oral dosing or vice versa. In other cases a PK/PD difference in men and women may be seen after an intramuscular injection but cannot be found after injection by the intravenous route. An excellent article previously published in this Journal has provided several examples that illustrate sex-related differences in the clearance of cytochrome P450 (CYP) 3A4 substrates after intravenous and oral administration.10 As indicated, depending on whether the CYP3A4 substrate is given intravenously or orally, the clearance of a drug may be faster, slower, or comparable in women relative to men. The topic of potential sex-related differences in pharmacokinetics resulting from the route of administration has also been reviewed recently9 and thus will not be discussed in detail here.

As with the route of administration, changes in dosing regimen may have an impact on the PK/PD outcome. For example, the PK/PD profiles of a drug are usually different after single-dose versus multiple-dose administration. These profiles may be linear or nonlinear with dosage increment after either regimen. Drug accumulation may occur after repeated administrations. Whereas linear kinetics (including absorption, distribution, metabolism, and elimination) may prevail at the low concentrations of a drug after a single dose, nonlinear kinetics may occur at the higher concentrations
after multiple doses. Furthermore, the metabolizing enzymes, transporters, and receptors may be saturated, inhibited, or induced with the high level of drugs on board or the long duration of dosing. It is thus possible that PK/PD responses after single doses differ from those after multiple doses.

Another complicating factor lies in the dosing regimen with or without food. Some drugs should be taken orally under fasting conditions, whereas others are to be administered with food. Men and women are known to differ in gastrointestinal (GI) pH and motility. The GI transit time in women has been shown to be significantly longer, which appears to be linked to the level of sex hormones such as progesterone and estradiol. These intrinsic differences, coupled with the food effect on GI physiologic characteristics and possibly on drug absorption, may further influence the PK or PD responses between the sexes.

To investigate the effect of dosing regimen on the PK outcome in relation to sex, Table I compiles the result of a literature survey on the oral clearance of drugs that are known to be (1) only metabolized by CYP3A4 with or without P-glycoprotein (P-gp) transport or (2) primarily either metabolized by CYP3A4 or transported by P-gp (or both) with minor contribution from other CYP enzymes. As shown, in most cases, although the clearance might be different between men and women after a single dose, no sex difference in the oral clearance was found after multiple doses except in 2 verapamil studies; one had a peculiar dosage form (controlled onset, extended release), whereas the other included an immediate-release product and various brands of modified-release products. The observed sex-related differences in the oral clearance are thus likely to derive from an interaction between the characteristics of sex and single-dose (but not multiple-dose) administration. These differences in the clearance of CYP3A4/P-gp substrates have been attributed to the differential activities of CYP3A4 and P-gp in the liver and intestine between the sexes. Furthermore, it is possible that at high concentrations of the drug, saturation of the enzymes or transporters occurs such that the observed sex differences disappear from single-dose to multiple-dose administration.

Notably, the impact of dosing regimen on the sex-related difference in PK/PD is also shown with tirilazad, a drug previously rejected by the Food and Drug Administration because it could be demonstrated to be efficacious in men but not in women. The systemic clearance of tirilazad after a single intravenous dose was about 40% to 60% higher in women compared with men. This sex difference in pharmacokinetics appeared to be clinically relevant because tirilazad significantly reduced the mortality rate in male patients but the effect in women was minimal. On multiple-dose administration, however, the sex effect on tirilazad kinetics became modest or negligible. Among others, enzyme saturation after multiple doses might be one of the factors that contributed to the disparity observed between single and multiple dosing of this drug.

It should be noted that, when the same route of administration and dosing regimen are followed, discrepancies in the dosage form can also bring about different PK or PD responses in men and women. The interplay of sex and dosage form characteristics (ie, sex–by–dosage form interaction) could arise such that a sex difference in PK or PD is observed with the use of one dosage form but not the other. For example, higher plasma levels of acetylsalicylic acid and salicylic acid have been reported in women after single oral doses of aspirin. However, similar bioavailability was found in men and women after oral administration of aspirin as a lysine salt, although the drug was absorbed more quickly in women. In contrast, women demonstrated greater delays in gastric emptying and aspirin absorption from enteric-coated tablets after a meal. These PK differences resulting from the ingestion of different dosage forms of aspirin may be of clinical importance in view of the indication of pain relief from the drug.

Likewise, differences in dosage form may help explain the seemingly contrary results obtained from the single-dose studies listed in Table I. In these studies, as shown for immediate-release dosage forms, men tended to have lower or similar clearance compared with that in women, except in 2 studies; one reported free prednisolone clearance, and the other reported on verapamil, for which CYP1A2 might also play a key role in the metabolism. Women have been shown to have higher CYP3A4 content and lower P-gp levels in the intestine, although there is a paucity of information on the CYP3A4 or P-gp activity with respect to sex, a recent study has indicated no sex difference in the duodenum for both. Hence, the information supports the observed direction of sex differences in oral clearance from immediate-release dosage forms (Table I). Unfortunately, there is difficulty in discerning the direction of sex difference for modified-release dosage forms because only a few studies are available for evaluation. CYP3A4 has the highest content in the duodenum, followed by the jejunum, ileum, and colon, whereas P-gp efflux activity is ranked highest in the ileum, followed by the colon, jejunum, and colon.
Table I. Influence of dosing regimen on oral clearance of either CYP3A4 substrates or P-gp (or both)

<table>
<thead>
<tr>
<th>Drug</th>
<th>Substrate*</th>
<th>Single dose</th>
<th>Multiple dose</th>
<th>Dosage form</th>
<th>Comment</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alprazolam</td>
<td>3A4</td>
<td>CLm = CLf</td>
<td>CLm &lt; CLf</td>
<td>IR</td>
<td></td>
<td>18, 19</td>
</tr>
<tr>
<td>Atorvastatin</td>
<td>3A4, P-gp</td>
<td>CLm &lt; CLf</td>
<td></td>
<td>IR</td>
<td>No body weight or surface area adjustment</td>
<td>20</td>
</tr>
<tr>
<td>Bromazepam</td>
<td>3A4</td>
<td>CLm = CLf</td>
<td></td>
<td>IR</td>
<td></td>
<td>22</td>
</tr>
<tr>
<td>Buspirone</td>
<td>3A4</td>
<td>CLm = CLf</td>
<td></td>
<td>IR, ER</td>
<td></td>
<td>23, 24</td>
</tr>
<tr>
<td>Cyclosporine</td>
<td>3A4, P-gp</td>
<td>CLm &lt; CLf</td>
<td></td>
<td>Solution</td>
<td>Black subjects</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CLm = CLf</td>
<td>White subjects</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CLm = CLf</td>
<td></td>
<td>27</td>
</tr>
<tr>
<td>Diltiazem</td>
<td>3A4, P-gp</td>
<td>CLm = CLf</td>
<td></td>
<td>IR</td>
<td>No body weight or surface area adjustment</td>
<td>28, 29</td>
</tr>
<tr>
<td>Ebastine</td>
<td>3A4, P-gp</td>
<td>CLm = CLf</td>
<td></td>
<td>IR</td>
<td>No body weight or surface area adjustment</td>
<td>30</td>
</tr>
<tr>
<td>Felodipine</td>
<td>3A4</td>
<td>CLm = CLf</td>
<td>IR and ER</td>
<td></td>
<td></td>
<td>31</td>
</tr>
<tr>
<td>Lovastatin</td>
<td>3A4, P-gp</td>
<td>CLm = CLf</td>
<td></td>
<td>IR</td>
<td>No body weight or surface area adjustment</td>
<td>32</td>
</tr>
<tr>
<td>Methadone</td>
<td>3A4, P-gp</td>
<td>CLm = CLf</td>
<td></td>
<td>IR</td>
<td></td>
<td>33</td>
</tr>
<tr>
<td>Midazolam</td>
<td>3A4</td>
<td>CLm &lt; CLf</td>
<td></td>
<td>Solution</td>
<td></td>
<td>34, 35</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CLm = CLf</td>
<td></td>
<td>36, 37</td>
</tr>
<tr>
<td>Nefazodone</td>
<td>3A4</td>
<td>CLm = CLf</td>
<td></td>
<td>IR</td>
<td></td>
<td>10, 38</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CLm = CLf</td>
<td></td>
<td>10, 38</td>
</tr>
<tr>
<td>Nifedipine</td>
<td>3A4</td>
<td>CLm &lt; CLf</td>
<td></td>
<td>CR</td>
<td>Mixed brands (Adalat CC, nifedipine-GITS, and Procardia XL)</td>
<td>39</td>
</tr>
<tr>
<td>Nimodipine</td>
<td>3A4</td>
<td>CLm &lt; CLf</td>
<td></td>
<td>CR</td>
<td>Adalat Retard</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CLm = CLf</td>
<td></td>
<td>17, 41</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>3A4, P-gp</td>
<td>CLm = CLf</td>
<td></td>
<td>IR</td>
<td>Total clearance</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CLm &gt; CLf</td>
<td>Free clearance</td>
<td>42</td>
</tr>
<tr>
<td>Simvastatin</td>
<td>3A4, P-gp</td>
<td>CLm = CLf</td>
<td></td>
<td>IR</td>
<td></td>
<td>32</td>
</tr>
<tr>
<td>Trazodone</td>
<td>3A4</td>
<td>CLm &lt; CLf</td>
<td></td>
<td>IR</td>
<td></td>
<td>17, 43</td>
</tr>
<tr>
<td>Triazolam</td>
<td>3A4</td>
<td>CLm &lt; CLf</td>
<td></td>
<td>IR</td>
<td></td>
<td>17, 44–47</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CLm = CLf</td>
<td>Women taking oral contraceptives</td>
<td>48, 49</td>
</tr>
<tr>
<td>Verapamil</td>
<td>3A4, P-gp,</td>
<td>CLm = CLf</td>
<td></td>
<td>IR</td>
<td>Young women not taking oral contraceptives</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>1A2</td>
<td>CLm &gt; CLf</td>
<td></td>
<td>SR</td>
<td>Elderly subjects</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CLm &gt; CLf</td>
<td>Controlled onset</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CLm = CLf</td>
<td></td>
<td>53</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CLm &lt; CLf</td>
<td>Mixed brands (Covera SR, Calan SR, Verelan, Isoptin SR, and others)</td>
<td>54</td>
</tr>
</tbody>
</table>

CLm, Clearance for men; CLf, clearance for women; IR, immediate release; P-gp, P-glycoprotein; ER, extended release; CR, controlled release; SR, sustained release; MR, modified release (may include ER, CR, SR, and so on).

Adalat CC and Adalat Retard, Bayer, Leverkusen, Germany; Procardia XL and Covera SR, Pfizer, New York, NY; Calan SR, GD Searle, Skokie, Ill; Verelan, Schwarz, Milwaukee, Wis; Isoptin SR, Abbott Laboratories, North Chicago, Ill.

*Minor metabolic pathways are shown in parentheses.
Sex differences in PK and PD

It is still unknown whether a sex difference in CYP3A4 or P-gp activities exists in the lower intestine. As mentioned, the gastric emptying time, as well as small intestinal and colonic transit time, in women has been shown to be significantly longer compared with that in men.11-16 The longer GI residence time for sustained-release dosage forms may further facilitate more drugs to be absorbed in the gut for women. This seems to be consistent with the observation that the PK of a diltiazem extended-release formulation administered once a day is sensitive to the GI transit time.72

With the advances in pharmacoeconomic science and technology, it is noteworthy that each modified-release dosage form may possess its own unique release mechanism and release profile of the drug. For example, the GI therapeutic system (GITS) for Procardia XL (Pfizer, New York, NY), nifedipine extended-release tablets, is fabricated to provide a drug delivery rate independent of pH or motility of the GI tract.73 In contrast, the delayed-release dosage form, such as enteric-coated tablets of omeprazole, is intended to release the drug only after the tablet leaves the stomach.74 Yet the controlled-onset, extended-release verapamil formulation is designed to initiate the drug release 4 to 5 hours after ingestion at bedtime.52,74 On the other hand, Adalat CC tablets (another nifedipine extended-release dosage form) (Bayer, Leverkusen, Germany) consist of an external coat and an internal core, with the coat as a slow-release formulation and the core as a fast-release formulation.74 In addition, some modified-release products are designed to have a controlled lag time (in hours) for drug absorption, whereas others may rely on the degradation of polymeric membranes to achieve pulsatile drug delivery.75 In view of the complex features of modern pharmaceutic designs, it is likely that the direction and magnitude of a sex difference, if it occurs, depend on the specific type of modified-release dosage form used in the PK/PD study. Therefore, to facilitate a better understanding of the mechanistic basis for sex differences in PK/PD, it is advisable to use a single brand of a modified-release drug product in a study. The use of mixed brands of dosage form, as is sometimes seen in population studies,39,54 inevitably confounds the data analysis and renders it difficult to interpret the study results.

Similarly, within the same dosage form, discrepancies in formulation may also result in a sex-by-formulation interaction, because of the interplay between the differences in sex-related physiologic characteristics and formulation makeup. This type of interaction has been reported from the PK analysis of bioequivalence trials.76 For instance, a statistically significant sex-related difference in PK parameters was found for one brand of nitroglycerin patches but not for the other. An advantage of using bioequivalence studies to investigate sex-related differences in pharmacokinetics is that these studies generally have a crossover design and each subject can serve as his or her own control. Another example can be illustrated by a previous case study from the Food and Drug Administration database, where bioequivalence was evaluated between 2 orally administered extended-release products of a drug.77 The drug is a calcium channel antagonist and is known to be a substrate of both CYP3A4 and P-gp. In this study both products had the same active ingredient, dosage form, and route of administration. However, a sex-dependent difference in pharmacokinetics was found after the oral administration of product A but not product B. In both studies, although the mean plasma levels of product B were similar between the sexes, those of product A were significantly higher in women compared with men. A sex-by-formulation interaction was thus found for product A but not for product B. Further in vitro dissolution testing revealed a pronounced difference in the dissolution behavior for the 2 products. In the case of product A, a lower fraction of the drug dissolved at pH 4.5 and a higher fraction dissolved at pH 6.8, reflecting more drug in the ileum and perhaps in the colon. For product B, however, most of the drug dissolved at pH 4.5, possibly reflecting a rapid release at the duodenum and jejunum in vivo. The labeling of the drug indicates that absorption occurs throughout the dosing interval of 24 hours. Hence, the higher plasma levels in women obtained from product A could be theorized as a result of different in vivo release profiles from the drug products and a longer GI transit time in women.11-16 in addition to the potential disparity in the metabolizing enzymes or transporters.

Apart from the specifics in the route of administration, dosing regimen, dosage form, and formulation of the pharmaceutic product given to subjects, several factors can complicate the analysis of sex-based PK/PD data. These factors have been extensively discussed elsewhere.1-8 The sample size of the study should be sufficient enough to allow for the conclusion of a sex-based difference (or no difference) statistically. The conditions of the subjects who participate in the study are critical to the results of the study. For example, it is crucial to include the following factors in the selection criteria for a study: healthy subjects or patients, young or elderly patients, women taking or not taking oral contraceptives, smoking status, fasting or nonfasting state, dietary content, and concurrent medications. It is
also important to control the intake of alcohol, vitamins, hormones, and nutraceuticals by the participants. All of the pertinent factors should be considered when one is designing a sex-related study or interpreting sex-based data.

Although sex-related discrepancies in pharmacokinetics have been identified for a number of drugs, they may or may not translate into differences in pharmacodynamics or clinical outcome.\textsuperscript{3,5,6,8} As shown in Table I, the body weight–normalized free prednisolone oral clearance was higher in men compared with women, whereas total clearances were comparable between the sexes. This study was performed with women in the luteal phase of the menstrual cycle and was based on the premise that perturbation caused by hormonal differences would be apparent in this phase. Yet the 50% inhibitory concentration values for prednisolone effects on cortisol secretion and T-helper lymphocytes or neutrophil trafficking were not statistically different between men and women. On the other hand, it should be noted that, on the basis of the literature data, several mechanisms relevant to drug absorption and disposition have been shown to exhibit sex-related differences. Furthermore, these PK differences have been translated into different PD and pharmacologic responses of drugs (eg, calcium channel blockers, \(\beta\)-blockers, and selective serotonin reuptake inhibitors).\textsuperscript{3,5,6,8}

In conclusion, the issue of sex influence on pharmacokinetics and pharmacodynamics has received increasing attention in the past decade. Several potential effects of sex have been uncovered as a result of the efforts from research scientists in the field. Many intrinsic and extrinsic factors could contribute to the observed PK or PD differences in men and women. This commentary focuses on the importance of dosing regimen, dosage form, and formulation used in the studies. It is submitted that in the future incorporation of additional information regarding dosing regimen, dosage form, and formulation in sex-related PK/PD studies will further enhance the power of research scientists to analyze the study data and interpret the results more effectively and efficiently.

The author has no conflicts of interest relevant to this commentary.

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Rosuvastatin pharmacokinetics and pharmacogenetics in white and Asian subjects residing in the same environment

Background: Systemic exposure to rosuvastatin had been observed to be approximately 2-fold higher in Japanese subjects living in Japan compared with white subjects in Western Europe or the United States. The organic anion transporting polypeptide 1B1 contributes to the hepatic uptake of rosuvastatin. Polymorphisms in the \textit{SLCO1B1} gene can lead to reduced transport function in vitro (T521>C). This study was conducted to determine whether the pharmacokinetic differences between Japanese and white subjects extended to other Asian ethnic groups and to determine whether polymorphisms in the \textit{SLCO1B1} gene contribute to any pharmacokinetic differences observed.

Methods: Rosuvastatin pharmacokinetics was studied in an open-label, parallel-group, single-oral dose (40 mg) study in 36 white, 36 Chinese, 35 Malay, and 35 Asian-Indian subjects living in Singapore. Plasma concentrations of rosuvastatin and metabolites were determined by HPLC–mass spectrophotometry. Two \textit{SLCO1B1} polymorphisms (A388>G and T521>C) were genotyped.

Results: Ratios for rosuvastatin area under the plasma concentration–time curve from time 0 to the time of the last quantifiable concentration were 2.31, 1.91, and 1.63 and ratios for maximum plasma concentration were 2.36, 2.00, and 1.68 in Chinese, Malay, and Asian-Indian subjects, respectively, compared with white subjects. Similar increases in exposure to \textit{N}-desmethyl rosuvastatin and rosuvastatin-lactone were observed. \textit{SLCO1B1} genotypes did not account for the observed pharmacokinetic differences between Asians and white subjects.

Conclusions: Plasma exposure to rosuvastatin and its metabolites was significantly higher in Chinese, Malay, and Asian-Indian subjects compared with white subjects living in the same environment. (Clin Pharmacol Ther 2005;78:330-41.)

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Rosuvastatin is a 3-hydroxy-3-methylglutaryl–coenzyme A reductase inhibitor (statin) that has been developed for the treatment of dyslipidemia. Pharmacokinetic studies conducted in healthy white volunteers living in England or the United States and by Japanese investigators studying healthy Japanese volunteers living in Japan showed an increase of approximately 2-fold in systemic exposure to rosuvastatin in the Japanese subjects compared with the white subjects.\textsuperscript{1,3} A population pharmacokinetic analysis showed that rosuvastatin apparent oral clearance was approximately 50% lower in Japanese subjects living in Japan than in white subjects living in Europe or the United States.\textsuperscript{4} In
view of these findings, we carried out a prospective study to determine whether the pharmacokinetic differences between Japanese subjects and white subjects extended to other Asian groups compared with white subjects living in the same environment.

Metabolic transformation plays a minor role in rosuvastatin clearance.\(^5,6\) and 90% of an orally administered dose of rosuvastatin is recovered as unchanged drug primarily in the feces.\(^3\) After intravenous administration, renal clearance and nonrenal (hepatic) clearance account for 28% and 72% of total systemic clearance, respectively.\(^7\) The values for absolute bioavailability of rosuvastatin are 29% (AstraZeneca, data on file) and 20%\(^7\) in Japanese subjects and white subjects, respectively. Thus ethnic differences in metabolism are unlikely to make an important contribution to the observed data.

In humans, organic anion transporting polypeptide C (OATP1B1, also known as OATP-C) is expressed in the basolateral membrane of hepatocytes and contributes to hepatic uptake of statins including rosuvastatin\(^8\) and pravastatin.\(^9,10\) Atorvastatin, simvastatin acid, and lovastatin are effective inhibitors of the uptake of pravastatin and rosuvastatin by OATP1B1 and may be substrates for this transporter.\(^9,11\)

Several single-nucleotide polymorphisms (SNPs) in the gene encoding OATP1B1 (SLCO1B1) have been described.\(^12,13\) One common SNP in SLCO1B1, T521>C, predicts the substitution of alanine for valine at amino acid 174 (Val174Ala). Another prevalent SNP, A388>G, affects the amino acid at position 130 (Asn130Asp). Together, these 2 SNPs define 4 SLCO1B1 haplotypes (alleles) that code for OATP1B1 peptides containing 130Asn and 174Val (*1a allele), 130Asp and 174Val (*1b), 130Asn and 174 Ala (*5), and 130Asp and 174 Ala (*15).

Three in vitro studies have failed to detect a difference in substrate transport between the 2 most prevalent OATP1B1 alleles, *1a and *1b.\(^12,14\) A single in vivo study showed a nonsignificant trend toward higher pravastatin exposure in *1b/*1b and *1a/*1b subjects.\(^15\) Thus, to date, there is no convincing evidence that the *1b allele has functional significance.

In contrast, 2 of 3 in vitro studies have shown reduced function of OATP1B1 alleles containing 521C; that is, the *5 and *15 alleles. Transfected HeLa cells showed reduced uptake by the *5 allele (compared with *1a) of estrone sulfate and estradiol 17β-glucuronide.\(^12\) In another study, transfected HEK293 cells showed no difference in the uptake of estrone sulfate by the *5 allele compared with the *1a and *1b alleles.\(^13\) More recently, however, experiments with cultured Xenopus oocytes expressing OATP1B1 and use of 7-ethyl-10-hydroxycamptothecin, pravastatin, estrone sulfate, and estradiol-17β-glucuronide as substrates showed significantly reduced uptake in cells containing the *15 allele compared with the reference allele (*1a).\(^14\) This study also showed a nonsignificant trend toward reduced function of the *5 allele for all 4 substrates. Heterozygosity and homozygosity for 521C have also been associated with higher pravastatin plasma concentrations in vivo.\(^15,16\)

The frequency of OATP1B1 521C (ie, the proportion of OATP1B1 alleles containing 521C) has been reported as 11% to 16% in Japanese subjects, 14% in European Americans, and 0.02% in African Americans.\(^12,13\) Among Japanese subjects, most 521C alleles are part of a *15 haplotype.\(^13\) An early survey of genetic variation in OATP1B1 in European Americans and African Americans did not describe the *15 allele;\(^12\); however, it is not clear that the study distinguished persons with haplotype pair *1b/*15 from those with haplotype pair *1a/*5. A more recent study of 41 Finnish volunteers included Bayesian haplotype estimation and identified 4 *15 alleles and 1 *5 allele.\(^17\) Thus it is not clear from the literature whether the frequency of *15 in white subjects differs from that in Japanese subjects. If so, then genetic variation within OATP-C might be one factor contributing to pharmacokinetic differences in rosuvastatin between Japanese subjects and white subjects. Thus we genotyped subjects in this study to explore the effects of these SLCO1B1 SNPs on any between- and within-group variation in rosuvastatin pharmacokinetics that might be observed.

**METHODS**

**Human pharmacokinetic trial**

**Subjects.** Subjects were healthy white, Chinese, Malay, and Asian-Indian volunteers identified from their medical history, physical examination, electrocardiogram, clinical chemical, and urinalysis findings. Race and ethnic group were self-reported by the volunteers for both parents and all 4 grandparents. For Singaporean Chinese, Malay, and Asian-Indians, the ethnic group as defined by National Registration Identity Cards provided additional confirmation of ethnicity. One hundred forty-one volunteers entered and completed the trial; all gave informed consent. There were 106 men and 35 women who participated in this study. In white subjects, gender has no significant effect on rosuvastatin pharmacokinetics; therefore no effort was made to balance the study with respect to gender.\(^18\)
**Trial design.** The trial (AstraZeneca Trial 4522IL/0101) was designed and monitored in accordance with Good Clinical Practice guidelines and the Declaration of Helsinki. The trial was carried out at 2 centers in Singapore, Singapore (National University Hospital and Changi General Hospital), after protocol approval by their institutional review boards. Volunteers were recruited from the Singapore metropolitan area. White subjects were required to have resided in Singapore for at least 6 months before participation in the trial. Asian subjects were permanent residents of the region.

The trial was an open-label, parallel-group, single-dose study. Volunteers fasted for 8 hours before and 4 hours after administration of a single oral 40-mg dose of rosuvastatin on day 1. They were required to refrain from strenuous exercise, smoking, caffeine-containing drinks and food, alcohol, grapefruit-containing products, and other medications.

To reduce potential dietary influences on rosuvastatin exposure, dietary histories of the subjects were assessed before entry into the study. Subjects on extreme diets such as weight reduction diets were excluded. Subjects with diets in which the percent saturated fat content was less than 10% and daily cholesterol intake was less than 300 mg were also excluded. The intent was to have all subjects on similar diets at the time of dosing. Each subject recorded his or her food intake in a diary for 3 days before rosuvastatin administration. The diet diary records were analyzed with Dietplan5 (Forestfield Software, Horsham, United Kingdom), a validated nutrition analysis software program. Dietary parameters, estimated as the mean of the 3-day evaluation, included daily caloric intake; fraction of caloric intake as protein, carbohydrate, and fat; and daily cholesterol intake.

**Blood sampling.** Venous blood samples (7 mL) for rosuvastatin, N-desmethyl rosuvastatin, and rosuvastatin-lactone assays were taken before and at 0.5, 1, 2, 3, 4, 5, 6, 8, 10, 12, 18, 24, 30, 48, 72, and 96 hours after rosuvastatin administration. Samples were collected into tubes containing lithium-heparin anticoagulant and centrifuged within 30 minutes; plasma was then harvested and mixed 1:1 with sodium acetate buffer, 0.1 mol/L (pH 4.0), and stored at −70°C until assay.

**Determination of plasma rosuvastatin, N-desmethyl rosuvastatin, and rosuvastatin-lactone concentrations.** Plasma samples were analyzed for rosuvastatin, N-desmethyl rosuvastatin, and rosuvastatin-lactone by use of a method (HPLC with mass spectrometric detection) developed and validated at AstraZeneca, Wilmington, Del (data on file). A robotic liquid-handling system was used to perform the sample preparation in a 96-well format. Plasma proteins were precipitated via simple protein precipitation and filtration. Analysis of the filtrate was accomplished by multiple-reaction monitoring via positive electrospray ionization–tandem mass spectrometric detection.

The lower limit of quantitation for rosuvastatin was 0.100 ng/mL; the upper limit was 100 ng/mL but was extended by dilution. The lower limit of quantitation for N-desmethyl rosuvastatin and rosuvastatin-lactone was 0.250 ng/mL; the upper limit was 25.0 ng/mL. The accuracy and precision of the analytic method were ensured on the basis of the results for spiked quality control samples, which were assayed on each day of trial analysis. For rosuvastatin, accuracy averaged 101.5% (7.3% relative SD [RSD]) at 0.750 ng/mL, 97.0% (3.9% RSD) at 7.5 ng/mL, and 96.1% (3.4% RSD) at 25 ng/mL. For N-desmethyl rosuvastatin, accuracy averaged 102.0% (6.5% RSD) at 0.750 ng/mL, 95.6% (4.1% RSD) at 7.50 ng/mL, and 99.3% (4.1% RSD) at 15 ng/mL. For rosuvastatin-lactone, accuracy averaged 97.1% (9.6% RSD) at 0.750 ng/mL, 98.3% (7.7% RSD) at 7.50 ng/mL, and 97.5% (8.2% RSD) at 15 ng/mL.

**Pharmacokinetic evaluation.** The primary pharmacokinetic parameter of this trial was the area under the plasma concentration–time curve (AUC) or, if fewer than 29 subjects per ethnic group had estimable AUC values, the AUC from time 0 to the time of the last quantifiable concentration (AUC_{0-t}). Other pharmacokinetic parameters included maximum plasma concentration (C_{max}), time to C_{max} (t_{max}), and terminal elimination half-life (t_{1/2}) of rosuvastatin and AUC_{0-\infty}, C_{max}, and t_{1/2} of N-desmethyl rosuvastatin and rosuvastatin-lactone. The apparent terminal half-life was calculated as 0.693/\lambda_{z}, where \lambda_{z} is the terminal elimination rate constant calculated by log-linear regression of the terminal portion of the plasma concentration–time curve. AUC_{0-t} was determined by use of the linear trapezoidal rule, and AUC was determined as AUC_{0-t} + C_{last}/\lambda_{z} (where C_{last} is the last measurable plasma concentration).

**Genotyping of SLC01B1 polymorphisms**

Deoxyribonucleic acid (DNA) was extracted from blood samples by use of the QIAamp DNA Blood Maxi kit (Qiagen, Hilden, Germany). SLC01B1 polymorphisms A388>G and T521>C were genotyped by use of TaqMan MGB technology, marketed as Assays-by-Design (Applied Biosystems, Foster City, Calif). Applied Biosystems–designed and –synthesized primers and probes, supplied at \times 40, were diluted in 1× Universal Master Mix (Applied Biosystems), to \times 0.5. Primer sequences were as follows (5’−3’): N130D poly-
merase chain reaction (PCR) forward TTTAATTCAGTGATGTTCTTACAGTTACAGGT, PCR reverse GAGTGATAAAATTTGATTAATTAAACAGTGATGATAAAATTTGATTAATTAAACAGTGATGTTCTTACAGTTACAGGT, FAM probe AAAGAAACTATATCGATTCAT, and VIC probe CTAAAGAAACTATATCAATTCAT; and V174A PCR forward AGGTTGTTTAAAGGAATCTGGGTCATAC, PCR reverse CTCCCCTATTTCCACGAAGCATATT, FAM probe CCATGAACGCATATAT, and VIC probe CCCATGAACACATATAT. Two microliters of reaction mix was added to dried-down DNA (10 ng/well) in ABgene Thermofast 384-well plates (ABgene, Epsom, United Kingdom). Plates were sealed by use of an ALPS 300 sealer (ABgene) with Clear Seal Strong (ABgene) and cycled in a DT-108 Super-Duncan water bath cycler (KBiosystems, Basildon, United Kingdom). The cycling conditions were 92°C for 10 minutes (92°C for 15 seconds and 57°C for 1 minute) ×45 cycles. After PCR, FAM, VIC, and ROX fluorescence intensities were measured in an ABI 7900HT Sequence Detection System (Applied Biosystems). Cluster analysis was performed manually.

**Statistical methods.** Differences in diet (total daily caloric intake, daily cholesterol intake, and total fat, saturated fat, carbohydrate, and protein as a percent of total calories) were analyzed by ANOVA and results given as the least squares mean difference and 90% confidence interval (CI) between each Asian ethnic group and the white group.

Summary statistics for AUC$_{0-t}$ and C$_{max}$ are presented as geometric means and 95% CIs. Half-life values are given as least square means and SEs, and t$_{max}$ values are given as mean, median, and range. Three primary comparisons of rosuvastatin AUC$_{0-t}$ were made as follows: Chinese versus white subjects, Asian-Indian versus white subjects, and Malay versus white subjects. A sample comprising 29 volunteers per ethnic group had greater than 90% power to ensure that the 90% CI for the ratio of AUC$_{0-t}$ for each of the 3 comparisons would be contained within the interval 0.7 to 1.43, with the assumption that the true underlying ratio was 1. No adjustment for multiple comparisons was made.

Rosuvastatin AUC$_{0-t}$ was log-transformed before analysis. ANOVA was used to determine geometric mean ratios and 90% CIs for each Asian group versus the white group. Rosuvastatin C$_{max}$ and AUC$_{0-t}$ and C$_{max}$ for N-desmethyl rosuvastatin and rosuvastatin-lactone were analyzed in a similar manner. Rosuvastatin and metabolite half-lives (untransformed) were analyzed by ANOVA, and results are given as the least squares mean difference and 90% CI between each Asian ethnic group and the white group.

In contrast to the pharmacokinetic analyses, all pharmacogenetic analyses were post hoc and hence exploratory. Chi-square analysis was used to test for Hardy-Weinberg equilibrium (HWE) for each SNP within each population. SLCO1B1 haplotypes were assigned by use of an expectation maximization method as implemented in the software package SNPHAP. The pharmacokinetic measurements AUC$_{0-t}$ and C$_{max}$ of rosvustatin were log-transformed before analysis, and summary statistics reported are the geometric means and 95% CIs. The effects of the T521C and A388G SNPs and of SLCO1B1 diplotypes on log-transformed C$_{max}$ and AUC$_{0-t}$ were examined by ANOVA. Tukey-Kramer analysis was used to locate nominally significant genotypic and diplotypic differences within each ethnic group for $\alpha = .05$.

**Tolerability.** Adverse event reports, medical examinations, and clinical laboratory data were assessed to evaluate tolerability.

**RESULTS**

**Subject demographic characteristics**

The demographic characteristics of the volunteers are shown in Table I. Body weight and height were greater in white subjects compared with those in each of the Asian groups. However, body mass index values were similar among the 4 groups. Substantially more
men than women were enrolled in the white, Chinese, and Asian-Indian groups compared with the Malay group.

**Dietary parameters**

Total daily caloric intake; total fat, saturated fat, carbohydrate, and protein as a percent of total calories; and daily cholesterol intake in each of the groups are presented in Table II. Total daily caloric intake was 14% higher in the Chinese subjects compared with the white subjects. Cholesterol intake was 29% higher in the Malay subjects compared with the white subjects. No other significant differences were observed between the Asian groups and the white subjects.

**Rosuvastatin pharmacokinetic parameters**

The mean rosuvastatin plasma concentration–time profiles for each ethnic group are shown in Fig 1. Error bars indicating the SDs for mean plasma concentrations are shown for white subjects and Chinese subjects. SDs were similar among Malay and Asian-Indian subjects but are not shown because of the substantial overlap in variability and resulting lack of clarity in the figure.

Rosuvastatin pharmacokinetic parameters are summarized by ethnic group in Table III. The table also presents the statistical comparisons for each Asian ethnic group relative to the white group.

Rosuvastatin geometric mean AUC$_{0-t}$ was 2.31-fold higher in Chinese subjects, 1.63-fold higher in Asian-Indian subjects, and 1.91-fold higher in Malay subjects compared with white subjects. These differences were all statistically significant. Geometric mean C$_{max}$ was 2.36-fold higher in Chinese subjects, 1.68-fold higher in Asian-Indian subjects, and 2.00-fold higher in Malay subjects compared with white subjects. These differences were also statistically significant. The ANOVA model included weight as a covariate. Adjustment of the pharmacokinetic parameters by weight resulted in less than a 10% change in C$_{max}$ or AUC$_{0-t}$ in any ethnic group. The terminal slope ($\tau$) and t$_{1/2}$ could not be estimated in all subjects because of either the presence of multiple peaks or insufficient data (or both) in the terminal phase of the plasma concentration–time profiles. Among subjects with reliable estimates, the mean t$_{1/2}$ values were approximately 3 hours shorter in the Chinese and Asian-Indian groups compared with the Malay group.

<table>
<thead>
<tr>
<th>Table II. Dietary characteristics by ethnic group</th>
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<tbody>
<tr>
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<td>White (n = 36)</td>
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<td>Daily caloric intake (kcal)</td>
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<td>Difference*</td>
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<tr>
<td>90% CI</td>
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<tr>
<td>% Total fat↑</td>
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<tr>
<td>Least squares mean and SD</td>
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<tr>
<td>Difference*</td>
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<tr>
<td>90% CI</td>
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<tr>
<td>% Saturated fat↑</td>
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<tr>
<td>Least squares mean and SD</td>
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<tr>
<td>Difference*</td>
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<tr>
<td>90% CI</td>
</tr>
<tr>
<td>% Carbohydrate↑</td>
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<tr>
<td>Least squares mean and SD</td>
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<td>Difference*</td>
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<td>90% CI</td>
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<tr>
<td>% Protein↑</td>
</tr>
<tr>
<td>Least squares mean and SD</td>
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<tr>
<td>Difference*</td>
</tr>
<tr>
<td>90% CI</td>
</tr>
<tr>
<td>Daily cholesterol intake (mg)</td>
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<tr>
<td>Least squares mean and SD</td>
</tr>
<tr>
<td>Difference*</td>
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<td>90% CI</td>
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</tbody>
</table>

CI, Confidence interval.
*Difference of Asian ethnic group compared with white subjects derived from least squares mean (ethnic group effect).
†Percent of total daily caloric intake.
value in white subjects, but the difference did not reach statistical significance. The mean \( t_{1/2} \) in the Malay group was similar to that of white subjects.

**N-desmethyl rosuvastatin**

Systemic exposure to the active metabolite of rosuvastatin mirrored the pattern observed for rosuvastatin. The mean exposure \( AUC_{0-t} \) and \( C_{max} \) values (not adjusted for body weight) were highest in Chinese volunteers, followed by Malay, Asian-Indian, and white volunteers. Geometric means for \( AUC_{0-t} \) in these ethnic groups were 61.2 ng · h/mL (95% CI, 50.1-74.7 ng · h/mL), 54.3 ng · h/mL (95% CI, 44.5-66.3 ng · h/mL), 45.1 ng · h/mL (95% CI, 36.6-55.5 ng · h/mL), and 28.3 ng · h/mL (95% CI, 23.2-34.5 ng · h/mL), respectively. Geometric means for \( C_{max} \) were 8.08 ng/mL (95% CI, 6.7-9.73 ng/mL), 7.48 ng/mL (95% CI, 6.21-9.01 ng/mL), 5.95 ng/mL (95% CI, 4.89-7.22 ng/mL), and 3.80 ng/mL (95% CI, 3.15-4.58 ng/mL), respectively.

The increase in exposure among Asians relative to white subjects was similar to that observed for rosuvastatin: Geometric mean ratios for \( AUC_{0-t} \) were 2.17 (90% CI, 1.71-2.74), 1.92 (90% CI, 1.52-2.43), and 1.59 (90% CI, 1.25-2.03) in Chinese, Malay, and Asian-Indian subjects respectively. Geometric mean ratios for \( C_{max} \) in Asians relative to white subjects were 2.13 (90% CI, 1.70-2.65), 1.97 (90% CI, 1.58-2.45), and 1.56 (90% CI, 1.25-1.96) in Chinese, Malay, and Asian-Indian subjects, respectively.

Values for \( t_{max} \) and \( t_{1/2} \) were similar among all 4 ethnic groups.

**Rosuvastatin-lactone**

Mean \( AUC_{0-t} \) and \( C_{max} \) (not adjusted for body weight) for rosuvastatin-lactone were also greater in Asian subjects relative to white subjects. Geometric mean values for \( AUC_{0-t} \) were 112 ng · h/mL (95% CI, 94.0-133 ng · h/mL), 99.1 ng · h/mL (95% CI, 83.4-118 ng · h/mL), 91.9 ng · h/mL (95% CI, 76.7-110 ng · h/mL), and 52.1 ng · h/mL (95% CI, 43.9-61.9 ng · h/mL) in Chinese, Malay, Asian-Indian, and white subjects, respectively. Geometric mean values for \( C_{max} \) were 7.53 ng/mL (95% CI, 6.24-9.10 ng/mL), 6.61 ng/mL (95% CI, 5.48-7.98 ng/mL), 5.32 ng/mL (95% CI, 4.37-6.49 ng/mL), and 3.14 ng/mL (95% CI, 2.60-3.79 ng/mL), respectively.

The geometric mean ratios for the relative exposure to the lactone in each Asian group relative to white subjects were 2.14 (90% CI, 1.75-2.63), 1.90 (90% CI, 1.55-2.33), and 1.76 (90% CI, 1.43-2.17) for Chinese, Malay, and Asian-Indian subjects, respectively. The geometric mean ratios for \( C_{max} \) in Asians relative to white subjects were 2.40 (90% CI, 1.92-3.00), 2.11 (90% CI, 1.68-2.63), and 1.70 (90% CI, 1.35-2.13) in Chinese, Malay, and Asian-Indian subjects, respectively.
Values for \( t_{\text{max}} \) were similar among the groups. Values for \( t_{1/2} \) were significantly longer in white subjects compared with the Asian groups.

### Metabolite ratios

The AUC\(_{0-t}\) ratios for N-desmethyl rosvastatin to rosvastatin were 0.13, 0.12, 0.13, and 0.13 for white, Chinese, Malay, and Asian-Indian subjects, respectively. For the ratio of rosvastatin-lactone to rosvastatin, these values were 0.24, 0.22, 0.24, and 0.26, respectively. Similar results were obtained for C\(_{\text{max}}\).

### Results of genetic analysis

Tables IV and V also show geometric means of AUC\(_{0-t}\) and C\(_{\text{max}}\) in the 4 study groups according to \( SLCO1B1 \) genotypes (T521>C and A388>G) and diplotype. Among white subjects, there was a significant effect of T521>C genotype on AUC\(_{0-t}\) (\( P < .0001 \)). AUC\(_{0-t}\) was higher in 521C homozygotes (CC) than in heterozygotes (TC) and in 521T homozygotes (TT), but AUC\(_{0-t}\) in heterozygotes did not differ significantly from that of 521T homozygotes (TT) (Table IV and Fig...
The effects of the T521>C genotype on C\textsubscript{max} in white subjects was similar to that seen for AUC\textsubscript{0-t} (\(P < .0001\)). There was a marginally significant effect of the A388>G genotype on C\textsubscript{max} (\(P = .0456\)), with a higher mean in 388G homozygotes (GG) than in 388A homozygotes (AA).

No effect of the T521>C genotype on systemic exposure to rosuvastatin was evident in the Chinese, Malay, and Asian-Indian subjects. The A388>G genotype also showed no association with AUC\textsubscript{0-t} or C\textsubscript{max} in any of the Asian groups (Table IV).

In white subjects analysis of SLCO1B1 diplotypes showed significant effects on both AUC\textsubscript{0-t} and C\textsubscript{max} (\(P = .028\) and .002, respectively). AUC\textsubscript{0-t} and C\textsubscript{max} were higher among subjects with diplotypes *15/15 (which contains 2 copies of the 521C allele) than in those with wild-type diplotypes *1a/1a.

A comparison of AUC\textsubscript{0-t} in all subjects homozygous for 521T (TT) showed a significant effect of race (\(P < .0001\)), with a lower AUC\textsubscript{0-t} in white subjects than in Chinese, Malay, and Asian-Indian subjects. There was a similar effect of race on AUC\textsubscript{0-t} in subjects with the heterozygous (TC) genotype (\(P = .001\)), with a lower AUC\textsubscript{0-t} in white heterozygotes than in Chinese and Malay heterozygotes (Fig 2).

Rosuvastatin was well tolerated by all volunteers after administration of the single 40-mg dose, and clin-

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### Table IV. Rosuvastatin pharmacokinetic parameters according to SLCO1B1 genotype

<table>
<thead>
<tr>
<th>Genotype</th>
<th>TT</th>
<th>TC</th>
<th>CC</th>
<th>AA</th>
<th>AG</th>
<th>GG</th>
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<td>8</td>
<td>19</td>
<td>9</td>
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<tr>
<td>Frequency</td>
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<td>0.14</td>
<td>0.22</td>
<td>0.53</td>
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<tr>
<td>AUC\textsubscript{0-t}</td>
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<td>204†</td>
<td>416‡</td>
<td>183</td>
<td>207</td>
<td>274</td>
</tr>
<tr>
<td>(165 to 224)</td>
<td>(131 to 318)</td>
<td>(257 to 672)</td>
<td>(152 to 221)</td>
<td>(165 to 260)</td>
<td>(182 to 411)</td>
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<tr>
<td>C\textsubscript{max}</td>
<td>21.4*</td>
<td>23.8†</td>
<td>58.6‡</td>
<td>18.7</td>
<td>24.3</td>
<td>34.4‡</td>
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<td>(36.8 to 93.3)</td>
<td>(13.9 to 25.2)</td>
<td>(19.1 to 31.0)</td>
<td>(16.7 to 52.0)</td>
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<td>5</td>
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<tr>
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<td>579</td>
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<td>508</td>
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<td>400</td>
<td>454</td>
<td>402</td>
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<td>419</td>
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<td>(318 to 502)</td>
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<tr>
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</tr>
<tr>
<td>AUC\textsubscript{0-t}</td>
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<td>378</td>
<td>322</td>
<td>322</td>
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<tr>
<td>(288 to 421)</td>
<td>(245 to 582)</td>
<td>(256 to 404)</td>
<td>(249 to 417)</td>
<td>(307 to 636)</td>
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<td><strong>Asian-Indian (n = 35)</strong></td>
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<tr>
<td>Frequency</td>
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<td>AUC\textsubscript{0-t}</td>
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<td>378</td>
<td>322</td>
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<td>(288 to 421)</td>
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</tr>
<tr>
<td>C\textsubscript{max}</td>
<td>42.5</td>
<td>39.5</td>
<td>37.6</td>
<td>37.7</td>
<td>55.3</td>
<td></td>
</tr>
<tr>
<td>(34.6 to 52.1)</td>
<td>(22.1 to 70.9)</td>
<td>(28.5 to 49.7)</td>
<td>(28.4 to 49.9)</td>
<td>(37.7 to 81.2)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Summary values of AUC\textsubscript{0-t} and C\textsubscript{max} are given as geometric mean and 95% CI calculated from log-transformed data. Differences were tested by use of an ANOVA model; where a genotypic effect was significant (\(P < .05\)), the Tukey-Kramer test (\(\alpha = .05\)) was used to identify significant pairwise differences.

*Within TT homozygotes for T521>C, the values in white subjects were lower than those in Chinese, Asian-Indian, and Malay subjects.
†Within TC heterozygotes for T521>C, the values in white subjects were lower than those in Chinese and Malay subjects.
‡Within white subjects values greater than those for T521>C genotypes TT and TC.
§Within white subjects values greater than those for A388>G genotype AA.
ically significant changes in laboratory parameters were not observed.

**DISCUSSION**

Plasma exposure to rosvastatin was significantly increased in the 3 Asian groups compared with the white subjects living in the same environment for at least 6 months. The increases in AUCₜ₀-ₜ were approximately 2.3-fold in Chinese subjects, 2.0-fold in Malay subjects, and 1.6-fold in Asian-Indian subjects. Body weight differences contributed less than 10% to the pharmacokinetic differences noted between the Asian ethnic groups and the white subjects.

Previous phase I studies conducted in Japanese subjects living in Japan suggested that plasma exposure in these subjects is approximately 2-fold greater compared with white subjects living in England or the United States.¹-³ A population pharmacokinetic analysis including dyslipidemic Japanese subjects living in Japan confirmed the approximate 2-fold increase in exposure in these subjects compared with dyslipidemic white subjects living in Europe and the United States.⁴ The results of our study and the previously conducted studies indicate that the pharmacokinetic difference between white subjects and Asians extends across several Asian ethnic groups.

Conceivably, differences in specific dietary constituents not captured in the diaries may have contributed to the observed differences in exposure. However, the dietary intake of the Asian groups was similar to that of the white subjects in terms of total calories and fractional contribution of fat, protein, and carbohydrate to total caloric intake. Cholesterol intake was 29% higher in the Malay subjects compared with the white subjects.

### Table V. Rosuvastatin pharmacokinetic parameters according to SLCO1B1 diplotype

<table>
<thead>
<tr>
<th>Diplotype</th>
<th>*1a/*1a</th>
<th>*1a/*1b</th>
<th>*1b/*1b</th>
<th>*1a/*15</th>
<th>*1b/*15</th>
<th>*15/*15</th>
<th>*15/*5</th>
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<tbody>
<tr>
<td>White (n = 36)</td>
<td>8</td>
<td>13</td>
<td>4</td>
<td>5</td>
<td>1</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Count</td>
<td>183</td>
<td>191</td>
<td>216</td>
<td>214</td>
<td>159</td>
<td>397†</td>
<td>499</td>
</tr>
<tr>
<td>AUC₀₋ₜ</td>
<td>(152 to 221)</td>
<td>(147 to 249)</td>
<td>(105 to 445)</td>
<td>(122 to 376)</td>
<td>(200 to 789)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cₘ₉ₜ-max</td>
<td>18.7</td>
<td>22.2</td>
<td>23.7</td>
<td>23.6</td>
<td>24.9</td>
<td>54.1‡</td>
<td>80.9</td>
</tr>
<tr>
<td>Chinese (n = 35)</td>
<td>5</td>
<td>12</td>
<td>12</td>
<td>1</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Count</td>
<td>538</td>
<td>466</td>
<td>482</td>
<td>577</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>AUC₀₋ₜ</td>
<td>(260 to 1110)</td>
<td>(386 to 562)</td>
<td>(391 to 594)</td>
<td>(380 to 876)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Cₘ₉ₜ-max</td>
<td>66.6</td>
<td>54.4</td>
<td>54.0</td>
<td>68.1</td>
<td>77.2</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Malay (n = 35)</td>
<td>0</td>
<td>8</td>
<td>18</td>
<td>4</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Count</td>
<td>183</td>
<td>191</td>
<td>216</td>
<td>214</td>
<td>159</td>
<td>397†</td>
<td>499</td>
</tr>
<tr>
<td>AUC₀₋ₜ</td>
<td>(152 to 221)</td>
<td>(147 to 249)</td>
<td>(105 to 445)</td>
<td>(122 to 376)</td>
<td>(200 to 789)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cₘ₉ₜ-max</td>
<td>18.7</td>
<td>22.2</td>
<td>23.7</td>
<td>23.6</td>
<td>24.9</td>
<td>54.1‡</td>
<td>80.9</td>
</tr>
<tr>
<td>Asian-Indian (n = 35)</td>
<td>7</td>
<td>15</td>
<td>8</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Count</td>
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<td>325</td>
<td>425</td>
<td>307</td>
<td>516</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>AUC₀₋ₜ</td>
<td>(256 to 404)</td>
<td>(238 to 444)</td>
<td>(265 to 682)</td>
<td>(163 to 577)</td>
<td>(113 to 2370)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cₘ₉ₜ-max</td>
<td>37.6</td>
<td>39.8</td>
<td>53.4</td>
<td>28.8</td>
<td>63.5</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

Summary values of AUC₀₋ₜ and Cₘ₉ₜ are given as geometric mean and 95% CI calculated from log-transformed data. Differences were tested by use of an ANOVA model; where a diplotypic effect was significant (P < .05), the Tukey-Kramer test (α = .05) was used to identify significant pairwise differences.

†Within white subjects values greater than those for *1a/*1a and *1a/*1b diplotypes.
‡Within white subjects values greater than those for *1a/*1a diplotypes.
but similar cholesterol intake was noted for the Chinese and Asian-Indian groups compared with the white subjects. Thus variation in these parameters cannot account for the differences in exposure observed in our study.

It is of interest to note that the relative increase in plasma concentrations of N-desmethyl rosuvastatin and rosuvastatin-lactone was similar to that of rosuvastatin in the Asian groups relative to the white subjects. It is also of interest that the plasma AUC_{0-t} and C_{max} metabolite/parent ratios were similar across all ethnic groups for each metabolite. N-desmethyl rosuvastatin formation is thought to occur primarily in the liver via cytochrome P450 2C9. The lactone may also be formed in the liver. These observations suggest that a greater fraction of the rosuvastatin dose reaches the liver in the Asian groups compared with the white subjects and that the formation and elimination clearances of the metabolites may not differ between groups. Differences in the hepatic clearance of rosuvastatin may also play a role in racial variation in rosuvastatin exposure. Biliary excretion is likely to be the major process for the hepatic clearance of rosuvastatin, and reduced biliary clearance in Asians compared with white subjects could lead to higher systemic plasma concentrations. The identity of the biliary transporter(s) for rosuvastatin has not been defined, but studies to do so are ongoing. The absorptive mechanism for rosuvastatin is unknown, but rosuvastatin is a high-solubility, low-permeability compound (AstraZeneca, data on file) and active transport processes (both uptake and efflux in nature) are likely to be involved. Recently, the organic anion transporter OATP2B1 (OATP-B) has been shown to be expressed in the microvilli of the human small intestine. Pravastatin has been shown in vitro to be a substrate for this transporter. The role of this transporter in rosuvastatin absorption has not been established. Rosuvastatin is not a substrate for P-glycoprotein; thus this intestinal transporter is unlikely to affect the absorption of rosuvastatin or account for the pharmacokinetic differences observed in this study. Further studies are required to assess whether variation in intestinal absorption underlies differences in rosuvastatin exposure in Asians compared with white subjects.

The mean renal clearance of rosuvastatin was 13.6 L/h in white volunteers after intravenous administration and 11.6 L/h in Japanese subjects living in Japan (AstraZeneca, data on file). Greater than 90% of the renal clearance was a result of tubular secretion. Differences in the renal clearance of rosuvastatin between Asians and white subjects cannot explain the differences in exposure after oral administration because the kidney is responsible for only about 30% of rosuvastatin systemic clearance and the Japanese renal clearance values are approximately 85% of that of the white subjects.

The inclusion in our study of 5 white subjects homozygous for the SLCO1B1 521C allele and the associated deviation from HWE in white subjects is somewhat surprising. Other reports, public database information (dbSNP), and our own unpublished data indicate that the homozygous 521C genotype is uncommon (less than 4%) in white subjects and Japanese populations. We suspect that the disproportionate number of white 521C homozygotes in this study reflects random sampling variation. No white subject had a relative who was also enrolled, and we have not been able to identify an alternative, satisfactory explanation for biased recruitment of white subjects with this genotype.

Our observation of higher rosuvastatin exposure in the 5 white subjects with the homozygous 521C genotype compared with that in white heterozygotes and 521T homozygotes is consistent with the finding of Nishizato et al, who studied the effects of SLCO1B1 SNPs on pravastatin pharmacokinetics. The single 521C homozygote in that study showed a higher C_{max} and a lower estimated nonrenal clearance than the other subjects. In contrast, in the study of Niemi et al,
pravastatin exposure in 521C homozygotes (N = 2) fell between that of TT homozygotes and TC heterozygotes.

The possibility that homozygosity for the 521C allele results in higher exposure to certain drugs, particularly those for which hepatic clearance accounts for a large proportion of systemic clearance, is biologically plausible. However, the genetic results should be interpreted cautiously because of the deviation from HWE in the white group, the small number of white 521C homozygotes, and the post hoc nature of the genetic analysis. This hypothesis could be tested in an adequately powered prospective study, with selection of subjects of known SLCO1B1 genotype.

We found no significant effect of heterozygosity for the T521>C SNP on rosuvastatin pharmacokinetic parameters in any of the 4 groups. This finding contrasts with the results of several studies of pravastatin in which higher plasma concentrations were seen in heterozygous (TC) subjects compared with homozygotes for the 521T allele. Our results, of course, do not exclude the possibility that a larger sample could show an effect of heterozygosity on rosuvastatin plasma concentration. It is also possible that there are significant differences in the hepatic transport of rosuvastatin and pravastatin such that rosuvastatin transport is unaffected by heterozygosity for the T521>C SNP.

Regarding the A388>G SNP, white subjects homozygous for the 388G allele had higher Cmax but not AUC0-t values compared with white subjects homozygous for 388A. This influence of the A388>G SNP on Cmax, however, was less than that described previously for the T521>C SNP, and no effect of the former SNP was seen in the Asian groups. Moreover, we did not detect a significant difference (in any of the study groups) in rosuvastatin pharmacokinetic parameters in subjects with diplotype *1a/*1b or *1b/*1b compared with those with diplotype *1a/*1a. These observations suggest that the apparent effect of the 388G SNP on rosuvastatin Cmax in white subjects in our study reflects linkage disequilibrium between the A388>G and T521>C SNPs. This interpretation is consistent with the in vitro findings of Tirona et al. which showed no functional differences between the *1b and *1a alleles. It contrasts, however, with the proposal of Mwinyi et al. that the *1b allele is associated with “accelerated uptake” of pravastatin. Their conclusion was prompted by the observation of diminished urinary excretion, as well as a trend toward lower plasma concentrations of pravastatin in subjects with 1 or 2 copies of the *1b allele (diplotypes *1a/*1b or *1b/*1b) compared with *1a/*1a homozygotes. However, the differences were of marginal statistical significance.

Neither the T521>C nor the A388>G SNPs nor the SLCO1B1 diplotypes that they define account for the higher rosuvastatin exposure we have observed in Chinese, Malay, and Asian-Indian subjects compared with white subjects. Whether these differences result from additional genetic influences or from environmental factors remains unclear, although the similarities in macroenvironment and in consumption of saturated fat, cholesterol, and other dietary constituents across the 4 groups provide indirect support for additional genetic effects.

The finding of increased exposure to rosuvastatin in Asian subjects relative to white subjects should be considered when rosuvastatin treatment is initiated or doses are increased in dyslipidemic Asian patients.

We acknowledge Laura Snyder and the AZ Clinical Genotyping Group.

Stephen Ryan, Bruce Birmingham, Dennis Schneck, Ruth March, Helen Ambrose, Rachael Moore, and Yusong Chen are employees of AstraZeneca and hold stock in the company.

References


Effect of OATP1B1 (SLCO1B1) variant alleles on the pharmacokinetics of pitavastatin in healthy volunteers

Background: Pitavastatin is a potent, newly developed 3-hydroxy-3-methylglutaryl–coenzyme A reductase inhibitor for the treatment of hyperlipidemia. We characterized the effects of organic anion transporting polypeptide 1B1 (OATP1B1) alleles *1a, *1b, and *15 on the pharmacokinetics of pitavastatin.

Methods: Twenty-four healthy Korean volunteers who had previously participated in a pharmacokinetic study of pitavastatin (single oral dose, 1-8 mg) were further investigated. Subjects were grouped according to OATP1B1 genotype. Dose-normalized area under the plasma concentration–time curve (AUC) and peak plasma concentration (Cmax) values were analyzed, because different dosages were administered to subjects, whereas the pharmacokinetics showed linear characteristics.

Results: Dose-normalized pitavastatin AUCs for *1b/*1b (group 1), *1a/*1a or *1a/*1b (group 2), and *1a/*15 or *1b/*15 (group 3) were 38.8 ± 13.3, 54.4 ± 12.4, and 68.1 ± 16.3 ng·h·mL⁻¹·mg⁻¹ (mean ± SD), respectively, with significant differences between all 3 groups (P = .008) and between subjects carrying and those not carrying the *15 allele (P = .004). Dose-normalized pitavastatin Cmax values were 13.2 ± 3.3, 18.2 ± 5.7, and 29.4 ± 9.6 ng·mL⁻¹·mg⁻¹ in groups 1, 2, and 3, respectively, and also showed significant differences (P = .003) in a manner similar to that shown by AUC. No significant differences were found between the genotype groups in terms of dose-normalized AUC or Cmax values of pitavastatin lactone.

Conclusion: OATP1B1 variant haplotypes were found to have a significant effect on the pharmacokinetics of pitavastatin. These results suggest that the *15 allele is associated with decreased pitavastatin uptake from blood into hepatocytes and that OATP1B1 genetic polymorphisms have no effect on the pharmacokinetics of pitavastatin lactone. (Clin Pharmacol Ther 2005;78:342-50.)

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Pitavastatin is a potent inhibitor of 3-hydroxy-3-methylglutaryl–coenzyme A (HMG-CoA) reductase and was developed for the treatment of hypercholesterolemia.¹ In humans, pitavastatin is only minimally me-tabolized by the cytochrome P450 2C9 isozyme.² The major metabolic pathway of pitavastatin involves its initial glucuronidation by uridine diphosphate–glucuronosyltransferase and then spontaneous lactonization by the elimination of the glucuronide moiety.³ Moreover, the lactone form can be reversibly converted to the parent drug.⁴ Given the specific distribution of pitavastatin in the liver,⁵ the hepatic uptake of pitavastatin through membrane transporters is likely to be a major determinant of drug disposition, as is the case for other HMG-CoA reductase inhibitors (pravastatin, ro-suvastatin, and cerivastatin).

Human organic anion transporting polypeptide 1B1 (OATP1B1/OATP-C/OATP2, SLCO1B1) is expressed at the basolateral membrane of hepatocytes, and OATP1B1 plays an important role in transporting a broad range of compounds including bile acids, sulfate,
and glucuronide conjugates. Moreover, it makes a substantial contribution to the hepatic uptake of HMG-CoA reductase inhibitors (statins). Recently, the transporter responsible for the hepatic uptake of pitavastatin in humans was identified as OATP1B1, which could account for 90% of the total hepatic uptake of pitavastatin.

Because the OATP1B1-mediated hepatic uptake of statins is important in terms of enhancing therapeutic efficacy and minimizing excess systemic exposure, which could cause adverse reactions, the presence of a functionally deleterious polymorphism in the OATP1B1 gene may increase the risk of statin-mediated rhabdomyolysis. Recently, a number of single-nucleotide polymorphisms (SNPs) were identified in this gene, and most of these SNPs were associated with a significant reduction in transporter activity in vitro. Moreover, commonly occurring SNPs such as A388G (N130D) and T521C (V174A) were found to cause a marked alteration in the disposition of pravastatin in several in vivo studies. Specifically, the OATP1B1*15 (A388G and T521C) allele was associated with increased pravastatin plasma levels in humans as compared with the OATP1B1*1b (A388G) allele, which is most common (approximately 46%) in Japanese subjects. In another study the OATP1B1*5 (T521C) allele, a variant seen in European Americans, was found to have delayed pravastatin hepatocellular uptake and increased plasma levels, whereas the OATP1B1*1b allele showed accelerated transporter activity versus the *1a allele, also known as the wild type.

Although pitavastatin is less hydrophilic than pravastatin or rosuvastatin, genetic polymorphisms of OATP1B1 would be expected to affect the pharmacokinetics of pitavastatin because of its high liver specificity and OATP1B1-dependent hepatic uptake. In this study we investigated the relationships between polymorphisms in the OATP1B1 gene and the pharmacokinetics of pitavastatin (and pitavastatin lactone) in healthy Korean subjects to determine to what extent its pharmacokinetic (PK) variability is explained by known OATP1B1 alleles.

METHODS

Subjects. Twenty-four healthy, unrelated Korean male subjects were enrolled in a PK study, in which subjects were randomly allocated to 1 of 4 groups as follows: 1 mg, 2 mg, 4 mg, or 8 mg pitavastatin (n = 6 for each group). Genotypes were assessed retrospectively and were related to the PK results. All subjects were ascertained to be healthy by medical history, a physical examination, vital signs, 12-lead electrocardiography, and routine clinical laboratory tests performed within 3 weeks before the start of this study. Regular heavy drinkers, smokers of more than 10 cigarettes per day, and subjects with a body weight differing by more than 20% from the ideal weight were excluded. A urinary drug-screening analysis by use of REMEDI HS (Bio-Rad Laboratories, Hercules, Calif) was used to exclude drug abusers. No medications, herbal drugs, alcohol, beverages containing caffeine, or grapefruit products were permitted from 7 days before the study and for the duration of the study. This study was approved by the Institutional Review Board of Seoul National University Hospital, Seoul, Korea. All procedures were performed in accordance with the recommendations of the Declaration of Helsinki on biomedical research involving human subjects and with the International Conference on the Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use–Good Clinical Practice guidelines. Written informed consent for participation in the study and genotyping was obtained from all subjects before enrollment.

Clinical study. This was a dose-rising, parallel-group study. Subjects were randomly assigned to 1 of 4 dose groups taking 1, 2, 4, or 8 mg. After an overnight fast, all subjects were given a single dose of pitavastatin (Libalo tablet; Choongwae Pharma, Seoul, Korea) or a placebo with 200 mL water at approximately 9 AM. Subjects were kept in a fasting state until 4 hours after drug administration, except for 200 mL water at 2 and 4 hours after dosing. Venous blood samples for PK analysis (8 mL) were collected via an intravenous catheter before and at 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 24, and 32 hours after dosing. Blood sampling for genotyping was also done before drug administration, and OATP1B1 genotyping was done after the end of the study. Alcohol, soft drinks, smoking, drugs, and beverages containing caffeine were prohibited during the study.

OATP1B1 genotyping. Genomic deoxyribonucleic acid (DNA) was extracted from ethylenediaminetetraacetic acid–treated venous blood by use of a QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). The National Center for Biotechnology Information reference sequence used for the OATP1B1 (SLCO1B1) gene was NM_006446. To determine the presence or absence of the 3 alleles, OATP1B1*1a, *1b, and *15, DNA near 2 polymorphic sites, A388G and T521C, was amplified by polymerase chain reaction (PCR), which was followed by SNaPshot analysis (Applied Biosystems, Foster City, Calif). The sequences of the
forward and reverse primers used were as follows: 5'-GGGGAAGATAATGGTGCAAA-3' and 5'-CGGCAGGTTTATCATCCAGT-3', respectively, for A388G SNP and 5'-CAGCATAAGAATGGACTA-ATACACC-3' and 5'-TGGACCAATCATTGCTAT-TG-3', respectively, for T521C SNP. PCR reactions were performed in a volume of 20 μL consisting of 1.5-mmol/L magnesium chloride, 250-μmol/L deoxyribonucleoside triphosphates, 0.5 pmol of each primer, and 0.25 U of AmpliTaq DNA polymerase (Applied Biosystems). After an initial denaturation at 94°C for 10 minutes, DNA was amplified over 30 cycles (denaturation at 95°C for 30 seconds, annealing at 60°C for 1 minute, and extension at 72°C for 1 minute), and this was followed by an extension at 72°C for 7 minutes. For SNaPshot analysis, PCR products were purified with exonuclease I and shrimp alkaline phosphatase (USB, Cleveland, Ohio) and mixed with AmpliTaq DNA polymerase and 4 fluorescently labeled deoxyribonucleoside triphosphates in the reaction buffer contained in an ABI Prism SNaPshot multiplex kit (Applied Biosystems) according to the manufacturer’s protocol. The internal reverse primer sequences used for the single-base extension were 5'-GTGATGTTGAATTT TCTGATGAAT-3' and 5'-CCACGAACATATTA CCCATGAAC-5' to detect A388G and T521C, respectively. The primers were extended over 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 30 seconds. Amplicons were then purified by use of exonuclease I and shrimp alkaline phosphatase and analyzed on an ABI Prism 3700 Automated Sequencer (Applied Biosystems). DNA sequences near polymorphic sites were confirmed by direct sequencing.

**Drug concentration analysis and pharmacokinetics.** Plasma concentrations of pitavastatin were determined by HPLC with ultraviolet detection. An aliquot of 1 mL plasma was mixed with 225 μL 2N potassium phosphate and 6 mL methyl tert-butyl ether, which included 100 μL of internal standard (isopropyl pitavastatin at 0.1 μg/mL). After centrifugation for 10 minutes, 100 μL of the supernatant was injected into an HPLC system (Nanospace SI-2; Shiseido Fine Chemicals, Tokyo, Japan). A mobile phase of 0.2-mol/L acetate/acetonitrile (50:50 [vol/vol], pH 4.0) was used at a flow rate of 0.8 mL/min through a C18 column (150 × 4.6-mm internal diameter, 5.0-μm particle size) (Cosmosil AR-II; Nacalai Tesque, Kyoto, Japan). The lower limit of quantification was 0.5 ng/mL, and calibration curves were linear over the concentration range 0.5 to 200 ng/mL (r > 0.98). The accuracy of this assay was within the range of 96.4% to 116.8%, and the interbatch coefficient of variation was less than 9.2% over the calibrated range.

PK parameters were calculated by use of actual sampling times. The maximum drug concentration in plasma (Cmax) was determined from the observed values. Plasma concentrations of the terminal phase were fitted to a log-linear line by the least squares method to obtain the terminal half-life. The area under the time-concentration curve (AUC) was calculated by use of a combination of the trapezoidal rule and extrapolation to infinity by use of the elimination rate constant. AUC and Cmax were normalized with respect to administered dose to allow PK parameters to be compared for the different genotypes. Linear dose proportionality of dose-normalized PK parameters was confirmed by 1-way ANOVA (P > .05) and linear regression (Fig 1). WinNonlin, version 4.0.1 (Pharsight, Mountain View, Calif) was used for the PK analysis.

**Statistical analysis and modeling.** Comparisons of the PK parameters between genotype groups were made nonparametrically by use of the Kruskal-Wallis test for multigroup comparisons and the Mann-Whitney test for 2-group comparisons. P < .05 was considered statistically significant.
To quantify the proportional contributions made by the individual alleles to PK parameters, we constructed a linear additive model as follows:

\[
\text{AUC} = Q_1 \cdot \theta_{1a} + Q_2 \cdot \theta_{1b} + Q_3 \cdot \theta_{15} \tag{1}
\]

in which Q1, Q2, and Q3 are variables dependent on genotypes. The AUC of the 5 OATP1B1 genotypes (*1a/*1a, *1a/*1b, *1b/*1b, *1a/*15, and *1b/*15) was modeled as the sum of 2 partial THETAs values of the 3 alleles (\(\theta_{1a}, \theta_{1b}\), and \(\theta_{15}\) for the OATP1B1 *1a, *1b, and *15 allele–related AUC, respectively). For example, for genotype *1a/*1a, Q1 equals 2, Q2 equals 0, and Q3 equals 0, and for genotype *1b/*15, Q1 equals 0, Q2 equals 1, and Q3 equals 1. In this manner we calculated the proportional contribution to AUC made by each allele. In the case of \(C_{\text{max}}\), the same method was applied in the analysis. Model fitting was done by use of NONMEM, version V (GloboMax, Hanover, Md), by use of PRED, which enables flexible model coding.

**RESULTS**

Subjects were grouped by allele pairs of OATP1B1 *1a, *1b, or *15 or by OATP1B1 T521C SNP pairs to investigate the effect of the OATP1B1 genotypes (Table I). Subjects were grouped into 3 groups according to allele pairs (*1b homozygous, *1a/*1b heterozygous + *1a homozygous [only 1 subject], and *15 heterozygous) or into 2 groups by T521C SNP (TT or TC). These groupings were based on the fact that only 1 subject with *1a/*1a was studied, and considerations of statistical comparative power were taken into account. Also, it had been previously reported that the *15 allele (or T512C SNP) has a significant impact on the pharmacokinetics of pravastatin.12

Demographic characteristics of subjects and dosages administered were not significantly different among the genotype groups according to OATP1B1 haplotype pairs or T512C SNP. Representative pitavastatin and pitavastatin lactone plasma concentration–time profiles versus genotypes are shown in Fig 2.

Mean dose-normalized AUC and \(C_{\text{max}}\) values in genotype group 3 (OATP1B1*15/*1a or OATP1B1*15/*1b) were 68.1 \(\text{ng} \cdot \text{h} \cdot \text{mL}^{-1} \cdot \text{mg}^{-1}\) and 29.4 \(\text{ng} \cdot \text{h} \cdot \text{mL}^{-1} \cdot \text{mg}^{-1}\), respectively, which were 1.8- and 2.2-fold higher than those values (38.8 \(\text{ng} \cdot \text{h} \cdot \text{mL}^{-1} \cdot \text{mg}^{-1}\) and 13.2 \(\text{ng} \cdot \text{mL}^{-1} \cdot \text{mg}^{-1}\), respectively) in group 1 (OATP1B1*1b/*1b) (Table II and Fig 3). Tendencies toward an increase in AUC and \(C_{\text{max}}\), as well as a decreasing trend in the volume of distribution, were noted on moving from genotype group 1 to 2 to 3 in sequence, although no significance difference was observed between groups 1 and 2.

Statistically significant differences for dose-normalized AUC and \(C_{\text{max}}\) values of pitavastatin were found among the 3 genotype groups (\(P = .008\) and \(P = .003\), respectively), between groups 1 and 3 (\(P = .003\) and \(P = .006\), respectively), and between groups 2 and 3 (\(P = .038\) and \(P = .009\), respectively). A statistically significant difference was also found between OATP1B1 512TC and TT groups (AUC, 46.6 \(\pm 14.3\) \(\text{ng} \cdot \text{h} \cdot \text{mL}^{-1} \cdot \text{mg}^{-1}\) and 68.1 \(\pm 16.3\) \(\text{ng} \cdot \text{h} \cdot \text{mL}^{-1} \cdot \text{mg}^{-1}\) \([P = .004]\), respectively; \(C_{\text{max}}\), 16.5 \(\pm 5.5\) \(\text{ng} \cdot \text{mL}^{-1} \cdot \text{mg}^{-1}\) and 29.4 \(\pm 9.6\) \(\text{ng} \cdot \text{mL}^{-1} \cdot \text{mg}^{-1}\) \([P = .001]\), respectively). No significant difference was observed between groups 1 and 2 (Table II).

**Table I.** Demographic summary of subjects according to OATP1B1 genotype

<table>
<thead>
<tr>
<th>Genotype group</th>
<th>SNP T521C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype</td>
<td>TT</td>
</tr>
<tr>
<td>*1b/*1b (n = 4)</td>
<td>521TT (n = 13)</td>
</tr>
<tr>
<td>*1a/*1a (n = 1)</td>
<td>6</td>
</tr>
<tr>
<td>*1a/*15 (n = 5)</td>
<td>.081§</td>
</tr>
<tr>
<td>*1b/*1b (n = 8)</td>
<td>2</td>
</tr>
<tr>
<td>*1b/*15 (n = 6)</td>
<td>2</td>
</tr>
</tbody>
</table>

Data are given as mean ± SD. Haplotypes *1a, *1b, and *15 are determined by SNPs 388A and 521T, 388G and 521T, and 388G and 521C, respectively. OATP1B1, Organic anion transporting polypeptide 1B1; SNP, single-nucleotide polymorphism.

†ANOVA for 3 groups.

‡Two-sample \(t\) test.

§Fisher exact test.
In the case of pitavastatin lactone, no significant difference were observed for dose-normalized AUC and C_{max} values among the 3 genotype groups or between the OATP1B1 512TC and TT groups (Table III).

Estimated allele- and genotype-specific PK parameters were obtained for all possible genotypes. The SEs of parameter estimations were less than 20% in terms of coefficient of variation in all cases. All estimated genotype-specific values were consistent with actual mean values according to genotypes ($R^2 = 0.85$ and 0.52 for AUC and C_{max}, respectively, by linear regression). Mean estimated values of AUC and C_{max} for the OATP1B1*15/*15 genotype (simulated), which were the highest recorded, were 2.1- and 3.4-fold higher than those of the OATP1B1*1b/*1b genotype, respectively, which were the lowest recorded (Table IV).

**DISCUSSION**

We investigated the functional significance of the OATP1B1 genetic polymorphism on the pharmacokinetics of pitavastatin in humans. Significant PK differences were observed according to OATP1B1 genotype. The dose-normalized AUC and C_{max} of pitavastatin were 1.4- and 1.8-fold higher, respectively, in subjects...
Table II. Effects of OATP1B1 genotypes on pharmacokinetic parameters of pitavastatin in 24 healthy Korean male subjects

<table>
<thead>
<tr>
<th>Group 1: *1b/*1b (n = 4)</th>
<th>Group 2: *1a/*1a (n = 1)</th>
<th>Group 2: *1a/*1b (n = 8)</th>
<th>Group 3: *1a/*15 (n = 5)</th>
<th>Group 3: *1b/*15 (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNAUC (ng · h · mL⁻¹ · mg⁻¹)</td>
<td>DNAUC (ng · h · mL⁻¹ · mg⁻¹)</td>
<td>DNAUC (ng · h · mL⁻¹ · mg⁻¹)</td>
<td>DNAUC (ng · h · mL⁻¹ · mg⁻¹)</td>
<td>DNAUC (ng · h · mL⁻¹ · mg⁻¹)</td>
</tr>
<tr>
<td>38.8 ± 13.3</td>
<td>45.1</td>
<td>55.6 ± 12.7</td>
<td>68.8 ± 15.6</td>
<td>67.4 ± 18.3</td>
</tr>
<tr>
<td>DNCmax (ng · mL⁻¹ · mg⁻¹)</td>
<td>DNCmax (ng · mL⁻¹ · mg⁻¹)</td>
<td>DNCmax (ng · mL⁻¹ · mg⁻¹)</td>
<td>DNCmax (ng · mL⁻¹ · mg⁻¹)</td>
<td>DNCmax (ng · mL⁻¹ · mg⁻¹)</td>
</tr>
<tr>
<td>13.2 ± 3.3</td>
<td>15.3</td>
<td>18.5 ± 6.0</td>
<td>34.4 ± 6.9</td>
<td>25.2 ± 10.0</td>
</tr>
<tr>
<td>Half-life (h)</td>
<td>Half-life (h)</td>
<td>Half-life (h)</td>
<td>Half-life (h)</td>
<td>Half-life (h)</td>
</tr>
<tr>
<td>7.7 ± 1.3</td>
<td>10.6 ± 2.9</td>
<td>10.0 ± 0.9</td>
<td>10.0 ± 0.9</td>
<td>10.0 ± 0.9</td>
</tr>
<tr>
<td>Vz/F (L)</td>
<td>Vz/F (L)</td>
<td>Vz/F (L)</td>
<td>Vz/F (L)</td>
<td>Vz/F (L)</td>
</tr>
<tr>
<td>212.2 ± 66.2</td>
<td>194.8 ± 41.2</td>
<td>153.7 ± 34.9</td>
<td>153.7 ± 34.9</td>
<td>153.7 ± 34.9</td>
</tr>
</tbody>
</table>

Data are given as arithmetic mean ± SD.
DNAUC, Dose-normalized area under plasma concentration–time curve from time 0 to infinity; DNCmax, dose-normalized peak plasma concentration; NS, not significant; Vz/F, volume of distribution based on terminal phase.
*Comparison between *1a/*15 and *1b/*15 within group 3 by Mann-Whitney test; P > .99 for DNAUC, and P = .126 for DNCmax.
†Kruskal-Wallis test for differences across all 3 groups.
§Mann-Whitney test for 2 groups.

heterozygous for the OATP1B1*15 allele (or 521T>C SNP) versus subjects not carrying this allele (or SNP). We estimated values of the PK parameters in all possible haplotype pairs, and dose-normalized AUC and Cmax were predicted to be 2- to 3-fold higher for the OATP1B1*15/*15 genotype than for the OATP1B1*1b/*1b genotype.

In a recent in vitro study, OATP1B1 was suggested to be the most important transporter with respect to the hepatic uptake of pitavastatin in humans. OATP1B1 is a major hepatic statin transporter and is known to act on pravastatin, cerivastatin, and rosuvastatin. However, only the association between polymorphisms in OATP1B1 and the pharmacokinetics of pravastatin has been reported. In recent studies in humans, significantly higher systemic exposures to pravastatin were identified in subjects carrying the OATP1B1*15 allele (containing 388A>G and 521T>C SNPs) in Japanese subjects or the OATP1B1*5 (521T>C SNP) allele and OATP1B1 −11187G>A and 521T>C SNPs in white subjects. These findings are consistent with the results of our study.

No subjects carrying the OATP1B1*5 allele were found among our Korean subjects, which is consistent with the results of the previously mentioned Japanese study. The allele frequency of OATP1B1*5 was 0% in 120 Japanese subjects, whereas it was 14% in European Americans and 0.02% in black subjects. However, both OATP1B1*5 and *15 contain 521T>C SNP, and the sum of these allele frequencies was found to be similar in Asian and white subjects (15% in Japanese, 20% in Koreans, and 14% in European Americans), implying an ethnic insensitivity with regard to pitavastatin in these 2 ethnic groups. The pharmacokinetics of pitavastatin appear to be similar among Asian ethnic groups. The geometric mean AUC values after a 2-mg oral dose of pitavastatin in healthy volunteers were 118.0 ng · h/mL in Korean subjects, 121.2 ng · h/mL in Chinese subjects, and 104.7 ng · h/mL in Japanese subjects (unpublished data), respectively. More studies are needed on OATP1B1 in black subjects to determine the impact of 521T>C SNP and ethnic differences on pitavastatin and other statins.

The pharmacokinetics of pitavastatin lactone showed no difference for the OATP1B1 genotype groups, although the plasma lactone level is determined by complex processes, perhaps because both intracellular transportation and the metabolism (glucuronidation and lactonization) of pitavastatin are not associated with OATP1B1 genotypes.

This study was conducted in a relatively small group of subjects, at different dosages, and the haplotype-pair genotype groupings adopted appear to be somewhat arbitrary, because this study was not prospectively designed to evaluate the effects of OATP1B1 genotypes. Nevertheless, the effects of OATP1B1*15 or T512C SNP were clearly observed with statistical significance. The dose normalizations of PK parameters are probably biased; however, we believe that this is justified because pitavastatin PK parameters show a linear dose proportionality, which was confirmed by linear regression and ANOVA.
Fig 3. Box-and-whiskers plot of pharmacokinetic parameters of pitavastatin grouped by OATP1B1 genotypes. The horizontal lines with solid circles within each box represent the median. The box edges show lower (25th) and upper (75th) quartiles, respectively. The whiskers extend from the 25th and 75th quartiles to the furthest data points within a distance of 1.5 interquartile ranges from the 25th and 75th quartiles. Individual data points are given as triangles. The horizontal lines with solid boxes outside whiskers represent outliers.

Table III. Effects of OATP1B1 genotypes on pharmacokinetic parameters of pitavastatin lactone in 24 healthy Korean male subjects

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>P value: All groups†</th>
<th>P value‡: Group 1 versus group 2</th>
<th>P value‡: Group 1 versus group 3</th>
<th>P value‡: Group 2 versus group 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype</td>
<td><em>(b)</em>/<em>b</em> (n = 4)</td>
<td><em>(a)</em>/<em>a</em> (n = 1) or <em>(b)</em>/<em>b</em> (n = 8)</td>
<td><em>(a)</em>/<em>a</em>5 (n = 5) or <em>(b)</em>/<em>b</em>5 (n = 6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNAUC (ng·h·mL⁻¹·mg⁻¹)</td>
<td>55.2 ± 13.5</td>
<td>98.4 ± 52.3</td>
<td>72.3 ± 25.5</td>
<td>.26</td>
<td>.14</td>
<td>.34</td>
<td>.41</td>
</tr>
<tr>
<td>DNCmax (ng·mL⁻¹·mg⁻¹)</td>
<td>9.4 ± 3.1</td>
<td>12.2 ± 4.1</td>
<td>11.7 ± 2.9</td>
<td>.63</td>
<td>.41</td>
<td>.49</td>
<td>.88</td>
</tr>
</tbody>
</table>

†Kruskal-Wallis test for differences across all 3 groups.
‡Mann-Whitney test for 2 groups.
Table IV. Estimated allele- and genotype-specific pharmacokinetic parameters

<table>
<thead>
<tr>
<th>Allele-specific parameter</th>
<th>AUC estimate (ng · h · mL⁻¹ · mg⁻¹) Mean</th>
<th>SE†</th>
<th>Cₘₐₓ estimate (ng · mL⁻¹ · mg⁻¹) Mean</th>
<th>SE†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>*1b/*1b</td>
<td>44.1</td>
<td>13.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>*1a/*1b</td>
<td>51.0</td>
<td>17.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>*1a/*1a</td>
<td>58.8</td>
<td>22.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>*1b/*15</td>
<td>65.2</td>
<td>26.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>*1a/*15</td>
<td>72.0</td>
<td>31.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>*15/*15</td>
<td>86.2</td>
<td>40.4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

†Asymptotic SE as calculated by NONMEM.

To the best of our knowledge, this is the first study on the association between genetic polymorphisms of OATP1B1 and the pharmacokinetics of pitavastatin in humans. No in vitro data are available on the transport function of the OATP1B1 variant with the use of pitavastatin as substrate. However, Iwai et al.17 recently investigated the in vitro function of the 4 major OATP1B1 alleles (OATP1B1*1a, *1b, *5, and *15) constructed by the 2 SNPs (A388G and T521C). The normalized Vₘₐₓ (concentration/time) value (picomoles per minute per milligram of protein) for OATP1B1*15 was decreased to less than 30% compared with OATP1B1*1a, which is consistent with our results. Further in vitro functional studies may clarify the mechanism underlying the in vivo PK results obtained.

In conclusion, the OATP1B1*15 allele (containing the 388A>G and 521T>C SNPs) and 521T>C SNP were identified as single major determinants of the pharmacokinetics of pitavastatin. About 2-fold higher pitavastatin concentrations were observed in subjects carrying the OATP1B1*15 or OATP1B1 512C alleles than in those without them. These results suggest that the *15 allele is associated with the reduced uptake of pitavastatin by OATP1B1 from blood. No significant effects of OATP1B1 genetic polymorphisms were observed on pitavastatin lactone formation, which implies that pitavastatin has a similar therapeutic efficacy for all OATP1B1 genotype groups. However, plasma concentration–dependent adverse events such as rhabdomyolysis may be related to the OATP1B1 polymorphism, especially in the presence of a drug interaction; for example, it was reported that gemfibrozil increases plasma pitavastatin concentrations when the 2 drugs are concomitantly administered.18 A large-scale study including a patient population is necessary to confirm the effect of the OATP1B1 polymorphisms on the clinical efficacy and adverse events associated with pitavastatin treatment.

All authors have no conflict of interest regarding this study.

References


The impact of uridine diphosphate–glucuronosyltransferase 1A9 (UGT1A9) gene promoter region single-nucleotide polymorphisms T–275A and C–2152T on early mycophenolic acid dose-interval exposure in de novo renal allograft recipients

**Background:** Mycophenolic acid (MPA), an effective immunosuppressive drug used in renal transplantation, is extensively glucuronidated by several uridine diphosphate–glucuronosyltransferases (UGTs) into an inactive 7-O-glucuronide and, to a lesser extent, into a pharmacologically active acyl-glucuronide. Experiments using human liver microsomes have shown that T–275A and C–2152T single-nucleotide polymorphisms (SNPs) of the UGT1A9 promoter region are associated with higher hepatic expression of UGT1A9 and increased in vitro glucuronidation activity for MPA.

**Methods:** The distribution of UGT1A9 promoter region T–275A and C–2152T SNPs and the less frequent UGT1A9*3 coding region mutation, which results in decreased in vitro activity, was determined in 95 de novo renal recipients. The impact of these UGT1A9 SNPs on early clinical MPA pharmacokinetics was evaluated.

**Results:** Only in patients taking 2 g mycophenolate mofetil daily was a decreased MPA exposure observed in those who carried either the T–275A or the C–2152T polymorphism (or both) compared with those who did not (area under concentration-time curve [AUC] from 0 to 12 hours, 31.7 ± 17.6 mg · h/L versus 63.6 ± 30.9 mg · h/L [P = .009]; predose trough plasma concentration, 1.23 ± 1.25 mg/L versus 2.84 ± 1.64 mg/L [P = .04]). The partial MPA AUC from 6 to 12 hours (AUC6–12)—an estimate of MPA enterohepatic recirculation—and the ratio between partial MPA AUC6–12 and dose-interval AUC from 0 to 12 hours decreased when either or both UGT1A9 promoter region SNPs were present (AUC6–12, 6.2 ± 5.4 mg · h/L versus 21.5 ± 14.9 mg · h/L [P = .002]; ratio, 18.4% ± 7.8% versus 31.7% ± 8.8% [P = .002]).

**Conclusion:** The T–275A and C–2152T SNPs of the UGT1A9 gene promoter are associated with significantly lower MPA exposure in renal recipients treated with 2 g mycophenolate mofetil daily, and part of this effect is caused by interruption of enterohepatic recirculation of MPA. (Clin Pharmacol Ther 2005;78:351-61.)

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Mycophenolic acid (MPA) is a cornerstone immunosuppressive drug effectively used in renal transplantation1-3 that is mainly metabolized by uridine diphosphate–glucuronosyltransferases (UGTs) to the inactive 7-O-glucuronide (MPAG) metabolite and, to a lesser extent, to the pharmacologically active acyl-glucuronide (AcMPAG).4,5 The latter metabolites are excreted via the kidney, at least in part by multidrug resistance protein 2 (MRP2)–mediated tubular transport.4,6 MPA and MPAG are subject to enterohepatic circulation and recirculation, which can account for up to 40% of the total dose-interval MPA area under the
Contrary to calcineurin inhibitors, MMF, the inactive intraindividual variability in pharmacokinetics. Con- sur and explain in part the large interindividual and patic UGT activity by tacrolimus.9

AcMPAG metabolite,18 which has been associated with in the liver, is responsible for generation of the active drugs.16,17

recently reported effects of MPA exposure: These effects are analogous to the subsequent excretion in the intestine and urine) are, ling the level of UGT-mediated MPA metabolism (and least partially unexplained, and genetic factors control- large variability in MPA kinetics, therefore, remains at 4% to 50% of intestinal MPAG production.18 At present, the impact of genetic polymorphisms of different UGTs involved in MPA metabolism on clinical pharmacokinetics has not been evaluated. In human liver microsomes, UGT1A9 expression varies by 17-fold, and this correlates with specific single-nucleotide polymorphisms (SNPs) in the gene promoter region (T−275A, C−2152T) that result in significantly higher glucuronidation rates of MPA compared with those in wild-type individuals.22 More than 15% of liver samples examined were found to carry the T−275A and C−2152T SNPs.22 Conversely, the glucuronosyltrans- ferase activity for MPA was lower in 1 individual carrying the UGT1A9*3 allele (T98C), indicating a decreased enzymatic activity caused by this mutation in the coding region (codon 33) of the UGT1A9 gene.22 Contrary to the promoter region SNPs, the coding region polymorphism UGT1A9*3 is present in less than 5% of white populations.16,17 Whether functional poly- morphisms of other UGT genes, such as, for example, UGT2B7,23 lead to clinically relevant changes in MPA glucuronidation and subsequent AcMPAG production is currently not known.

The frequency distributions of specific SNPs of the UGT1A9 gene that respectively resulted in increased in vitro glucuronidation activity (T−275A and C−2152T)22 and decreased enzymatic activity (UGT1A9*3 mutation)16,19 were determined in 95 de novo renal transplant recipients. This group of patients was part of a larger study cohort (N = 100) of primary renal transplant recipients in whom long-term changes in MPA exposure were prospectively studied within the first year after transplantation.10 The impact of the carrier state of these SNPs on clinical MPA pharmacokinetics and exposure was evaluated.

METHODS Study population. Ninety-five de novo white recipients (57 male and 38 female patients) out of the original study cohort of 100 patients described elsewhere10 were enrolled in this pharmacogenetic study. Patients were treated with a standard immunosuppressive drug regimen of oral tacrolimus (Prograf; Fujisawa, Munich, Germany). 0.1 mg/kg twice daily, in combination with 1 g MMF (n = 63) or 2 g MMF (n = 32) (CellCept; Hoffmann-La Roche, Basel, Switzerland) divided into 2 doses and oral methylprednisolone (Medrol; Upjohn, Kalamazoo, Mich). The daily tacrolimus dose was adjusted to achieve target predose trough blood concentrations between 8 and 15 ng/mL. Five patients from the original 100-patient cohort10 were excluded from this pharmacogenetic study because of the inability to extract sufficient deoxyribonucleic acid (DNA) for analysis.

Inclusion and exclusion criteria. The minimum age for inclusion was 18 years, and only recipients of a
single primary or secondary cadaveric donor kidney were eligible. Exclusion criteria were as follows: medical or surgical gastrointestinal disorders, including active peptic ulcer disease and diabetes mellitus, that could interfere with the absorption, distribution, metabolism, or excretion of MMF; chronic infectious or inflammatory diseases; and current therapy with bile sequestrants. Patients with a history of noncompliance leading to graft loss were also excluded, as were recipients with a known current drug, nicotine, or alcohol addiction. Finally, because this was a primary pharmacokinetic study, all substances that were documented to have a significant clinical effect on the absorption, distribution, metabolism, and excretion of MMF were prohibited. If the use of one of the latter drugs was considered to be essential, the patient was excluded from the study.

Approval was obtained from the ethics committee from the University of Leuven Faculty of Medicine, Leuven, Belgium (Nos. ML 1307 and ML 2956), and each patient gave his or her informed consent.

**Pharmacokinetic studies.** MPA AUC plasma samplings were performed on days 7, 42, 90, and 360 after transplantation. A full dose-interval (12-hour) AUC profile was obtained on day 7 after transplantation when steady-state concentrations were reached. Abbreviated 2- and 4-hour profiles were obtained at week 6 and months 3 and 12, respectively, as described elsewhere, and previously validated algorithms were used to calculate the corresponding total dose-interval AUC from the abbreviated concentration profiles.

Because the impact of UGT1A9 polymorphisms was postulated to affect enterohepatic recirculation of MPA, only the full dose-interval AUC_{0-12} determined on day 7 after transplantation was withheld for further analysis.

**MPA concentration measurements.** MPA plasma concentrations were measured by an immunoassay method (Emit Mycophenolic Acid Assay; Dade Behring, Deerfield, Ill). Compared with HPLC, the immunoassay method measures approximately 10% higher MPA concentrations because of cross-reactivity with the active AcMPAG metabolite generated mainly by hepatic UGT2B7 activity. The analytic performance of the immunoassay for MPA was ensured through participation in the Mycophenolic Acid Proficiency Testing Scheme provided by Analytical Services International (London, United Kingdom). Adjustments of the MMF dose were made strictly on the basis of clinical indications.

**Pharmacokinetic modeling.** Pharmacokinetic parameters and exposure indices were calculated by use of WinNonlin 3.2 Pro software (Pharsight, Mountain View, Calif) and the SAS 8.02 statistical program (SAS Institute, Cary, NC). Model-independent noncompartmental pharmacokinetic parameters for MPA were calculated and dose-corrected when appropriate: The dose-interval AUC (AUC_{0-12}) was estimated by use of the "linear up/log down" trapezoidal rule, and the partial AUC from 6 to 12 hours (AUC_{6-12}) as an estimate of enterobacterial circulation and recirculation, maximum plasma concentration (C_{max}), predose trough plasma concentration (C_{0}), and time to reach C_{max} (t_{max}) were determined. Estimates of apparent steady-state total body clearance (CL/F) were computed.

**Statistical analysis.** The distribution of continuous data was evaluated, and parametric tests and nonparametric tests were consequently applied when appropriate. Genotype groups were compared by use of the Mann-Whitney U test and Kruskal-Wallis test with post hoc testing for multiple comparisons. Categoric and ordinal variables were analyzed by use of nonparametric tests. ANOVA followed by multiple regression analysis was applied to assess the contribution of UGT1A9 promoter region SNPs and other clinical covariates (recipient age and gender, graft function, serum albumin concentration, hematocrit level, and liver function and dysfunction) to the interindividual variability of MPA pharmacokinetic parameters. UGT1A9 SNPs and other categoric variables were coded with a dummy variable set arbitrarily at 1 or 2 depending on the absence or presence of the respective trait. Significant covariates of MPA pharmacokinetics were selected by the backward regression procedure. A P value < .05 was considered statistically significant.

**Identification of genotypes.** Genomic DNA was extracted from ethylenediaminetetraacetic acid–treated whole blood by dehydration and precipitation with a saturated sodium chloride solution. All patients were genotyped for the UGT1A9*3, UGT1A9 C→2152T, and UGT1A9 T→275A mutation by use of a polymerase chain reaction (PCR)–restriction fragment length polymorphism method. Forward primers and reverse primers were 5’-gttctctgtgctgc-3’ and 5’-atgecc cctgagaatgagtt-3’, respectively, for the UGT1A9*3 mutation, 5’-ttagagcagactgtcgtttg-3’ and 5’-agttca agtggtcctgatc-3’, respectively, for the UGT1A9 C→2152T mutation, and 5’-ctgctgctgctgctgctttg-3’ and 5’-ccagtagttcagtaac3’, respectively, for the UGT1A9 T→275A mutation. For all gene polymorphisms, the PCR mixture (for 96 wells) contained 60 pmol forward and 60 pmol reverse primer, 300-mmol/L deoxyribonucleoside triphosphate (ABgene; Applied Biosystems, Foster City, Calif), and 40 U AmpliTaq Gold (Applied Biosystems). Then 150 μL of PCR
buffer (Applied Biosystems) was added to the PCR mixture, with 2.5-mmol/L magnesium chloride for the UGT1A9 C–2152T mutation and 1.5-mmol/L magnesium chloride for the UGT1A9*3 and UGT1A9 T–275A polymorphisms. The UGT1A9 T–275A reverse primer contained a mismatch, creating an XhoI restriction site in the allele PCR product. For the UGT1A9 C–2152T SNP, the PCR conditions were as follows: 12 minutes at 95°C; 35 cycles of 30 seconds at 93°C, 35 seconds at 63°C, and 30 seconds at 72°C; and, finally, 5 minutes at 72°C. For the UGT1A9*3 and UGT1A9 T–275A polymorphisms, the PCR amplification conditions were as follows: 12 minutes at 95°C; 35 cycles of 30 seconds at 93°C, 35 seconds at 60°C, and 30 seconds at 72°C; and, finally, 5 minutes at 72°C. After amplification, the PCR products for UGT1A9 C–2152T, UGT1A9 T–275A, and UGT1A9*3 were digested with MseI, XhoI, and StyI, respectively. DNA fragments were separated on 2% agarose gels (UGT1A9 C–2152T and UGT1A9*3) and 3% agarose gels (UGT1A9 T–275A) and visualized by ethidium bromide.

RESULTS

Demographics and transplantation-related results. Recipient demographics, transplantation-related characteristics, and biochemical and therapeutic parameters on day 7 after transplantation are summarized in Table I.

Identification and frequency distribution of UGT1A9 T–275A and C–2152T and UGT1A9*3 SNPs. The T–275A promoter region mutation occurred 16 times (16/95 [16.8%]) in this study population, whereas the C–2152T mutation was identified 12 times (12/95 [12.6%]). Eleven recipients (11/95 [11.6%]) carried both mutations, with only 1 individual being homozygous for both mutations. The UGT1A9*3 (T98C) mutation was encountered less frequently in this study group: Only 3 heterozygous individuals (3/95 [3.1%]) carrying this SNP were identified; none of the latter had associated promoter region T–275A and C–2152T mutations. All SNPs were in Hardy-Weinberg equilibrium ($\chi^2 = 0.091$ and $P = .76$ for T–275A, $\chi^2 = 0.79$ and $P = .37$ for C–2152T, and $\chi^2 = 0.024$ and $P = .87$ for UGT1A9*3) (Table II).

Clinical pharmacokinetics of MPA according to UGT1A9 T–275A and C–2152T SNPs. Because of the relatively low frequency of T–275A and C–2152T SNPs and the strong linkage of both mutations, all recipients carrying either or both promoter region polymorphisms (heterozygous or homozygous) were grouped together and compared with the noncarriers in the respective MMP dosing groups. Separation of both UGT1A9 promoter region mutations into distinct subgroups (carriers of T–275A and carriers of C–2152T) did not alter pharmacokinetic results; these data are summarized in Table III.

In recipients taking 1 g MMF per day, the presence of the UGT1A9 promoter region SNPs T–275A and C–2152T did not cause detectable changes in MPA

Table I. Baseline demographic and transplantation-related characteristics of renal recipients and biochemical parameters and immunosuppressive therapy on day 7 after transplantation (N = 95)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline demographics</td>
<td></td>
</tr>
<tr>
<td>Recipient gender (female/male) (n)</td>
<td>38/57</td>
</tr>
<tr>
<td>Age (y)</td>
<td>51.3 ± 14.1</td>
</tr>
<tr>
<td>Body weight at transplantation (kg)</td>
<td>68.7 ± 13.4</td>
</tr>
<tr>
<td>Time receiving renal replacement (mo)</td>
<td>30.3 ± 29.6</td>
</tr>
<tr>
<td>Type of renal replacement therapy (n)</td>
<td>79</td>
</tr>
<tr>
<td>Hemodialysis</td>
<td></td>
</tr>
<tr>
<td>Peritoneal dialysis</td>
<td>14</td>
</tr>
<tr>
<td>Pre-emptive</td>
<td>2</td>
</tr>
<tr>
<td>Transplantation-related characteristics</td>
<td></td>
</tr>
<tr>
<td>Retransplantation (n)</td>
<td>13</td>
</tr>
<tr>
<td>Mean No. of HLA mismatches</td>
<td>2.31 ± 1.16</td>
</tr>
<tr>
<td>Donor gender (female/male) (n)</td>
<td>37/58</td>
</tr>
<tr>
<td>Donor age (y)</td>
<td>42.7 ± 16.2</td>
</tr>
<tr>
<td>Cold ischemia time (h)</td>
<td>16.8 ± 4.31</td>
</tr>
<tr>
<td>Delayed graft function (n)*</td>
<td>6</td>
</tr>
<tr>
<td>Patients with biopsy-proven acute rejection (n)†</td>
<td>15</td>
</tr>
<tr>
<td>Biochemical parameters and therapy on day 7 after transplantation</td>
<td></td>
</tr>
<tr>
<td>Serum creatinine (mg/dL)</td>
<td>2.83 ± 2.29</td>
</tr>
<tr>
<td>Calculated creatinine clearance (mL·min⁻¹·1.73 m²⁻¹)‡</td>
<td>36.9 ± 15</td>
</tr>
<tr>
<td>Serum albumin (g/dL)</td>
<td>34.2 ± 4.36</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>31.4 ± 4.89</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>10.2 ± 1.59</td>
</tr>
<tr>
<td>Tacrolimus daily dose (mg)</td>
<td>9.4 ± 4.2</td>
</tr>
<tr>
<td>Body weight–corrected tacrolimus daily dose (mg/kg)</td>
<td>0.14 ± 0.06</td>
</tr>
<tr>
<td>MMF daily dose (n)</td>
<td></td>
</tr>
<tr>
<td>1 g</td>
<td>63</td>
</tr>
<tr>
<td>2 g</td>
<td>32</td>
</tr>
<tr>
<td>Steroid dose (mg/d)</td>
<td>17.5 ± 4.5</td>
</tr>
</tbody>
</table>

HLA, Human leukocyte antigen; MMF, mycophenolate mofetil.

*Delayed graft function defined as the need for dialysis after transplantation.
†Biopsy-proven acute rejection at 7 ± 7 days.
‡Creatinine clearance calculated according to the formula of Cockcroft and Gault.26
Table II. Frequency distribution of UGT1A9 T–275A and C–2152T and UGT1A9*3 polymorphisms in 95 renal graft recipients

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Noncarriers</td>
<td>79</td>
<td>83</td>
<td>NA</td>
<td>92</td>
</tr>
<tr>
<td>Total No. of carriers</td>
<td>16 (16.8%)</td>
<td>12 (12.6%)</td>
<td>11 (11.6%)</td>
<td>3 (3.1%)</td>
</tr>
<tr>
<td>Heterozygous carriers (1/2)</td>
<td>15 (15.8%)</td>
<td>11 (11.6%)</td>
<td>10 (10.5%)</td>
<td>3 (3.1%)</td>
</tr>
<tr>
<td>Homozygous carriers (2/2)</td>
<td>1 (1%)</td>
<td>1 (1%)</td>
<td>1 (1%)</td>
<td>0</td>
</tr>
</tbody>
</table>

UGT, Uridine diphosphate–glucuronosyltransferase; NA, not applicable.

Table III. UGT1A9 genotype and steady-state mycophenolic acid pharmacokinetic parameters and exposure indices on day 7 after transplantation in renal recipients (N = 95)

<table>
<thead>
<tr>
<th>UGT1A9 T–275A and C–2152T SNP carriers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carriers of</td>
</tr>
<tr>
<td>T–275A or</td>
</tr>
<tr>
<td>both</td>
</tr>
<tr>
<td>All</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Group receiving 1 g MMF (n)</td>
</tr>
<tr>
<td>AUC_{0-12} (mg · h/L)</td>
</tr>
<tr>
<td>Partial AUC_{0-12} (mg · h/L)</td>
</tr>
<tr>
<td>EHC (%)</td>
</tr>
<tr>
<td>C₀ (mg/L)</td>
</tr>
<tr>
<td>C_{max} (mg/L)</td>
</tr>
<tr>
<td>t_{max} (h)</td>
</tr>
<tr>
<td>CL/F (L/h)</td>
</tr>
<tr>
<td>Group receiving 2 g MMF (n)</td>
</tr>
<tr>
<td>AUC_{0-12} (mg · h/L)</td>
</tr>
<tr>
<td>Partial AUC_{0-12} (mg · h/L)</td>
</tr>
<tr>
<td>EHC (%)</td>
</tr>
<tr>
<td>C₀ (mg/L)</td>
</tr>
<tr>
<td>C_{max} (mg/L)</td>
</tr>
<tr>
<td>t_{max} (h)</td>
</tr>
<tr>
<td>CL/F (L/h)</td>
</tr>
</tbody>
</table>

SNP, Single-nucleotide polymorphism; AUC_{0-12}, area under concentration-time curve from 0 to 12 hours; AUC_{6-12}, area under concentration-time curve from 6 to 12 hours; EHC, enterohepatic circulation and recirculation; C₀, predose trough plasma concentration; C_{max}, maximum plasma concentration; t_{max}, time to reach maximum plasma concentration; CL/F, total body clearance.

*P < .01 between carriers and noncarriers of UGT1A9 T–275A and C–2152T SNPs.

†P < .05 between carriers and noncarriers of UGT1A9 T–275A and C–2152T SNPs.

There was no difference in MPA enterohepatic recirculation, estimated as the ratio of the partial MPA AUC_{0-12} and the dose–interval AUC_{0-12}, between carriers and noncarriers of the T–275A and C–2152T mutations. In contrast, for patients taking 2 g MMF per day, a significant decrease in MPA exposure was observed in those who carried either the T–275A or the C–2152T promoter region polymorphism (or both) compared with those who did not (MPA AUC_{0-12}, 31.7 ± 17.6 mg · h/L versus 63.6 ± 30.9 mg · h/L [P = .009]; C₀, 1.23 ± 1.25 mg/L versus 2.84 ± 1.64 mg/L [P = .041]) (Fig. 1). C_{max} was not significantly different between carriers and noncarriers, but t_{max} was significantly shorter (0.68 ± 0.05 hour versus 1.22 ± 0.57 hour, P = .02). Estimated total body clearance was significantly higher for recipients with the T–275A and C–2152T promoter region polymorphisms (40.3 ± 20.1 L/h versus 19.5 ± 10.7 L/h, P = .009). Interestingly, the partial MPA AUC_{6-12}, reflecting an estimate of MPA enterohepatic recirculation, was significantly decreased when either or both UGT1A9 promoter re-
region mutations were present (6.2 ± 5.4 mg·h/L versus 21.5 ± 14.9 mg·h/L, P = .002). Similarly, the ratio between the partial MPA AUC_{6-12} and total dose-interval AUC_{0-12} was significantly lower in recipients with T_{275A} and C_{2152T} polymorphisms (18.4% ± 7.8% versus 31.7% ± 8.8%, P = .002).

The lower MPA exposure and estimates of MPA enterohepatic recirculation and the increased MPA clearance in the group of recipients taking 2 g MMF and carrying the T_{275A} and C_{2152T} promoter region polymorphisms were not affected by recipient gender (P > .99, Fisher exact test), the occurrence of delayed graft function (defined as the need for dialysis therapy after transplantation) (P > .99), or the presence of liver function disturbances (P = .22) (data not shown). Biopsy-proven acute rejection was diagnosed on day 7 in 1 of 6 patients (16.6%) carrying a promoter region SNP, whereas 2 noncarriers (7.7%) were treated for acute rejection on day 7 (P = .48); during later follow-up (12 months), no additional biopsy-proven acute rejection episodes occurred in either group. Documented episodes of noninfectious diarrhea were reported in 8 of 26 recipients (30.8%) not carrying a UGT1A9 promoter region SNP, whereas diarrhea did

**Fig 1.** Mycophenolic acid (MPA) area under concentration-time curve from 0 to 12 hours (AUC_{0-12}), apparent steady-state total body clearance (CL), predose trough plasma concentration (C_{0}), time to reach maximum plasma concentration (t_{max}), and estimate of enterohepatic recirculation in carriers (n = 6) and noncarriers (n = 26) of uridine diphosphate–glucuronosyltransferase 1A9 (UGT1A9) promoter region T_{275A} and C_{2152T} single-nucleotide polymorphisms (2-g daily dose of mycophenolate mofetil). *Horizontal solid lines* indicate median values of MPA pharmacokinetic parameters.
not develop in any of the recipients carrying a promoter region SNP ($P = .29$, Fisher exact test). Leukopenia occurred in 50% of patients in the first year after transplantation, with equal distribution in carriers (3/6) and noncarriers (13/26) of the UGT1A9 promoter region SNP. Patients with $T^\text{275A}, C^\text{2152T}$ polymorphisms did not differ from noncarriers in terms of age ($P = .28$), body weight ($P = .71$ for serum creatinine level and $P = .28$ for calculated creatinine clearance according to the Cockcroft-Gault formula$^{26}$), serum albumin concentration, liver dysfunction, and hematocrit level. The backward elimination procedure was performed with $P < .05$ for retaining covariate in the model.

### Clinical pharmacokinetics of MPA according to UGT1A9*3 SNP

Three heterozygous carriers (3.1%) of the UGT1A9*3 SNP were identified, 2 recipients in the group receiving 1 g MMF and 1 patient in the group receiving 2 g MMF.

In the group receiving 1 g MMF, a higher MPA AUC$_{0-12}$ was measured on day 7 after transplantation in the 2 individuals who carried the UGT1A9*3 SNP compared with those who did not (78.7 ± 18.7 mg · h/L vs. 42.5 ± 23.4 mg · h/L, $P = .04$) whereas C$_\text{0}$ did not differ ($2.72 ± 1.61$ mg/L vs. 2.1 ± 1.54 mg/L, $P = .52$). The partial MPA AUC$_{6-12}$, reflecting an estimate of MPA enterohepatic recirculation, was significantly higher for the UGT1A9*3 SNP carriers (31.28 ± 6.47 mg · h/L versus 14.6 ± 10.46 mg · h/L, $P = .02$), but the ratio between the partial MPA AUC$_{6-12}$ and total dose-interval AUC$_{0-12}$ did not differ from that in patients not carrying this SNP (39.9% ± 1.2% versus 31.9% ± 9.4%, $P = .10$).

The one recipient in the group receiving 2 g MMF carrying the UGT1A9*3 SNP displayed similar changes in MPA exposure compared with those in noncarriers receiving 2 g MMF daily (MPA AUC$_{0-12}$, 73.82 mg · h/L versus 57.13 ± 31.69 mg · h/L; C$_\text{0}$, 4.5 mg/L versus 2.47 ± 1.67 mg/L; AUC$_{6-12}$, 22.28 mg · h/L versus 18.7 mg · h/L).

### Table IV. Determinants of MPA pharmacokinetic parameters in recipients taking 2 g MMF daily (n = 32)

<table>
<thead>
<tr>
<th>Dependent variables</th>
<th>Independent variables</th>
<th>Partial $r^2$</th>
<th>$P$ value</th>
<th>Model $r^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPA AUC$_{0-12}$ (mg · h/L)</td>
<td>UGT1A9 T$^\text{275A}$ and C$^\text{2152T}$ SNP</td>
<td>0.16 (neg)</td>
<td>.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gender</td>
<td>0.12 (pos)</td>
<td>.04</td>
<td>0.27</td>
</tr>
<tr>
<td>MPA AUC$_{6-12}$ (mg · h/L)</td>
<td>UGT1A9 T$^\text{275A}$ and C$^\text{2152T}$ SNP</td>
<td>0.16 (neg)</td>
<td>.02</td>
<td>0.16</td>
</tr>
<tr>
<td>EHC (%)</td>
<td>UGT1A9 T$^\text{275A}$ and C$^\text{2152T}$ SNP</td>
<td>0.27 (neg)</td>
<td>.002</td>
<td>0.27</td>
</tr>
<tr>
<td>C$_\text{0}$ (mg/L)</td>
<td>UGT1A9 T$^\text{275A}$ and C$^\text{2152T}$ SNP</td>
<td>0.14 (neg)</td>
<td>.007</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gender</td>
<td>0.12 (pos)</td>
<td>.04</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Calculated creatinine clearance</td>
<td>0.14 (neg)</td>
<td>.03</td>
<td>0.36</td>
</tr>
<tr>
<td>C$_{\text{max}}$ (mg/L)</td>
<td>No covariates retained in the model</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$t_{\text{max}}$ (h)</td>
<td>UGT1A9 T$^\text{275A}$ and C$^\text{2152T}$ SNP</td>
<td>0.14 (neg)</td>
<td>.02</td>
<td>0.14</td>
</tr>
<tr>
<td>CL/F (L/h)</td>
<td>UGT1A9 T$^\text{275A}$ and C$^\text{2152T}$ SNP</td>
<td>0.29 (pos)</td>
<td>.002</td>
<td></td>
</tr>
</tbody>
</table>

The independent variables were carrier state of UGT1A9 T$^\text{275A}$ and C$^\text{2152T}$ promoter region SNPs, recipient gender and age, graft function (calculated creatinine clearance), serum albumin concentration, liver dysfunction, and hematocrit level. The backward elimination procedure was performed with $P < .05$ for retaining covariate in the model.

MPA, Mycophenolic acid; neg, negative slope; pos, positive slope.
versus 18.54 ± 15.11 mg·h/L; and AUC_{6-12}/AUC_{0-12} ratio, 30.1% versus 29.2% ± 10.2%).

**DISCUSSION**

This study has demonstrated for the first time that $T−275A$ and $C−2152T$ SNPs of the $UGT1A9$ gene promoter region, producing higher in vitro glucuronidation rates, are associated with significantly lower MPA exposure (AUC_{0-12}, C_{0}) in de novo renal transplant recipients treated with 2 g MMF per day. Failure to recognize the impact of this genetic variability in $UGT1A9$ glucuronidation activity could lead to MPA underexposure and loss of clinical efficacy. Interruption of the enterohepatic recirculation of MPA is at least partially responsible for decreased MPA exposure associated with these SNPs.

In vitro experiments demonstrated that $T−275A$ and $C−2152T$ SNPs correlate with higher hepatic expression of UGT1A9 and increased in vitro glucuronidation activity for MPA compared with those of wild-type individuals. The fact that these in vitro observations were confirmed by measuring a reduced MPA exposure in renal transplant recipients carrying these polymorphisms underlines the clinical importance of genetic variability in the promoter region of the $UGT1A9$ gene for in vivo MPA metabolism. In accordance with in vitro data, we found the $T−275A$ and $C−2152T$ SNPs to occur relatively frequently (16.8% for $T−275A$ and 12.6% for $C−2152T$) in white renal transplant recipients treated with 2 g MMF per day. Failure to recognize the impact of this genetic variability in $UGT1A9$ glucuronidation activity could lead to MPA underexposure and loss of clinical efficacy. Interruption of the enterohepatic recirculation of MPA is at least partially responsible for decreased MPA exposure associated with these SNPs.

In vitro experiments demonstrated that $T−275A$ and $C−2152T$ SNPs correlate with higher hepatic expression of $UGT1A9$ and increased in vitro glucuronidation activity for MPA compared with those of wild-type individuals. The fact that these in vitro observations were confirmed by measuring a reduced MPA exposure in renal transplant recipients carrying these polymorphisms underlines the clinical importance of genetic variability in the promoter region of the $UGT1A9$ gene for in vivo MPA metabolism. In accordance with in vitro data, we found the $T−275A$ and $C−2152T$ SNPs to occur relatively frequently (16.8% for $T−275A$ and 12.6% for $C−2152T$) in white renal transplant recipients treated with 2 g MMF per day. Failure to recognize the impact of this genetic variability in $UGT1A9$ glucuronidation activity could lead to MPA underexposure and loss of clinical efficacy. Interruption of the enterohepatic recirculation of MPA is at least partially responsible for decreased MPA exposure associated with these SNPs.

The strong decrease in the partial MPA AUC_{6-12}, indicating loss of enterohepatic recirculation, adds to the complexity of the $T−275A$ and $C−2152T$ mutation effect on MPA metabolism. Increased $UGT1A9$-mediated glucuronidation of MPA into MPAG would theoretically lead to an increase in enterohepatic recirculation rather than a decrease. A possible explanation for the decreased MPA AUC_{6-12} could lie in the simultaneously increased activity of both hepatic and intestinal $UGT1A9$ (the latter being responsible for approximately 40%-50% of intestinal MPAG production), which could lead to increased blood and intestinal concentrations of MPAG, thereby counteracting the deglucuronidation of MPAG to MPA in the distal gut by the microbial flora. This complex mechanism could result in lower effective MPA reabsorption from the distal small bowel and hence less enterohepatic recirculation. Recently, it was shown that rifampin (INN, rifampicin), an inducer of UGTs, caused a more than 2-fold reduction in MPA exposure (AUC_{0-12}) when coadministered in a lung transplant recipient and reduced the estimated enterohepatic recirculation of MPA to a similar extent. Although the effects of a pleiotropic inducer such as rifampin are not completely comparable to those of a $UGT1A9$ promoter region SNP resulting in increased glucuronidation activity, this case strengthens the hypothesis for explaining the mechanism of the obvious decrease in enterohepatic recirculation in recipients carrying the $T−275A$ and $C−2152T$ mutation. The higher apparent MPA clearance and the shorter time to reach maximum MPA plasma concentration ($t_{max}$) in carriers of the $T−275A$
and C\textsuperscript{2152T} SNPs also concur with this proposed mechanism.

The fact that the carrier state of \textit{UGT1A9} promoter region SNPs was only functionally apparent in recipients taking 2 g MMF daily suggests underlying dose-dependent processes and might indicate that \textit{UGT1A9} promoter genotyping may not be useful when recipients take lower doses of MMF. A possible explanation for this dose-dependency of \textit{UGT1A9} promoter region SNP-induced changes in MPA exposure could lie in the fact that higher intestinal concentrations of MPAG obtained with 2 g MMF daily would eventually (after prior deglucuronidation by the gut flora) lead to even more renewed MPAG production by increased SNP-associated intestinal UGT1A9 activity and relatively lower net absorption of MPA through enterohepatic recirculation, as compared with a lower daily dose of the drug. At least in vitro, UGT1A9 exhibits a linear concentration-dependent glucuronidation activity for MPA under basal circumstances, achieving saturation kinetics at 2.4-mmol/L MPA.\textsuperscript{20} The latter concentration lies several hundred-fold above predose trough plasma concentrations of MPA achieved in renal recipients but could possibly be attained locally in the gut, at the mucosal intestinal barrier, where UGT1A9 is active. Conversely, the primary MRP2-mediated biliary excretion of MPAG\textsuperscript{5,28} could also be a rate-limiting step in MPA enterohepatic recirculation, which is analogous to effects of other compounds that depend on MRP2-mediated transport such as flavonoids (quercetin),\textsuperscript{29} and explain the dose-dependent changes in MPA kinetics in both basal circumstances\textsuperscript{10} and situations of increased UGT1A9 activity. Simultaneous measurements of plasma and urine MPA and MPAG concentrations could have shed more light on this potential mechanism but were not performed in our study. Another reason why the effects of \textit{UGT1A9} promoter region SNPs on MPA kinetics are dose-dependent could be the simultaneous influence of early allograft function and tacrolimus blood concentrations. Tacrolimus is capable of inhibiting hepatic UGT in a dose-dependent manner,\textsuperscript{9} and extrahepatic MPA glucuronidation can occur to an important extent within the transplanted kidney.\textsuperscript{7,18} Early after transplantation, when tacrolimus blood concentrations are still relatively high and renal allograft function (and possibly renal glucuronidation capacity) is still suboptimal, this could lead to a state of functional UGT1A9 inhibition, thereby counteracting the increased glucuronidation activity induced by \textit{UGT1A9} promoter region SNPs, especially when smaller amounts of substrate are administered. With higher concentrations of MPA, the SNP-induced increased UGT1A9 activity would overcome these initial inhibitory effects. Unfortunately, subsequent MPA concentration-time curve measurements performed in the original study population at 6 weeks and 3 and 12 months after transplantation were abbreviated (2- and 4-hour) profiles,\textsuperscript{10} lacking the terminal part of the dose-interval AUC\textsubscript{0-12}. Therefore the strong impact of \textit{UGT1A9} promoter region SNPs on enterohepatic recirculation (AUC\textsubscript{6-12}), as demonstrated on day 7 after transplantation, could not be confirmed at subsequent sampling time points. Similarly, the abbreviated concentration curves could not be used to test the effect of progressive improvement of graft function and decreasing blood concentrations of tacrolimus on MPA kinetics in carriers and noncarriers of \textit{UGT1A9} promoter region SNPs treated with different doses of MMF (data not shown). Trough plasma MPA concentrations at later time points after grafting (day 42, 90, and 360) were not different between carriers and noncarriers of \textit{UGT1A9} promoter region SNPs. This could be explained by time-related alterations in the functional (phenotypic) status of \textit{UGT1A9} \textit{T}\textsuperscript{\textmd{275A}} and \textit{C}\textsuperscript{\textmd{2152T}} SNPs or other UGTs involved in MPA metabolism. Alternatively, changes independent of UGT activity\textsuperscript{10} (eg, graft function, albumin binding) could have attenuated the genotypic effects on MPA pharmacokinetics.

The previously demonstrated lack of a strong correlation between MPA \textit{C}\textsubscript{0} and corresponding dose-interval MPA AUC\textsubscript{0-12}\textsuperscript{10} illustrates, at least in this study population, that predose trough plasma MPA concentrations do not adequately reflect (time-related) changes in total MPA exposure and might not prove to be the most reliable tool to by which assess the effects of \textit{UGT1A9} promoter region SNPs. Full dose-interval MPA concentration-time curve measurements obtained at later time points after transplantation would have helped to clarify this issue.

Reduced MPA exposure in carriers of \textit{UGT1A9} promoter region \textit{T}\textsuperscript{275A} and \textit{C}\textsuperscript{2152T} SNPs was not associated with clinical loss of efficacy in terms of acute graft rejection (16.6% versus 7.7%, \textit{P} = .48), probably because of the low absolute number of rejections in the respective MMF dosing subgroups and further because the carrier state of the patient was taken into account. Conversely, \textit{UGT1A9} promoter region SNPs causing higher plasma concentrations of MPAG could lead to displacement of MPA from albumin binding sites and increase the pharmacologically active free fraction of MPA,\textsuperscript{4,11} thereby attenuating the clinical effect of decreased total MPA exposure. MMF-related side effects (leukopenia, diarrhea) were not significantly less frequent in carriers of \textit{UGT1A9} promoter region SNPs.
region SNPs, although no gastrointestinal toxicity was reported in the latter group whereas 30% of noncarriers had at least 1 documented episode of diarrhea within the first year after transplantation.

The less frequent SNP located in the coding region of the UGT1A9 gene (T98C) resulting in decreased in vitro enzymatic activity\textsuperscript{16,30} was clearly associated with higher MPA exposure as one would expect from decreased in vitro glucuronidation activity. However, the expected\textsuperscript{22} low frequency with which this mutation occurred (3.1%) in our study cautions against a preliminary interpretation of these sparse data and underlines the need for analysis of larger patient populations.

This study has shown that de novo renal allograft recipients treated with 2 g MMF daily in combination with tacrolimus who carry the $T\rightarrow275A$ and $C\rightarrow2152T$ SNPs of the UGT1A9 gene promoter region exhibit a significantly lower MPA dose-interval exposure early after transplantation in comparison with recipients not carrying these mutations. The interruption of the enterohepatic recirculation of MPA seems partially responsible for decreased MPA exposure associated with these SNPs, but further clinical intervention studies are required to identify the exact mechanisms underlying these alterations in clinical MPA kinetics associated with UGT1A9 promoter region SNPs. The clinical efficacy of not only MPA but also other drugs that are strongly dependent on UGT1A9-mediated metabolism could be compromised if the impact of this genetic variability in UGT1A9 glucuronidation activity is not recognized.

None of the authors disclosed any conflict of interest with respect to the article.

References


Influence of CYP2C9 genotypes on the pharmacokinetics and pharmacodynamics of piroxicam

**Objective:** Our objective was to evaluate the influence of cytochrome P450 (CYP) 2C9 polymorphisms on the pharmacokinetics and pharmacodynamics of the nonsteroidal anti-inflammatory drug piroxicam.

**Methods:** Thirty-five healthy subjects with CYP2C9 genotypes *1/*1 (n = 17), *1/*2 (n = 9), and *1/*3 (n = 9) received a single oral dose of piroxicam (20 mg). Blood samples were collected at various time points up to 240 hours for measurements of the concentrations of piroxicam and thromboxane B2 (TXB2).

**Results:** Piroxicam's area under the plasma concentration–time curve from time 0 to infinity and oral clearance corrected for body weight were 154 ± 37 μg·mL⁻¹·h and 2.0 ± 0.5 mL·h⁻¹·kg⁻¹, respectively, in CYP2C9*1/*1 individuals, as compared with 256 ± 97 mL·h⁻¹ (P = .002) and 1.3 ± 0.4 mL·h⁻¹·kg⁻¹ (P = .002), respectively, in CYP2C9*1/*2 individuals and 259 ± 95 mL·h⁻¹ (P = .002) and 1.3 ± 0.4 mL·h⁻¹·kg⁻¹ (P = .002), respectively, in CYP2C9*1/*3 individuals. There were no significant differences between CYP2C9*1/*2 and CYP2C9*1/*3 individuals in these pharmacokinetic parameters (P = .95 for area under the plasma concentration–time curve from time 0 to infinity and P = .94 for oral clearance corrected for body weight). The formation of TXB2, reflecting cyclooxygenase type 1 activity, showed significant differences in the area above the effect-time curves (expressed as percent of baseline TXB2 · h) between CYP2C9*1/*1 (10,190 ± 2632) and either CYP2C9*1/*2 (19,255 ± 1,291 [P = .00003]) or CYP2C9*1/*3 (18,241 ± 2397 [P = .00003]). The minimum serum TXB2 concentration, however, did not differ among the different genotypes (P = .32, ANOVA).

**Conclusion:** Piroxicam’s oral clearance was impaired and its inhibitory effect on cyclooxygenase 1 activity was increased in CYP2C9*1/*2 or CYP2C9*1/*3 individuals, as compared with CYP2C9*1 homozygous individuals. (Clin Pharmacol Ther 2005;78:362-9.)

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The nonsteroidal anti-inflammatory (NSAID) therapeutic class includes a large number of drugs, often chemically unrelated, that are available as both prescription-only and over-the-counter medications and are largely consumed worldwide. The NSAIDs’ pharmacologic effects, whether therapeutic or adverse, result mainly from inhibition of cyclooxygenase 1 (COX-1) and cyclooxygenase 2 (COX-2), which are responsible for the production of thromboxanes, prostaglandins, and related autacoids. The NSAIDs are substrates for the cytochrome P450 (CYP) family of drug-metabolizing enzymes, predominantly CYP2C9, although CYP1A2, CYP3A4, and CYP2C8 may also contribute to the hydroxylation of some NSAIDs. The CYP2C9-codifying gene, denoted CYP2C9, is highly polymorphic, and more than 50 single-nucleotide polymorphisms have been described in its regulatory and coding regions. Two of the coding variants, namely, CYP2C9*2 (C430T) and CYP2C9*3 (A1075C), have been the most extensively investigated for their functional consequences in relation to various drugs, including the NSAIDs. Clinical studies detected significant effects of CYP2C9*3 but not *2 on either the disposition or the
pharmacodynamics (or both) of celecoxib, flurbiprofen, and ibuprofen, whereas neither variant allele affected the pharmacokinetics of diclofenac. In contrast, we recently reported that both CYP2C9*2 and CYP2C9*3 affected the clearance of tenoxicam, an enolic acid (or oxicam) NSAID. This apparent discrepancy prompted the current study, which has 2 major objectives, as follows: first, to verify the influence of alleles CYP2C9*2 and CYP2C9*3 on the pharmacokinetics of another widely used oxicam (ie, piroxicam), and second, to explore the functional consequences of these variant alleles on COX-1, a major pharmacodynamic target for piroxicam. Preliminary results have appeared in abstract form.

**METHODS**

**Study population.** The study protocol was approved by the Ethics Committee of the Brazilian National Cancer Institute. From a panel of more than 400 healthy volunteers previously genotyped for the polymorphic enzymes CYP2C9, CYP2A6, and thiopurine S-methyltransferase (TPMT), 35 individuals with CYP2C9 genotypes *1/*1 (n = 17), *1/*2 (n = 9), or *1/*3 (n = 9) agreed to participate in the study and provided written informed consent. These individuals (24 men and 11 women), who had not participated in our previous study with tenoxicam, answered a questionnaire about their ancestry and demographics and were categorized as white (n = 19), black (n = 4), or intermediate (n = 12), according to the classification scheme used in the 2000 Brazilian Census, which relies on self-perception of skin color. The potential pitfalls of this categorization as a result of the extensive admixture of the Brazilian population have been discussed in previous reports from our group.

**CYP2C genotyping.** Peripheral blood samples (3 mL) were collected in ethylenediaminetetraacetic acid–containing tubes, and genomic deoxyribonucleic acid was extracted by use of the DNAzol purification kit (Invitrogen, Carlsbad, Calif) according to the manufacturer’s instructions. CYP2C9 genotypes were identified, as described by Vianna-Jorge et al, and the CYP2C8*3 variant allele was detected as described by Dai et al.

**Pharmacokinetic studies.** A single oral dose (20 mg) of piroxicam (Feldene; Laboratório Pfizer, São Paulo, Brazil) was administered to each participant, after an overnight fast (>10 hours). The subjects remained in the clinical unit from 6 to 7 pm on the night preceding drug administration until the collection of the 24-hour postdosing blood sample and returned to the unit for collection of the subsequent samples. Blood samples (6-8 mL) were collected before and at 1, 2, 3, 4, 5, 6, 8, 11, 24, 48, 72, 96, 144, 192, and 240 hours after dosing. Each sample was centrifuged within 30 minutes of collection, and the plasma was separated and stored at −20°C until analysis.

Plasma concentrations of piroxicam were determined by a validated HPLC procedure, by use of tenoxicam as the internal standard. All samples from each volunteer were analyzed in the same run. The plasma samples were made acidic by the addition of 1N hydrochloric acid, and piroxicam was extracted with dichloromethane. The residues were analyzed by HPLC (Agilent 1100 Series; Agilent Technologies, Palo Alto, Calif) by use of 1% triethylamine-acetonitrile-tetrahydrofuran (70:25:5) as the mobile phase, at a flow rate of 1.0 mL/min, with a Waters Xterra RP-18 endcapped column (125 × 4 mm, 5 μm) (Merck, Darmstadt, Germany) and LiChrospher 100RP-18 guard column (4 × 4 mm, 5 μm) (Merck), at an oven temperature of 37°C. Detection was accomplished by fluorescence absorbance at 360 nm. The assay was linear over the studied range (100-4000 ng/mL) with R² > 0.99, and the mean recovery for low (300 ng/mL), medium (1000 ng/mL), and high (3000 ng/mL) quality-control samples was 81% (range, 76%-88%). The intra-assay and interassay precision showed coefficients of variation of less than 6% and less than 8%, respectively, and intra-assay and interassay accuracy was greater than 99% and greater than 98%, respectively, for the range of quality-control samples tested.

The software WinNonlin Professional, version 4.0.1 (Pharsight, Mountain View, Calif), was used for the pharmacokinetic analysis and simulations. Noncompartmental analysis of the plasma concentration data points after administration of a single dose of piroxicam provided the following pharmacokinetic parameters: maximal piroxicam plasma concentration (Cmax); area under the concentration-time curve (AUC) from time 0 to infinity (AUC0-), calculated from the composite of AUC from 0 to 240 hours measured by the trapezoidal rule and from extrapolation to infinity of the log-linear fit of the last 6 to 10 measured points, and total oral clearance corrected for body weight (CL/Fcor) = Dose · AUC0-−1 · Body weight−1. Compartmental analysis was applied to the individual plasma piroxicam concentrations to identify the most parsimonious model for fitting the data and to derive the corresponding model descriptive parameters for each individual. These parameters were then used for pharmacokinetic simulation of the individual plasma piroxicam concentrations during a 7-day regimen of 20 mg piroxicam once daily. The simulated concentrations after the seventh dose were timed to reproduce the sampling schedule of the bioequivalence study.
Thromboxane B<sub>2</sub> concentration analysis. To measure piroxicam-induced inhibition of thromboxane B<sub>2</sub> (TXB<sub>2</sub>) generation (presumed to be generated by constitutively expressed platelet COX-1), samples without anticoagulant were drawn from 19 subjects before and at 2, 6, 24, 48, 72, 96, 144, 192, and 240 hours after piroxicam dosing. Each sample was incubated for 1 hour at 37°C, and the serum was separated by centrifugation and stored at −70°C until assayed for TXB<sub>2</sub> by use of an enzyme immunoassay kit (Cayman Chemical, Ann Arbor, Mich). The TXB<sub>2</sub> measurement was performed by an automatic plate reader (Spectra Max; Molecular Devices, Sunnyvale, Calif) controlled by SoftMax software (Molecular Devices). The samples were diluted with assay buffer according to the manufacturer’s instructions to obtain concentrations in the defined calibration range. The limit of quantification was 0.016 ng/mL, and the interassay and intra-assay coefficients of variation ranged from 1.9% to 10.4% and from 1.1% to 13.5%, respectively, for TXB<sub>2</sub> concentrations between 0.016 and 1 ng/mL. The individual data for TXB<sub>2</sub> are expressed as percent inhibition of the baseline serum TXB<sub>2</sub> concentration, and these normalized (percent) values were used to construct time-effect curves from time 0 (administration of piroxicam) to 240 hours (last sample collected) and for calculation of 2 parameters of the piroxicam-induced inhibition of COX-1: trough serum concentration of TXB<sub>2</sub> (TXB<sub>2</sub><sub>Cmin</sub>) and area above the time-effect curve from 0 to 240 hours (TXB<sub>2</sub> AUC).

Statistical analysis. The individual values for the pharmacokinetic (C<sub>max</sub>, AUC<sub>0-∞</sub>, CL/F<sub>cor</sub>, and trough plasma concentration [C<sub>Cmin</sub>]) and pharmacodynamic parameters (TXB<sub>2</sub> C<sub>min</sub> and TXB<sub>2</sub> AUC) were log-transformed for statistical analysis. Comparisons of parameter values across the 3 different genotypes were performed by use of ANOVA. The coefficient of determination value (r<sup>2</sup>) generated by ANOVA was used to describe the degree of variability in the parameter of interest that could be explained by the CYP2C9 genotype. As appropriate, the Student-Newman-Keuls test was used to assess the presence of statistical differences between the genotype groups when a statistically significant association was described by ANOVA. The software package StatXact-5 (Cytel Software, Cambridge, Mass) was used for all statistical analyses, and the level of significance was set at P < .05.

RESULTS

Pharmacokinetic differences across CYP2C9 genotypes. The plasma piroxicam concentration–time curves, according to CYP2C9 genotype, are shown in Fig 1, and the pharmacokinetic parameters derived from noncompartmental analysis of individual data are presented in Table I. There were significant differences (ANOVA) across the 3 genotypes in relation to AUC<sub>0-∞</sub> (P = .0001) and CL/F<sub>cor</sub> (P = .0001) but not in the C<sub>max</sub> values (P = .44). Pairwise comparisons (Student-Newman-Keuls) revealed no significant difference between CYP2C9*1/*2 and CYP2C9*1/*3 in any of the pharmacokinetic parameters studied (P = .95 for AUC<sub>0-∞</sub>, P = .94 for CL/F<sub>cor</sub>, and P = .23 for C<sub>max</sub>). In contrast, both CYP2C9*1/*2 and CYP2C9*1/*3 genotypes were associated with significantly greater AUC<sub>0-∞</sub> (P = .002 and .002, respectively) and lower CL/F<sub>cor</sub> (P = .002 and .002, respectively) values than those in the homozygous wild-type individuals. Despite these significant differences, there was overlap of the individual values for each of these pharmacokinetic parameters across the 3 genotypes represented in the study (Fig 2). The degree of variability (r<sup>2</sup>, as previously described in the Methods section) in AUC<sub>0-∞</sub> and CL/F<sub>cor</sub> that could be explained by the CYP2C9 genotype was 0.43 (Table I).

A 1-compartmental model with no latency term was fitted to the observed plasma piroxicam concentrations after a single oral dose, and least squares linear regression indicated good correlation between observed and estimated concentrations (R = 0.95, SD = 0.27, P = .0001). This model was used to simulate the individual plasma piroxicam concentrations after 7 daily doses of piroxicam (20 mg orally) in 35 healthy subjects expressing different CYP2C9 genotypes (CYP2C9*1/*1, n = 17; CYP2C9*1/*2, n = 9; and CYP2C9*1/*3, n = 9). Data are presented as geometric mean ± 95% confidence interval. For better visualization, some data points were displaced along the x-axis and a break was inserted in the x-axis. The lines are the best linear fit for the data points in the elimination phase (48-240 hours).

Fig 1. Plasma concentration–time curves of piroxicam (20 mg orally) in 35 healthy subjects expressing different CYP2C9 genotypes (CYP2C9*1/*1, n = 17; CYP2C9*1/*2, n = 9; and CYP2C9*1/*3, n = 9). Data are presented as geometric mean ± 95% confidence interval. For better visualization, some data points were displaced along the x-axis and a break was inserted in the x-axis. The lines are the best linear fit for the data points in the elimination phase (48-240 hours).
piroxicam (20 mg orally), and the estimated pharmacokinetic parameters after the seventh dose are shown in Table 1. The data suggest that there were significant differences (ANOVA) across the 3 genotypes in relation to Cmin (P = .006), Cmax (P = .021), and AUC in the 24-hour period after the seventh dose (AUC144-168) (P = .011). Pairwise comparisons (Student-Newman-Keuls) revealed no significant differences between CYP2C9*1/*2 and CYP2C9*1/*3 genotypes (P = .87 for Cmin, P = .97 for Cmax, and P = .91 for AUC144-168) for any of these estimated pharmacokinetic parameters. In contrast, CYP2C9*1/*2 and CYP2C9*1/*3 were associated with significantly greater Cmin (P = .03 and P = .03, respectively), Cmax (P = .05 and P = .05, respectively), and AUC144-168 (P = .04 and P = .04, respectively) values than those in the homozygous wild-type individuals.

**Pharmacodynamic differences across CYP2C9 genotypes.** The baseline TXB2 concentration (93.4 ± 46.7 ng/mL) for the individuals enrolled in the pharmacodynamic study was within the range reported in the literature, and the piroxicam-induced inhibition of TXB2 generation followed an erratic time course (Fig 3), consistent with previous observations with other NSAIDs.11,13 As shown in Table II, there was a significant difference (ANOVA) across the 3 genotypes in relation to the effect of piroxicam on TXB2 AUC (P = .0001) but not in relation to TXB2 Cmin (P = .32). In pairwise comparisons, no difference in TXB2 AUC was detected between CYP2C9*1/*2 and CYP2C9*1/*3 (P = .76 for Cmin and P = .43 for AUC), but both genotypes were associated with significantly greater TXB2 AUC (P = .0003 and .0003, respectively) values than those in the homozygous wild-type individuals. Despite these significant differences, the range of individual values for TXB2 AUC across the 3 genotypes represented in the study (Fig 4) overlapped to some extent. The degree of variability (r²) in TXB2 AUC that could be explained by the CYP2C9 genotype was 0.78 (Table II).

**Table I. CYP2C9 genotypes and piroxicam pharmacokinetic parameters**

<table>
<thead>
<tr>
<th>Variable</th>
<th>CYP2C9 genotypes</th>
<th>ANOVA</th>
<th>P value</th>
<th>r²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>*1/*1 (n = 17)</td>
<td>*1/*2 (n = 9)</td>
<td>*1/*3 (n = 9)</td>
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<tr>
<td>Single oral dose</td>
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<tr>
<td>Cmax (µg · mL⁻¹)</td>
<td>2.5 ± 0.7</td>
<td>2.2 ± 0.5</td>
<td>2.4 ± 0.4</td>
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<tr>
<td>AUC₁₄₄₋₁₆₈ (µg · mL⁻¹ · h)</td>
<td>154 ± 37</td>
<td>256 ± 97‡</td>
<td>259 ± 95‡</td>
<td>.0001</td>
</tr>
<tr>
<td>CL/Fcor (mL · h⁻¹ · kg⁻¹)</td>
<td>2.0 ± 0.5</td>
<td>1.3 ± 0.4‡</td>
<td>1.3 ± 0.4‡</td>
<td>.0001</td>
</tr>
<tr>
<td>Simulated 7 repeated daily doses</td>
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</tr>
<tr>
<td>Cmin (µg · mL⁻¹)</td>
<td>4.6 ± 1.3</td>
<td>6.4 ± 1.7‡</td>
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<tr>
<td>Cmax (µg · mL⁻¹)</td>
<td>6.5 ± 1.6</td>
<td>8.3 ± 2.1‡</td>
<td>8.3 ± 1.7‡</td>
<td>.021</td>
</tr>
<tr>
<td>AUC₁₄₄₋₁₆₈ (µg · mL⁻¹ · h)</td>
<td>138 ± 36</td>
<td>183 ± 49‡</td>
<td>181 ± 38‡</td>
<td>.011</td>
</tr>
</tbody>
</table>

Data are given as mean ± SD.

r², Interindividual variability explained by CYP2C9 genotype; Cmax, maximal plasma concentration; AUC₀₋₃, area under plasma concentration-time curve from time 0 to infinity; CL/Fcor, oral clearance corrected for body weight; Cmin, trough plasma concentration; AUC₁₄₄₋₁₆₈, area under plasma concentration-time curve from 144 to 168 hours (24-hour period after seventh dose).

†ANOVA performed on log-transformed values.

‡P < .05, versus CYP2C9*1/*1 (Student-Newman-Keuls test).

**Fig 2.** Individual values of oral clearance of piroxicam corrected for body weight (CL/Fcor) in 35 healthy subjects expressing different CYP2C9 genotypes (CYP2C9*1/*1, n = 17; CYP2C9*1/*2, n = 9; and CYP2C9*1/*3, n = 9). The horizontal bars are the median for each CYP2C9 genotype. Individuals having 1 or 2 copies of the CYP2C9*3 allele are identified by gray and black circles, respectively. P values are for pairwise comparisons between CYP2C9*1/*1 and either CYP2C9*1/*2 or CYP2C9*1/*3 by use of the Student-Newman-Keuls test. NS, Nonsignificant.
Fig 3. Effect of piroxicam (20 mg orally) on serum concentration-time curves of thromboxane B2 (TXB2) in 19 healthy subjects expressing different CYP2C9 genotypes (CYP2C9*1/*1, n = 8; CYP2C9*1/*2, n = 6; and CYP2C9*1/*3, n = 5). Data are normalized to the baseline serum TXB2 concentration and presented as mean ± SEM.

**Influence of CYP2C8*3 allele.** The suggestion that the impairment of the metabolism of the NSAID ibuprofen in carriers of CYP2C9*2 was actually a result of the presence of the variant CYP2C8*3 allele among these individuals led us to explore whether a similar explanation could be extended to our piroxicam data. Genotyping for CYP2C8*3 confirmed the strong linkage disequilibrium between the CYP2C9*2 and CYP2C8*3 alleles previously reported in other populations. Thus all 9 subjects with the CYP2C9*1/*2 genotype were also carriers of CYP2C8*3, 7 being CYP2C8*1/*3 heterozygous and 2 CYP2C8*3/*3 homozygous. In contrast, CYP2C8*3 was detected in only 1 of the 9 CYP2C9*1/*3 individuals and in none of the 17 CYP2C9*1 homozygous individuals. The strong linkage disequilibrium between CYP2C9*2 and CYP2C8*3 in the subjects in this study forestalled attempts to distinguish which allele was the true cause of impairment of piroxicam’s metabolism in individuals with the CYP2C9*1/*2 genotype.

**DISCUSSION**

This study revealed that the genotypes CYP2C9*1/*2 and CYP2C9*1/*3 are associated with the pharmacokinetics and pharmacodynamics of piroxicam. The significant reduction of piroxicam’s oral clearance in individuals with either genotype most likely accounts for the observed increase in AUC0-t, whereas the lack of effect of the CYP2C9*2 and *3 polymorphisms on Cmax can be explained by the much faster rate of absorption of piroxicam relative to its elimination. A similar interpretation has been advanced for the influence of CYP2C9 polymorphisms on the pharmacokinetics of tenoxicam. CYP2C9 is the major pathway for in vivo disposition of the NSAID oxicams, and the in vitro clearance of piroxicam is reduced up to 30-fold by the CYP2C9*3 isofrom relative to CYP2C9*1. These observations provide a pharmacogenetic mechanistic interpretation for the reduced CL/Fc and the increased AUC0-t for single doses of piroxicam in individuals heterozygous for CYP2C9*3. The same interpretation might apply to individuals heterozygous for CYP2C9*2; however, to our knowledge, there are no in vitro kinetic data for the catalysis of piroxicam by the CYP2C9*2 isofrom.

The greater AUC0-t of piroxicam in individuals with the CYP2C9*1/*2 and CYP2C9*1/*3 genotypes provides a reasonable explanation for the increased TXB2 AUC in these heterozygous individuals, relative to wild-type CYP2C9*1 homozygous individuals, whereas the lack of influence of the CYP2C9 genotype on piroxicam’s Cmax is mirrored by the absence of significant differences in TXB2 Cmin across the 3 genotypes represented in this study. The ex vivo production of TXB2 is a surrogate marker for the activity of COX-1, a major target for the adverse gastrointestinal effects of NSAIDs. If it is assumed that the pharmacokinetic-pharmacodynamic correlations observed for single doses prevail during repeated dosing and that piroxicam does not induce CYP2C9 (for which there is no evidence), our pharmacokinetic simulations suggest that individuals with CYP2C9*1/*2 or CYP2C9*1/*3 genotypes are at higher risk for adverse drug effects associated with inhibition of COX-1 activity when repeatedly exposed to piroxicam, as in the clinical treatment of inflammatory conditions (as discussed later).

The observation of significant effects of both CYP2C9*1/*2 and CYP2C9*1/*3 genotypes on the pharmacokinetics of piroxicam is consistent with data on the in vivo CYP2C9-mediated clearance of the NSAID tenoxicam; the S-enantiomers of the anticoagulants warfarin, acenocoumarol, and phenprocoumon; and the hypoglycemic drug tolbutamide, and in most cases the effects of allele *3 on drug clearance were more marked than those of allele *2. In contrast, neither variant allele affected the pharmacokinetics of diclofenac, whereas the in vivo oral clearance of other CYP2C9 substrates (for example, the NSAIDs celecoxib, flurbiprofen, and ibuprofen, the statin fluvastatin; and the oral hypoglycemic agents glyburide (INN, glibenclamide) and nateglinide) is significantly reduced by CYP2C9*3 but not CYP2C9*2. The pharmacokinetic changes associated with the variant CYP2C9 alleles *2 and *3 do not
always result in clinically relevant pharmacodynamic differences. For example, CYP2C9 genotype had a minor influence or no influence on the effects of glyburide and nateglinide on the plasma glucose, glucagon, or insulin levels, whereas conflicting results were reported for tolbutamide. In contrast, the impact of CYP2C9 genotype on the disposition of coumarin anticoagulants is accompanied by pharmacodynamic effects, whether assessed by the induced international normalized ratio changes, by the dose requirement to achieve and maintain a target international normalized ratio, or by the risk of overanticoagulation. Our study is the first to show that both alleles CYP2C9*2 and *3 have a significant impact on a major pharmacodynamic target for NSAIDs, namely, COX-1 activity. The clinical relevance of this observation must take into account several factors, as follows: First is the overlap of the individual values of pharmacokinetic (AUC₀₋₂₄₀ and CL/F) and pharmacodynamic (TXB₂ AUC) parameters across the genotypes investigated. Accordingly, the CYP2C9 genotype accounted for 43% and 78% of the variability detected in these pharmacokinetic and pharmacodynamic parameters, respectively. For comparison, the CYP2C9 genotype accounted for 77% to 80% of the variability in the clearance of tolbutamide, a hypoglycemic drug that has been proposed as a probe for CYP2C9 polymorphisms. Second, there is the possibility of a CYP2C9 genotype–related effect of NSAIDs’ pharmacodynamics in addition to its influence on their catabolism. Third, the fact that individuals having the rare CYP2C9 *2/*2, *2/*3, and *3/*3 genotypes, each occurring in less than 1% of the Brazilian population, were not represented limits the scope of our observations.

Our results confirm, for this sample of Brazilians, the gene linkage between CYP2C9*2 and CYP2C8*3, which was previously reported for European populations. On the basis of pharmacokinetic results with ibuprofen, Garcia-Martín et al suggested that the presence of CYP2C8*3 is the true cause for impairment in ibuprofen metabolism among carriers of CYP2C9*2. This possibility cannot be excluded by our data, because CYP2C8*3 occurred in all CYP2C9*2 carriers and in none of the CYP2C9*1/*1 individuals. However, in subcellular preparations of the human liver, the hydroxylation of oxicams was observed to follow simple Michaelis-Menten kinetics, compatible with catalysis by a single enzyme, and piroxicam has been shown to be a substrate for purified CYP2C9. Collectively, these observations point to CYP2C9 as providing the major pathway for piroxicam catabolism and favor the notion that CYP2C9*2 is responsible for the impairment in piroxicam clearance that we observed in carriers of both CYP2C9*2 and CYP2C8*3. A definite answer to this question, however, requires additional in vivo studies.

Table II. CYP2C9 genotypes and piroxicam pharmacodynamic parameters

<table>
<thead>
<tr>
<th>Variable</th>
<th>CYP2C9 genotypes</th>
<th>ANOVA</th>
<th>P value†</th>
<th>r²</th>
</tr>
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<tr>
<td>TXB₂, Cmin (% of baseline)</td>
<td>*1/*1 (n = 8)</td>
<td></td>
<td>0.32</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td>*1/*2 (n = 6)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>*1/*3 (n = 5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TXB₂, AUC (% of baseline · h)</td>
<td>*1/*1 (n = 8)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>*1/*2 (n = 6)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>*1/*3 (n = 5)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data are given as mean ± SD.

TXB₂, Thromboxane B₂; Cmin, trough serum concentration; AUC, area above time-effect curve from 0 to 240 hours.

†ANOVA performed on log-transformed values.

‡P < .05, versus CYP2C9*1/*1 (Student-Newman-Keuls test).
vitro kinetic studies in systems expressing either CYP2C9*2 or CYP2C8*3.

We thank Vera Lucia Buy for help in recruiting the subjects and Fábio Schneider Ribeiro and Taline Ramos Conde for technical assistance.

There are no known conflicts of interest associated with this work.

References


CYP2C9, but not CYP2C19, polymorphisms affect the pharmacokinetics and pharmacodynamics of glyburide in Chinese subjects

*Background:* Although cytochrome P450 (CYP) 2C9 was thought to be the main pathway for glyburide (INN, glibenclamide) metabolism in vivo, studies in vitro indicated that CYP2C19 had a more dominant effect. This study investigated the relative influence of CYP2C9 and CYP2C19 genotypes on the pharmacokinetics and pharmacodynamics of glyburide in Chinese subjects.

*Methods:* Three groups of healthy male Chinese subjects (n = 6 per group) were enrolled, as follows: group I, CYP2C9*1/*1 and CYP2C19 extensive metabolizers (EMs); group II, CYP2C9*1/*1 and CYP2C19 poor metabolizers (PMs); and group III, CYP2C9*1/*3 and CYP2C19 EMs. Subjects received single oral doses of 5 mg glyburide. Multiple blood samples were collected, and the plasma glyburide concentrations were determined by an HPLC method. The plasma glucose and insulin concentrations were also measured up to 2 hours after dosing.

*Results:* No significant differences in glyburide pharmacokinetics were observed between CYP2C19 EM and PM subjects who had the CYP2C9*1/*1 genotype (group I versus group II). Their respective values for area under the plasma concentration–time curve from time 0 to infinity (AUC₀∞) and elimination half-life (t½) were 0.46 ± 0.13 μg · h/mL versus 0.57 ± 0.11 μg · h/mL (P = .569) and 2.09 ± 0.22 hours versus 2.24 ± 0.27 hours (P = .721). However, significant increases in AUC₀∞ (125% and 82%; P = .008 and .024, respectively) and t½ (71% and 60%; P = .003 and .007, respectively) were observed when CYP2C9*1/*3 subjects (group III) were compared with CYP2C9*1/*1 subjects in group I or II. Blood glucose reductions at 2 hours after dosing were 41.8%, 23.9%, and 27.7% in groups I, II, and III, respectively (P = .029), and hypoglycemia developed in 3 of 6 CYP2C9*1/*3 carriers and 2 of 12 CYP2C9*1/*1 carriers.

*Conclusion:* CYP2C9, but not CYP2C19, polymorphism appears to exert a dominant influence on glyburide pharmacokinetics and pharmacodynamics in vivo. Further studies in diabetic patients with long-term dosing are warranted to confirm these findings. (Clin Pharmacol Ther 2005;78:370-7.)

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Glyburide (INN, glibenclamide) is a second-generation sulfonylurea antidiabetic agent widely used for the treatment of type 2 diabetes mellitus. Glyburide manifests large interindividual variation in its pharmacokinetics and pharmacodynamics. In subjects with normal renal and liver function, the apparent oral clearance of glyburide is reported to range from 11.2 to 230 L/h, with an elimination half-life of 2 to 4 hours. The drug is extensively metabolized in the liver to form its 2 major metabolites, 4-trans-hydroxyglyburide and 3-cis-hydroxyglyburide. Glyburide is thought to be metabolized primarily via cytochrome P450 (CYP) 2C9, on the basis of clinical studies in healthy subjects. However, in recent in vitro studies, glyburide has been found to be a substrate
of CYP2C19 and CYP3A4 in addition to CYP2C9. In one study, as many as 4 metabolites were formed after glyburide was incubated with recombinant human CYP isoforms. However, it is unclear which isozyme (CYP2C9 versus 2C19) is primarily related to the formation of the specific metabolites because their structures were not identified. In another study, which investigated the disappearance of glyburide in microsomes containing recombinant CYP isoforms, CYP2C19 was found to exert a much greater effect as compared with CYP2C9 (19.9% metabolism by CYP2C19 versus 0.2% metabolism by CYP2C9). At present, the in vivo and in vitro data on the specific isozymes responsible for glyburide metabolism are incomplete and inconsistent.

Of the isozymes reported to be involved in glyburide metabolism, genetic polymorphisms are well documented for CYP2C9 and CYP2C19. The frequency of CYP2C9 common mutant variants (*2 and *3) is 6% to 13% in white populations but only 2% to 3% in Asian populations. On the other hand, the CYP2C19 poor metabolizer (PM) phenotype is much more frequent in Asians than in white subjects. The 2 primary mutant alleles, CYP2C19*2 (single base change 681G>A) and CYP2C19*3 (single base change 636G>A), occur in approximately 15% and 0.04% of white subjects and 30% and 5% of Asians, respectively. They account for almost all of the PMs in white (87%) and Asian (>99%) populations.

Although the significant effect of CYP2C9 polymorphism on glyburide metabolism has been documented in the previous clinical studies involving white subjects, the contribution of CYP2C19 in vivo has not been investigated. It is unknown whether there is any interaction between the effects of CYP2C9 and CYP2C19 polymorphisms and how any such interaction may affect the pharmacokinetics and pharmacodynamics of glyburide. In view of the relatively high prevalence of the CYP2C19 PM phenotype in the Asian population, we carried out this study in 18 Chinese subjects with different genotype combinations of CYP2C9 and CYP2C19 to examine this interaction. The purpose of the study was to determine whether different combinations of CYP2C9 and CYP2C19 genotypes in individual subjects affect the pharmacokinetics and pharmacodynamics of glyburide differently and whether there is one isozyme that phenotypically exerts a dominant effect.

METHODS

Subjects. The human study protocol was approved by the local Clinical Research Ethics Committee. Written informed consent was obtained from each subject before participation in the study.

Eighteen healthy male Chinese subjects (age range, 21-26 years; weight range, 52.6-70.6 kg) were recruited and divided into 3 groups (n = 6 in each group), as follows: group I, CYP2C9*1/*1 and CYP2C19 extensive metabolizers (EMs) (CYP2C19*1/*1, *1/*2, or *1/*3); group II, CYP2C9*1/*1 and CYP2C19 PMs (CYP2C19*2/*2 or *2/*3); and group III, CYP2C9*1/*3 and CYP2C19 EMs (CYP2C19*1/*1, *1/*2, or *1/*3). The mean ages (±SD) of subjects in groups I, II, and III were 22.0 ± 0.6, 21.5 ± 0.6, and 21.5 ± 1.4 years, respectively, and the mean body weights were 62.4 ± 2.0, 66.4 ± 7.6, and 64.6 ± 2.4 kg, respectively.

The subjects were all nonsmokers and in good health as determined from their medical history, physical examination, electrocardiographic evaluation, and routine laboratory test results (blood chemical evaluation, hemato logic testing, and urinalysis). All subjects were required not to take any prescription or nonprescription medication 2 weeks before and throughout the study. They were instructed to abstain from grapefruit, grapefruit juice, herbal dietary supplements, and caffeine-containing beverages including coffee and green tea 3 days before the study and during the study period.

Genotyping of CYP2C9 and CYP2C19. A 10-mL blood sample was obtained from each subject, and deoxyribonucleic acid was isolated by use of an extraction kit (QIAamp Blood Mini Kit; Qiagen, Hilden, Germany). The CYP2C9 wild type (*1) and mutant alleles CYP2C9*2 (Arg144Cys) and CYP2C9*3 (Ile359Leu) were determined by a polymerase chain reaction–restriction fragment length polymorphism method, as described previously. The CYP2C19 wild type (*1) and the 2 mutant alleles, CYP2C19*2 in exon 5 and CYP2C19*3 in exon 4, were identified by polymerase chain reaction amplification with allele-specific primers as described by de Morais et al. followed by digestion with Smal and BamHI restriction enzymes, respectively.

Study design. After an overnight fast, each subject received a single oral dose of 5 mg glyburide (one 5-mg Daonil tablet; Aventis Pharma, Frankfurt am Main, Germany) with 240 mL of water. Standardized meals were served 3 and 10 hours after dosing. Venous blood samples were collected before dosing (0 hours) and at 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 10, and 12 hours after dosing. All blood samples were collected in lithium heparin tubes and centrifuged immediately. The separated plasma samples were stored at −80°C until analysis.
For safety monitoring, blood glucose concentrations were measured immediately at each blood sampling time point, by use of an Advantage Blood Glucose Meter (Roche, Basel, Switzerland). Before each measurement, standard calibration and quality-control tests were carried out to ensure the accuracy of measurement. In subjects who had hypoglycemic symptoms (ie, sweating, dizziness, faintness, palpitation, nausea, or anxiety) and a blood glucose concentration lower than 3 mmol/L (54 mg/dL), 10 g of oral glucose was administered. To further evaluate the pharmacodynamic effect of glyburide, plasma insulin concentrations were also determined by use of a radioimmunoassay with an enzyme-linked immunosorbent assay kit, which has no cross-reactivity with C peptide and proinsulin.

**Determination of glyburide concentration in plasma.** Plasma concentrations of glyburide were determined by a previously described HPLC method, with slight modification. In brief, to each 1 mL of plasma sample, 25 µL of internal standard solution (20 µg/mL diclofenac), 100 µL of 2-mol/L hydrochloric acid, and 6 mL of dichloromethane were added. After mixing, each sample was centrifuged at 2500 rpm for 10 minutes. The organic layer was transferred to a new tube and evaporated to dryness under a stream of nitrogen at room temperature. The residue was reconstituted with 100 µL of methanol, and 60 µL was injected for HPLC analysis.

The HPLC system consisted of a Waters Alliance 2690 separation module, Millennium chromatography management system, and Waters 996 photodiode array detector (Waters, Milford, Mass). The analytic column was a Waters Spherisorb ODS C18 column (5 µm, 250 × 4.6 mm). A mixture of 0.04-mol/L dihydrogen sodium phosphate buffer (pH 7.4) and acetonitrile (68:32 [vol/vol]) was used as the mobile phase, eluted at a flow rate of 0.7 mL/min. The eluate was monitored at an ultraviolet wavelength of 211 nm.

The intraday and interday coefficients of variation of the assay were 2.9% to 10.7% and 5.7% to 13.1%, respectively. The accuracy of the assay ranged from 89.7% to 101.0%. The lower limit of detection was 10 ng/mL for glyburide.

**Pharmacokinetic-pharmacodynamic data analysis.** The pharmacokinetic parameters of glyburide were calculated via the noncompartmental method, with the aid of the WinNonlin program (version 2.1; Pharsight, Mountain View, Calif). The peak plasma concentration (Cmax) of glyburide was obtained directly from the observed concentration-time data. The terminal elimination rate constant (λz) was estimated by linear regression of the terminal portion of the concentration-time curve, and the elimination half-life (t1/2) was calculated as 0.693/λz. The area under the plasma concentration–time curve (AUC) was calculated by use of the linear trapezoidal rule and extrapolated to infinity (AUC0-∞). The apparent oral clearance (CL/F) of glyburide was calculated as Dose/AUC.

To compare the pharmacodynamic effects of glyburide in subjects with different genotypes, changes in blood glucose and plasma insulin concentrations over time were calculated by subtracting their baseline values from the observed values after dosing. The decremental area under the glucose concentration–time curve (AUECglyburide, presented as a positive value) and the incremental area under the insulin concentration–time curve (AUECinsulin) were calculated by use of the linear trapezoidal rule, in a similar manner as described earlier for AUC.

**Statistical analysis.** Calculation of the sample size was initially based on the variability of glyburide oral clearance reported in the literature: With a coefficient of variation of 20% assumed, a sample size of 6 per group would be able to detect a 30% difference in glyburide clearance, with a power of 80% and α level of .05.

All data from the study results were expressed as mean ± SD. The pharmacokinetic parameters of glyburide and the insulin and glucose response to glyburide among the 3 different groups were compared by use of 1-way ANOVA, followed by a post hoc Bonferroni test for multiple comparisons. The relationship of AUECglyburide or AUECinsulin versus AUC was evaluated by use of the Spearman rank correlation coefficient (r_s). P ≤ .05 was considered statistically significant for all tests. All analyses were performed with SPSS software (version 11.5; SPSS, Chicago, Ill).

**RESULTS**

All 18 subjects completed the study. Their genetic makeup consisted of 5 different combinations of CYP2C9 and CYP2C19 genotypes (Figure 1). Large overlaps were observed in the glyburide oral clearance values among subjects with CYP2C19 homozygous and heterozygous EM genotypes. The mean clearance was 2.97 ± 0.89 mL·min⁻¹·kg⁻¹ versus 3.00 ± 1.16 mL·min⁻¹·kg⁻¹ (P = .979) for CYP2C9*1/*1 and CYP2C19 homozygous EMs versus CYP2C9*1/*1 and CYP2C19 heterozygous EMs and 1.45 ± 0.63 mL·min⁻¹·kg⁻¹ versus 1.54 ± 0.71 mL·min⁻¹·kg⁻¹ (P = .868) for CYP2C9*1/*3 and CYP2C19 homozygous EMs versus CYP2C9*1/*3 and CYP2C19 heterozygous EMs. Because the clearance values between the CYP2C19 homozygous and
heterozygous EMs were similar, we combined these 2 genotypes into 1 group to simplify subsequent data presentation and analysis. (Thus all of the subjects were divided into 3 major genotype groups for comparison.)

The mean plasma glyburide concentration–time profiles of different CYP2C9 and CYP2C19 genotype groups are shown in Figure 2, with the corresponding pharmacokinetic parameters shown in Table I. When CYP2C19 EM and PM subjects who had the CYP2C9*1/*1 genotype (group I versus group II) were compared, the plasma concentrations and pharmacokinetic parameters of glyburide did not differ significantly. The mean values for $C_{\text{max}}$ and $AUC_{0-\infty}$ of glyburide were $0.12 \pm 0.05 \mu g/mL$ versus $0.13 \pm 0.03 \mu g/mL$ ($P = .850$) and $0.46 \pm 0.13 \mu g \cdot h/mL$ versus $0.57 \pm 0.11 \mu g \cdot h/mL$ ($P = .569$), respectively. The terminal elimination half-life ($t_{1/2}$) of glyburide was also similar, which averaged $2.09 \pm 0.22$ and $2.24 \pm 0.27$ hours ($P = .721$) in the respective groups.

When group III (CYP2C9*1/*3 and CYP2C19 EMs) was compared with group I (CYP2C9*1/*1 and CYP2C19 EMs) or group II (CYP2C9*1/*1 and CYP2C19 PMs), the $AUC_{0-\infty}$ in group III was significantly higher (by 124.7% and 81.9% [$P = .008$ and .024], respectively) and the oral clearance was significantly lower (by 49.9% and 43.1% [$P = .007$ and .032], respectively). The $t_{1/2}$ of glyburide was also significantly prolonged in group III subjects when compared with the other 2 groups ($P = .003$ and .007, respectively).

The pharmacodynamic responses (insulin and glucose response) up to 2 hours after glyburide administration among different genotype groups are shown in Table II. (The responses were evaluated only up to 2 hours after dosing because of the development of hypoglycemia, which required oral glucose administration in 5 subjects). The mean reductions in blood glucose level at 2 hours after dosing were similar for CYP2C9 EM and PM subjects who had the CYP2C9*1/*1 genotype (23.9% vs. 11.0% versus 27.7% vs. 8.9% for group I versus II; $P = .561$). Similar increases in plasma insulin concentration from baseline between these 2 groups were also observed (158.1% vs. 129.5% versus 198.9% vs. 132.8%, $P = .612$).

In contrast, the mean reductions in blood glucose level at 2 hours after dosing were 17.9% ($P = .012$) and 14.1% ($P = .040$) greater in the CYP2C9*1/*3 subjects (group III) compared with the CYP2C9*1/*1 subjects (group I or II). The insulin response also tended to be greater in the CYP2C9*1/*3 subjects (group I), but the difference was not statistically significant ($P = .204$), probably because of the large variations in insulin response, as well as the relatively small number of subjects included in each group (Table II).

In an examination of the relationship between glyburide pharmacokinetics and pharmacodynamics, glyburide ex-
posure (AUC0-2) in individual subjects was found to correlate well with the observed response on glucose (AUEC0-2, glucose) and insulin change (AUEC0-2, insulin), with $r_s$ values of 0.85 ($P < .001$) and 0.78 ($P < .001$), respectively.

Significant hypoglycemic reactions (ie, blood glucose concentrations <3 mmol/L with symptoms) occurred in 5 study subjects. They all received oral glucose administration. Of these 5 subjects, 3 belonged to group III (carriers of $CYP2C9^*1/*3$ and $CYP2C19$ EMs), 1 belonged to group I (carriers of $CYP2C9^*1/*1$ and $CYP2C19$ EMs), and 1 belonged to group II (carriers of $CYP2C9^*1/*1$ and $CYP2C19$ PMs). Thus hypoglycemia requiring oral glucose administration developed in 50% of $CYP2C9^*1/*3$ carriers and 17% of $CYP2C9^*1/*1$ carriers.

### DISCUSSION

In this study glyburide oral clearance was found to be significantly decreased and the elimination half-life significantly increased in $CYP2C9^*1/*3$ subjects compared with $CYP2C9^*1/*1$ subjects, whereas these pharmacokinetic parameters were not significantly different between $CYP2C19$ EM and PM subjects. The lack of significance in the pharmacokinetic parameters between $CYP2C19$ EM and PM subjects may be related.

![Fig 2. Mean plasma concentration (Conc)–time profiles of glyburide in different genotype groups.](image)

### Table I. Comparison of pharmacokinetic parameters of glyburide among different CYP2C9 and CYP2C19 genotype groups

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Genotype group</th>
<th>Multiple comparisons</th>
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<tbody>
<tr>
<td></td>
<td>CYP2C9$^*1/*1$ and CYP2C19 EMs (group I, n = 6)</td>
<td>Group II versus group I versus group II</td>
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<tr>
<td></td>
<td>CYP2C9$^*1/*1$ and CYP2C19 PMs (group II, n = 6)</td>
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<tr>
<td></td>
<td>CYP2C9$^*1/*3$ and CYP2C19 EMs (group III, n = 6)</td>
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<tr>
<td>$t_{max}$ (h)</td>
<td>2.75 ± 1.17</td>
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<tr>
<td>$C_{max}$ (ng/mL)</td>
<td>124.29 ± 52.25</td>
<td>$P = .850$ $P = .033$ $P = .047$</td>
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<tr>
<td>AUC$_{0-\infty}$ (ng · h/mL)</td>
<td>457.48 ± 133.65</td>
<td>$P = .569$ $P = .008$ $P = .024$</td>
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<td>$t\frac{1}{2}$ (h)</td>
<td>2.09 ± 0.22</td>
<td>$P = .721$ $P = .003$ $P = .007$</td>
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<tr>
<td>CL/F (mL · min$^{-1}$ · kg$^{-1}$)</td>
<td>2.98 ± 0.93</td>
<td>$P = .466$ $P = .007$ $P = .032$</td>
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</table>

EM, Extensive metabolizers; PM, poor metabolizers; $t_{max}$, time to reach peak plasma concentration; $C_{max}$, peak plasma concentration; AUC$_{0-\infty}$, area under plasma concentration–time curve from time 0 to infinity; $t\frac{1}{2}$, terminal elimination half-life; CL/F, apparent oral clearance.
to the small sample size of our study. Nevertheless, our study sample size (6 per group) would be able to provide 80% power for detecting a clinically significant difference of 30% in glyburide clearance. Our observed large magnitude of change in glyburide clearance (50%) between CYP2C9*1/*3 and CYP2C9*1/*1 subjects compared with the small change (12%) between CYP2C19 PM and EM subjects clearly suggests that the disposition of glyburide is markedly influenced by CYP2C9 but not the CYP2C19 polymorphism in vivo.

The results from this human study on the effect of CYP2C9 and CYP2C19 are in contrast to those from previous in vitro studies. The previous in vitro studies have shown similar hydroxylation activities for drugs that involve multiple CYP isozymes. Because many drugs have been shown to be substrates of both CYP2C9 and CYP2C19 based on in vitro studies, the in vivo relevance of these data requires careful study.

This study further illustrates that a significant relationship of genotypes to pharmacokinetics and pharmacodynamics can be demonstrated involving the polymorphism of the dominant CYP isozymes. Not only is the pharmacokinetics of glyburide markedly influenced by the CYP2C9 polymorphism (Table I), significant correlations of the change in plasma glyburide concentration to glucose or insulin response also exist. Thus these data demonstrate the existence of a gene-specific effect for glyburide and also provide an important example of pharmacogenetics of an antidiabetic agent in a Chinese population.

The significant effects of CYP2C9 genotype on glyburide pharmacokinetics observed in our study are consistent with those reported in previous studies. However, in contrast to our finding, previous studies showed an insignificant or marginal effect of CYP2C9 genotype on blood glucose response. Such differences between our study and previous studies are most likely related to differences in the study protocol. In the previous studies, early administration of food (at 15 minutes after glyburide administration) or oral glucose (at 1, 4.5, and 8 hours after glyburide administration) could have obscured the effect of glyburide on blood glucose. In our study all subjects had fasted for 10 hours before and 3 hours after glyburide administration, and thus the blood glucose response up to 2 hours after dosing should primarily reflect the effect of glyburide. Hence, our
study design allowed us to demonstrate the relationship of genotypes to pharmacokinetics and pharmacodynamics. This relationship has been supported by the good correlation observed between glyburide pharmacokinetics and pharmacodynamics among subjects with different genotypes.

The observed pharmacodynamic effect of CYP2C9 genotypes for glyburide could be clinically important. In this study in healthy subjects, a significantly higher rate of hypoglycemia (50% versus 17%), as well as a greater reduction in blood glucose concentration (at 2 hours), occurred in the CYP2C9*1/*3 subjects as compared with the CYP2C9*1/*1 subjects. The insulin response also tended to be greater in the CYP2C9*1/*3 carriers in comparison to CYP2C9*1/*1 subjects, although the difference was not statistically significant.

Given the small number of subjects in our study, the significance of the earlier observations requires further confirmation in a larger population. In addition, our study was conducted in healthy subjects by use of a single-dose design, so future studies in diabetic patients with long-term dosing will be warranted to verify whether the pharmacokinetics and pharmacodynamics of glyburide are affected by CYP2C9 and not CYP2C19 genotypes in the clinical setting.

Our study did not include homozygous PMs of CYP2C9 (ie, CYP2C9*3/*3 carriers) because of the extremely low prevalence in Chinese populations. In addition, we did not determine the pharmacokinetics and pharmacodynamics of glyburide in CYP2C9*1/*3 or CYP2C9*3/*3 in combination with CYP2C19 PMs. The prevalence of such genotype combinations is very low (we did not find any such carriers in our prestudy genotype screening in 100 subjects).

In this study we did not determine the active metabolites of glyburide (ie, 4-trans-hydroxyglyburide and 3-cis-hydroxyglyburide), which also possess hypoglycemic activity in humans.3 Because these metabolites are eliminated from the body more rapidly with much lower systemic concentration than the parent drug,4 they are expected to contribute only a low or minor pharmacodynamic response, especially in those slow or poor metabolizers of glyburide (CYP2C9*1/*3 or CYP2C9*3/*3).

In conclusion, the CYP2C9 but not the CYP2C19 polymorphism appears to exert a dominant influence on glyburide pharmacokinetics and pharmacodynamics in vivo. Further studies in diabetic patients with long-term dosing are warranted to confirm these findings, especially with regard to the clinical significance, because many patients are currently receiving glyburide therapy throughout the world.

We thank Evelyn Chau, Benny Fok, Ping-chuen Ho, and Emily Poon for their valuable technical assistance in this investigation.

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References

Impact of CYP2D6 genotype on adverse effects during treatment with metoprolol: A prospective clinical study

Objective: Our objective was to study the impact of the cytochrome P450 (CYP) 2D6 polymorphism on the tolerability of metoprolol in a real-life primary care setting. The adverse effects studied comprised effects related to the central nervous system, cardiovascular effects, and sexual dysfunction.

Methods: Patients in whom treatment with metoprolol was considered were enrolled into this prospective, 6-week multicenter study. The dosage of metoprolol was determined on an individual basis and could be freely adjusted on clinical grounds. The indication for treatment was hypertension in about 90% of cases. Systolic and diastolic blood pressure, resting heart rate, and plasma metoprolol and β-hydroxymetoprolol concentrations were measured. CYP2D6 genotyping covered alleles *3 to *10 and *41 and the duplications. Possible adverse effects of metoprolol were systematically assessed over a 6-week period by means of standardized rating scales and questionnaires.

Results: The final study population comprised 121 evaluable patients (all white patients); among them, there were 5 ultrarapid metabolizers (UMs) (4.1%), 91 extensive metabolizers (EMs) (75%), 21 intermediate metabolizers (IMs) (17%), and 4 poor metabolizers (PMs) (3.3%). Plasma metoprolol concentrations normalized for the daily dose and metoprolol/β-hydroxymetoprolol ratios at steady state were markedly influenced by CYP2D6 genotype and displayed a gene-dose effect. The median of the dose-normalized metoprolol concentration was 0.0088 ng/mL, 0.047 ng/mL, 0.34 ng/mL, and 1.34 ng/mL among UMs, EMs, IMs, and PMs, respectively (P < .0001). There was no significant association between CYP2D6 genotype–derived phenotype (EMs and UMs combined versus PMs and IMs combined) and adverse effects during treatment with metoprolol. There was a tendency toward a more frequent occurrence of cold extremities in the PM plus IM group as compared with the EM plus UM group (16.0% versus 4.2%, P = .056; relative risk, 3.8 [95% confidence interval, 1.03-14.3]).

Conclusions: CYP2D6 genotype–derived phenotype was not significantly associated with a propensity for adverse effects to develop during treatment with metoprolol. However, the results concerning tolerability of metoprolol in PMs were inconclusive because of the small number of PMs enrolled. (Clin Pharmacol Ther 2005;78:378-87.)

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Metoprolol is a lipophilic β1-selective adrenergic receptor antagonist that is well established as an effective and well-tolerated drug in various cardiovascular diseases.1-6 Metoprolol undergoes extensive hepatic metabolism and is primarily metabolized by cytochrome P450 (CYP) 2D6.7-9 The metabolites of meto-
propranolol lack clinically relevant β-blocking activity at concentrations observed in humans.\textsuperscript{10}

The \textit{CYP2D6} gene is highly polymorphic; currently, about 80 distinct alleles and allele variants have been described.\textsuperscript{11,12} Four distinct phenotypes are observed among white subjects, which differ substantially in the ability to metabolize the \textit{CYP2D6} probe drugs sparteine and debrisoquin (INN, debrisoquine), as well as other \textit{CYP2D6} substrates. These are referred to as ultrarapid metabolizers (UMs), extensive metabolizers (EMs), intermediate metabolizers (IMs), and poor metabolizers (PMs) and compose about 3% to 5%, 70% to 80%, 10% to 15%, and 7% to 10% of the white population, respectively.\textsuperscript{11-15} A PM phenotype results if both alleles are nonfunctional (null) alleles. Alleles with reduced function (eg, \textit{*10}, \textit{*41}, and \textit{*42}) contribute to the occurrence of IMs.\textsuperscript{11,12,16,17} The IM phenotype is associated with considerably reduced in vivo activity of \textit{CYP2D6} as compared with EMs.\textsuperscript{11,12,17}

\textit{CYP2D6} polymorphism has a major impact on the pharmacokinetics of metoprolol.\textsuperscript{2-9,18} In a recent study median dose-corrected plasma metoprolol concentrations during long-term treatment were 6.2- and 3.9-fold higher in \textit{CYP2D6} PMs and IMs, respectively, as compared with those with 2 fully functional alleles.\textsuperscript{19} The elimination half-life of metoprolol was about 7.5 hours in PMs and 2.8 hours in EMs.\textsuperscript{7}

Only a few studies have addressed the question of whether the elevated plasma metoprolol concentrations observed in PMs (and IMs) are associated with an increased occurrence of adverse effects, and the results are conflicting. In a retrospective study Wuttke et al\textsuperscript{20} found a significantly increased frequency of \textit{CYP2D6} PMs (38%) among 24 patients with metoprolol-associated adverse effects necessitating discontinuation of therapy or dosage reduction. In contrast, an early case-control study comparing patients who discontinued treatment with metoprolol because of adverse effects with a group of control subjects did not support a causal relationship between \textit{CYP2D6} phenotype and tolerability of metoprolol.\textsuperscript{21} However, this study had certain methodologic limitations; for example, it may have missed early adverse effects.\textsuperscript{21} IMs were not considered in these 2 studies. In the recent study of Zineh et al,\textsuperscript{22} \textit{CYP2D6} genotypes did not predict metoprolol adverse events among 50 hypertensive patients, including white, black, and Latino-Hispanic subjects.

This prospective, observational clinical study was carried out to investigate the consequences of the \textit{CYP2D6} polymorphism on adverse effects of metoprolol in outpatients during the first 6 weeks of treatment. The assessed adverse effects comprised central nervous system (CNS)–related effects, cardiovascular effects, and sexual dysfunction. This study was larger than the study of Zineh et al\textsuperscript{22} and comprised only white subjects. In our study adverse effects were examined in a standardized fashion, whereas Zineh et al relied on self-reporting of adverse events based on open-ended patient interviews. Zineh et al titrated the metoprolol dose upward according to a uniform dose-escalation protocol until the maximal daily dose was achieved, the diastolic blood pressure was reduced to less than 90 mm Hg, or adverse effects precluded an increase in the dose. A protocol for dose titration of metoprolol was not used in our study, which was carried out under real-life conditions.

\textbf{METHODS}

\textit{Study design and study population.} Adult patients in whom treatment with metoprolol was about to be started were enrolled into this multicenter trial by general practitioners, interns, or neurologists working in the primary care setting in Germany.\textsuperscript{23} Altogether 39 physicians working in the regions of Tübingen, Reutlingen, and Stuttgart recruited patients for this study. Pregnant women, patients with contraindications for β-blockers, patients having taken a β-blocker within the previous 4 weeks, and patients taking drugs that inhibit \textit{CYP2D6} were not included. The study protocol allowed any daily dose, any dosage scheme, and any preparation of metoprolol. Metoprolol dosage could be freely adjusted on clinical grounds. The patients were instructed to record intake of each metoprolol dose in the patient diary, together with the brand name and strength of the preparation, as well as the number of tablets taken on each study day. All patients gave written informed consent to participate in the study, and the study protocol was approved by the ethical review board of the Medical Faculty of the University of Tübingen (Tübingen, Germany).

Study day 1 was defined as the day when metoprolol was started. Pretreatment (baseline) measurements were obtained on a visit that preceded study day 1 and, occasionally, on study day 1 before intake of the first metoprolol dose. Blood pressure and resting heart rate were determined in a sitting position after a resting time of at least 5 minutes, and a blood sample for \textit{CYP2D6} genotyping and determination of plasma metoprolol and α-hydroxymetoprolol concentrations was obtained on a visit that could take place on any day between study days 4 and 42.

\textit{Collection of data on adverse effects.} During the pretreatment visit, blood pressure and resting heart rate were measured, and every patient was provided with a
diary containing rating scales and questionnaires about symptoms that represent possible adverse effects of metoprolol. The adverse drug reactions (ADRs) that were systematically assessed included headache, dizziness, fatigue or drowsiness, sleep disturbances (eg, nightmares), dyspnea, and cold extremities. Each of these symptoms was evaluated at baseline (before metoprolol treatment) and again in the evenings of study days 2, 4, 7, 14, 21, 28, 35, and 42 by use of a standardized, ordinal rating scale that ranged from a score of 0 (no effect) to 10 (very strong effect) at intervals of 1. The baseline (pretreatment) entries were made by the patient in the presence and under the guidance of his or her physician. Sexual dysfunction was characterized according to various dimensions of sexual function (libido, frequency of coitus, arousability, erection or lubrication, orgasm) by means of a gender-specific questionnaire consisting of 5 standardized questions. These questionnaires, filled in by the patients at the same time as the rating scales for the other ADRs, represented short versions of the International Index of Erectile Function questionnaire for men and the Female Sexual Function Index questionnaire for women. Each question was rated from 0 to 3 points, and thus the maximum score for the whole questionnaire for both sexes was 15 points (corresponding to an active and satisfactory sex life).

**Determination of metoprolol and α-hydroxymetoprolol concentrations and metabolic ratio.** Trough plasma concentrations of metoprolol and α-hydroxymetoprolol, a CYP2D6-dependent metabolite, were determined at steady state by reversed-phase HPLC, as described previously. The limit of quantification was 0.3 ng/mL for both compounds, and the intra-assay and inter-assay coefficients of variation were less than 10% at relevant concentrations.

The metabolic ratio was defined as the ratio between the plasma concentrations of metoprolol and α-hydroxymetoprolol. The metabolic ratio was considered valid if the following conditions were met: (1) the intake of metoprolol was documented on the day before blood sampling, (2) the dosage was constant for at least 2 days before blood sampling (to allow for attaining steady state also in PMs), and (3) the blood sample was drawn before the first metoprolol dose of the day.

**CYP2D6 genotype and genotype-derived phenotype assignment.** Deoxyribonucleic acid was isolated from ethylenediaminetetraacetic acid–blood by means of a whole-blood deoxyribonucleic acid extraction kit as specified by the manufacturer (Qiagen, Hilden, Germany). CYP2D6 genotyping detected the nonfunctional alleles *3, *4, *5, *6, *7, and *8 and the alleles *9, *10, and *41 associated with reduced CYP2D6 activity. Subjects were also assessed for the presence of gene duplications. If no sequence variations were detected, the allele assignment defaulted to *1 or *2 (fully functional CYP2D6 alleles). Genotyping for the alleles *3, *4, *6 to *8, and *10 was performed by use of predeveloped TaqMan Assay-Reagents Allelic Discrimination Kits (Applied Biosystems, Foster City, Calif). TaqMan assays established and validated in our laboratory were used for genotyping of *9 and *41. The CYP2D6*5 allele and gene duplications were determined as previously described. Individuals were classified as PMs if they carried any 2 of the nonfunctional alleles. Individuals were classified as IMs if they carried any 2 of the alleles *9, *10, and *41 or 1 of these alleles and 1 nonfunctional allele. Subjects carrying more than 2 CYP2D6 gene copies were classified as UMs. The remaining subjects were classified as EMs.

**Statistics.** For each patient and a particular ADR, the Wilcoxon signed rank test was used to determine whether an ADR occurred during the 6-week study period. These analyses were carried out by a person who had no knowledge about plasma metoprolol concentrations or CYP2D6 genotypes. To examine the association between CYP2D6 polymorphism and the occurrence (frequency) of an ADR, PMs and IMs were combined, because the number of PMs was small (n = 4). Likewise, the 5 UMs were included in the EM group. These analyses were performed by the Fisher exact test. For the analysis of sexual function data, the same statistical methods were used, but a valid phenotype was not required (except that use of CYP2D6 inhibitors was not allowed) and those with no sexual activity could not be included. Thus, whereas the basic study population with a valid and sufficient phenotype and ADR data comprised 121 patients, 89 patients were evaluable for sexual function.

One-way ANOVA followed by a posteriori testing with the Tukey test or the Kruskal-Wallis test followed by a posteriori testing with the Dunn multiple comparisons test, unpaired t test or Mann-Whitney test, and paired t test or Wilcoxon paired sample test (all 2-tailed) was used, as appropriate, for analysis of pharmacokinetic and hemodynamic data. All data were analyzed with GraphPad Instat, version 3.05 (GraphPad Software, San Diego, Calif).

**RESULTS**

Initially, 184 patients were enrolled into the study. Of these patients, 63 had to be excluded from data analyses because one of the following conditions was present: (1) withdrawn informed consent (n = 4), (2)
protocol violation (eg, noncompliance or wrong timing of blood sampling) or missing information that invalidated determination of phenotype (n = 28), (3) missing or incomplete patient diary (n = 12), or (4) concomitant use of a CYP2D6 inhibitor (paroxetine; n = 2). In addition, all 17 patients recruited by 1 physician were excluded on the grounds of suspected data fabrication. Our suspicions were aroused when this physician recruited patients at a very rapid rate and the handwriting on several of the patient diaries from her patients appeared identical. Furthermore, some of the diaries contained future dates. These suspicions were confirmed when the study coordinator (R.F.) contacted the patients, who did not know that they had been “enrolled” into the study. Careful evaluation of the data from the other investigators did not raise any such concerns. As a precaution, most of the patients from each investigator were contacted. All of them knew that they were participating in the study and had been adequately informed about it. There were no early discontinuations because of adverse effects, but 2 subjects discontinued taking metoprolol after 3 weeks and 1 subject discontinued it after 4 weeks (all EMs), reporting that they did not tolerate it. The final study population consisted of 121 white patients (57 men and 64 women) with a mean age (± SD) of 51.5 ± 13.5 years (Table I). Indications for treatment comprised hypertension (n = 111), arrhythmias (n = 7), coronary heart disease (n = 3), migraine (n = 6), and anxiety disorder (n = 1) (some patients had >1 indication). About half of the patients took a slow-release metoprolol preparation (eg, controlled release/extended release [CR/XL]), and about half took a normal-release preparation. Twenty-seven of the patients were treated with at least 1 other cardiovascular drug (most frequently an angiotensin-converting enzyme inhibitor or a diuretic).

CYP2D6 genotyping detected 4 PMs (3.3%), 21 IMs (17%), 91 EMs (75%), and 5 UMs (4.1%) among this white study population. All UMs were subjects carrying 3 CYP2D6 gene copies. The observed genotype frequencies were in Hardy-Weinberg equilibrium, with the exception of the CYP2D6*41 allele, for which the number of homozygous subjects was greater than expected (6 versus 2.4). Of note, our genotyping method for *41 has been thoroughly validated, including a sequencing strategy.17 Actual plasma metoprolol concentrations, metoprolol concentrations normalized for the corresponding daily dose, and metabolic ratios (metoprolol/α-hydroxymetoprolol), all determined in steady-state conditions, were markedly affected by the CYP2D6 genotype in a manner that was consistent with a gene-dose effect (Table II and Figs 1 and 2). Regarding the overlap seen in dose-normalized plasma metoprolol concentrations between the EM and PM groups (Fig 2), it should be noted that none of the subjects among the evaluable patient population (n = 121) was taking a CYP2D6-inhibiting drug, excluding the possibility of phenocopying. As for the metabolic ratio, the PM group did not overlap with any other phenotype group (Fig 1). The median plasma metoprolol concentration was 1.27 ng/mL, 4.28 ng/mL (interquartile range, 1.88-17.0 ng/mL), 33.3 ng/mL (interquartile range, 9.64-142 ng/mL), and 80.5 ng/mL in the UMs, EMs, IMs, and PMs, respectively (P < .0001). In the PM plus IM group, the median metoprolol concentration was 16.9-fold higher than in the EM plus UM group (67.7 ng/mL versus 4.01 ng/mL, P < .0001). The median dose-normalized metoprolol concentration was 0.0088 ng/mL, 0.047 ng/mL (interquartile range, 0.024-0.15 ng/mL), 0.34 ng/mL (interquartile range, 0.26-1.58 ng/mL), and 1.34 ng/mL among UMs, EMs, IMs, and PMs, respectively (P < .0001).

The frequencies of the studied ADRs in relation to CYP2D6 genotype–derived phenotype are given in Table III. The frequency of patients in whom headache, dizziness, fatigue or drowsiness, sleep disturbances, dyspnea, or cold extremities developed during this 6-week trial was not significantly different between the PM plus IM group and the EM plus UM group. However, there was a tendency toward a more frequent occurrence of cold extremities in the PM plus IM group as compared with the EM plus UM group (16.0% versus 4.2%, P = .056; relative risk, 3.8 [95% confidence interval, 1.03-14.3]). Impaired sexual function developed during metoprolol treatment in 23 (25.8%, 8 women and 15 men) of the 89 evaluable patients (41 women and 48 men). Among

<table>
<thead>
<tr>
<th>Table I. Characteristics of patients</th>
<th>PMs plus IMs (n = 25)</th>
<th>EMs plus UM (n = 96)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y) (mean ± SD)</td>
<td>51.4 ± 13.9</td>
<td>51.5 ± 13.5</td>
</tr>
<tr>
<td>Men</td>
<td>7 (28.0%)</td>
<td>50 (52.1%)</td>
</tr>
<tr>
<td>Women</td>
<td>18 (72.0%)</td>
<td>46 (47.9%)</td>
</tr>
<tr>
<td>Concomitant use of other antihypertensives</td>
<td>3 (12.0%)</td>
<td>24 (25.0%)</td>
</tr>
</tbody>
</table>

PM, Poor metabolizer; IM, intermediate metabolizer; EM, extensive metabolizer; UM, ultrarapid metabolizer.
the affected cases were no PMs, 1 IM, 21 EMs, and 1 UM. Sexual dysfunction was more frequent among EMs and UMs than among the PM plus IM group (31.9% versus 5.0%, \( P < .05 \)).

Metoprolol treatment was effective in reducing systolic and diastolic blood pressure and resting heart rate, except that it did not seem to affect heart rate in the UMs (Table IV). The mean reduction (±SD) in systolic blood pressure was 16.6 ± 22.3 mm Hg, 19.5 ± 20.1 mm Hg, 17.9 ± 18.3 mm Hg, and 24.5 ± 17.1 mm Hg and that in diastolic blood pressure was 16.6 ± 18.0 mm Hg, 11.4 ± 14.2 mm Hg, 12.0 ± 11.3 mm Hg, and 12.5 ± 11.9 mm Hg in UMs, EMs, IMs, and PMs, respectively, with no significant differences between the phenotypes (\( P = .92 \) and .88, respectively). The mean reduction in resting heart rate was –1 ± 15 beats/min in UMs, 10 ± 12 beats/min in EMs, 12 ± 17 beats/min in IMs, and 10 ± 9 beats/min in PMs (\( P = .30 \)). There was a weak (Pearson \( r = 0.19 \)) but significant (\( P < .05 \)) correlation between the percent reduction in resting heart rate from baseline and log plasma metoprolol concentration. There was no relationship between the percent change in systolic or diastolic blood pressure and log plasma metoprolol concentration. There were no confirmed occurrences of symptomatic bradycar-
dia or hypotension in our study, although 3 patients reported having had a syncope.

**DISCUSSION**

This prospective, short-term clinical trial was conducted to study the consequences of the CYP2D6 polymorphism on the tolerability of metoprolol in a real-life primary care setting. CYP2D6 genotype–derived phenotype was not significantly associated with a propensity for headache, dizziness, fatigue, sleep disturbances, dyspnea, or cold extremities to develop during treatment with metoprolol. There was, however, a trend toward a higher frequency of patients with cold extremities among the group comprising PMs and IMs as compared with EMs and UM. Of the 8 affected patients, 5 had a low plasma metoprolol concentration (<11 ng/mL) and 3 had a high plasma metoprolol concentration (>78 ng/mL). The results of this study also suggest that CYP2D6 activity is higher in IM subjects carrying any 2 of the alleles *9, *10, and *41 as compared with those carrying 1 of these alleles together with a nonfunctional allele.

β-Blockers, including metoprolol, have been associated with a low incidence (5%-10%) of CNS-related adverse effects. In our study fatigue developed during treatment with metoprolol in about 20% of the patients, whereas the frequencies of the other studied CNS symptoms were about 10% to 15%. Our results are in line with previous studies, considering also that adverse effects of metoprolol usually occur in the early stages of treatment and are often transient. Examination of possible adverse effects of metoprolol on sexual function was 1 major objective of this study, because only a few studies have addressed this issue and the results are conflicting. Of the 89 evaluable patients, about 26% reported sexual dysfunction during this short-term study. Curiously enough, sexual dysfunction was significantly more common among the EMs plus UM than among the PM plus IM group. We cannot offer any explanation for this observation. The frequencies of the ADRs observed in this study should not be used for estimation of true ADR frequencies because there was no placebo group. Furthermore, all of the studied ADRs represented symptoms that were subjectively measured, and, therefore, their frequencies may have been overestimated. On the other hand, one strength of this study was that the ADRs under study were examined in a systematic manner, by means of standardized rating scales and questionnaires, over the 6-week study period. In any event our results support the notion that sexual dysfunction may occur during treatment with metoprolol in both men and women.

The median plasma metoprolol concentration at steady state was about 17-fold higher in the PM plus IM group as compared with the EMs plus UM. That this marked difference was not translated into significantly higher ADR frequencies among PMs and IMs may be partly explained by the log-linear relationship between drug concentrations and effects. It should be noted in this context that the S-R-metoprolol concentration ratio is higher in EMs than in PMs, because the preferred elimination of the less active R-enantiomer is lost in the PMs. Therefore measurement of total metoprolol concentrations slightly overestimates the difference in active drug concentrations between PMs and EMs. Furthermore, it is acknowledged that our study may have lacked power to detect moderate or small differences even after combining PMs and IMs. The difference in the proportion of patients reporting cold extremities between the PM plus IM and EM plus UM groups would have been statistically significant in a study population of about 150, assuming unchanged frequencies for this ADR in the 2 groups.

The aim of this study was to examine the relationship between CYP2D6 genotype–derived phenotype and tolerability of metoprolol in a real-life setting, and it was not designed to investigate, in a systematic manner, the relationship between steady-state metoprolol concentrations and ADRs. Plasma metoprolol concentration was determined once (between study days 4 and 42), and adjustment of metoprolol dosage was possible at any time during the 6-week trial period. Given that the whole study period was taken into account when assessing whether a particular ADR occurred in a particular patient, the measured metoprolol concentration may not reflect exposure to metoprolol at the time of the patient having the ADR. In a recent study, designed
to analyze metoprolol adverse events in relation to metoprolol steady-state pharmacokinetic profile and CYP2D6 genotype in hypertension, adverse event rates were not influenced by differences in plasma metoprolol concentrations or CYP2D6 genotypes among 50 patients treated with metoprolol monotherapy.22 However, as expected, the observed differences in the pharmacokinetics of metoprolol were closely associated with the genotype-derived CYP2D6 phenotype.22 The same investigators recently demonstrated that differences in tolerability to metoprolol CR/XL in a heart failure population, assessed by clinical measures of decompensation, were also not related to differences in metoprolol pharmacokinetics or CYP2D6 genotype.37

Metoprolol was effective in lowering blood pressure, as was to be expected because the indication for treatment was predominantly hypertension. It should be noted, however, that about 20% of the patients took other drugs that lower blood pressure. There were no differences between the CYP2D6 genotype–derived phenotypes with respect to the effect of metoprolol on systolic or diastolic blood pressure. In the study of Zineh et al,22 antihypertensive response rates and blood pressure changes were not dependent on plasma metoprolol concentrations or CYP2D6 polymorphism among hypertensive patients, in line with a single-dose study showing no association between the blood pressure–lowering effect of metoprolol (100 mg) and CYP2D6 genotype or plasma metoprolol levels among 29 healthy subjects.38 The resting heart rate was also considerably decreased by metoprolol in EMs, IMs, and PMs but not in UMs. There was a positive correlation between the percent reduction in the resting heart rate and log metoprolol concentration, in line with previous studies showing that a reduction of both resting heart rate and heart rate during exercise by metoprolol displays a linear relationship with log metoprolol concentrations.10,38-40 It should be noted that β1-adrenergic receptor polymorphisms, which have been shown to be important determinants of antihypertensive

Table III. Frequencies for studied adverse effects in PMs and IMs and in EMs and UMs

<table>
<thead>
<tr>
<th>Adverse effect</th>
<th>PMs plus IMs (n = 25)</th>
<th>%</th>
<th>EMs plus UMs (n = 96)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Headache</td>
<td>3 (0 PMs)</td>
<td>12.0</td>
<td>11 (0 UMs)</td>
<td>11.5</td>
</tr>
<tr>
<td>Dizziness</td>
<td>5 (1 PM)</td>
<td>20.0</td>
<td>12 (2 UMs)</td>
<td>12.5</td>
</tr>
<tr>
<td>Fatigue or drowsiness</td>
<td>7 (2 PMs)</td>
<td>28.0</td>
<td>19 (2 UMs)</td>
<td>19.8</td>
</tr>
<tr>
<td>Sleep disturbances</td>
<td>1 (PM)</td>
<td>4.0</td>
<td>12 (0 UMs)</td>
<td>12.5</td>
</tr>
<tr>
<td>Dyspnea</td>
<td>1 (IM)</td>
<td>4.0</td>
<td>6 (2 UMs)</td>
<td>6.3</td>
</tr>
<tr>
<td>Cold extremities</td>
<td>4 (1 PM)</td>
<td>16.0</td>
<td>4 (0 UMs)</td>
<td>4.2</td>
</tr>
<tr>
<td>Sexual dysfunction*</td>
<td>1 (man) (IM)</td>
<td>5.0</td>
<td>22 (8 women and 14 men)</td>
<td>31.9</td>
</tr>
</tbody>
</table>

*The PM plus IM group and the EM plus UM group comprised 20 and 69 evaluable patients, respectively.

Table IV. Effects of metoprolol on systolic and diastolic blood pressure and resting heart rate according to CYP2D6 genotype–derived phenotype

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Systolic blood pressure (mm Hg)</th>
<th>At baseline</th>
<th>During metoprolol</th>
<th>Diastolic blood pressure (mm Hg)</th>
<th>At baseline</th>
<th>During metoprolol</th>
<th>Resting heart rate (beats/min)</th>
<th>At baseline</th>
<th>During metoprolol</th>
</tr>
</thead>
<tbody>
<tr>
<td>UM (n = 5)</td>
<td>164 ± 27.3</td>
<td>148 ± 10.4</td>
<td>104 ± 16.4</td>
<td>87.4 ± 6.8</td>
<td>72 ± 14</td>
<td>72 ± 8</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>EM (n = 89)</td>
<td>160 ± 17.7</td>
<td>140 ± 19.0*</td>
<td>96.2 ± 10.7</td>
<td>84.7 ± 11.5*</td>
<td>80 ± 11</td>
<td>70 ± 12*</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>IM (n = 21)</td>
<td>150 ± 22.0</td>
<td>132 ± 17.8*</td>
<td>92.7 ± 12.7</td>
<td>80.7 ± 12.1*</td>
<td>79 ± 11</td>
<td>67 ± 13†</td>
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</tr>
<tr>
<td>PM (n = 4)</td>
<td>164 ± 7.5</td>
<td>139 ± 14.5</td>
<td>95.0 ± 17.3</td>
<td>82.5 ± 14.4</td>
<td>77 ± 9</td>
<td>67 ± 10</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data are given as mean ± SD.

Table: *P < .001, versus baseline by paired t test.

†P < .01, versus baseline by paired t test.
response to β-blocker therapy,41-43 were not investigated in our study. Therefore the possibility that some of the measured effects might have been confounded by uneven distribution of β1-adrenergic receptor genotypes among the CYP2D6 phenotype groups cannot be excluded.

On the basis of the considerable impact of CYP2D6 polymorphism on the disposition of CYP2D6 substrates, it has often been suggested that CYP2D6 PMs are more susceptible to adverse effects than EMs at standard doses of CYP2D6 substrates such as antidepressive and antipsychotic drugs. The clinical studies that have examined this issue have yielded conflicting results, but the balance of evidence indicates that PMs are more likely to have adverse effects than EMs during treatment with CYP2D6 substrates.20,44-51 With regard to metoprolol, the CYP2D6 PM genotype was overrepresented among 24 patients with severe metoprolol-associated adverse effects in a retrospective study.20 Metoprolol is well tolerated in the majority of patients with cardiovascular diseases.1-6 The effect of β-blockers on quality of life in hypertensive patients has not been extensively studied, but quality of life in patients with mild or moderate hypertension did not deteriorate in most studies with metoprolol.52 In our study the PM plus IM group was overall no more likely to have ADRs than the EMs plus UMs, although the results concerning tolerability of metoprolol in PMs remained inconclusive because of the small number of PMs enrolled. The wide therapeutic range of metoprolol may explain why it is well tolerated in the majority of CYP2D6 PMs and IMs despite several-fold higher plasma concentrations. In addition, severe adverse effects during metoprolol administration also do occur in EMs.20

The valuable contribution of the physicians who recruited patients to this study is gratefully acknowledged.

The authors have no financial or personal conflicts of interest to disclose.

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Cyclosporine markedly raises the plasma concentrations of repaglinide

**Background and Objective:** Repaglinide is an antidiabetic drug metabolized by cytochrome P450 (CYP) 2C8 and 3A4, and it appears to be a substrate of the hepatic uptake transporter organic anion transporting polypeptide 1B1 (OATP1B1). We studied the effects of cyclosporine (INN, ciclosporin), an inhibitor of CYP3A4 and OATP1B1, on the pharmacokinetics and pharmacodynamics of repaglinide.

**Methods:** In a randomized crossover study, 12 healthy volunteers took 100 mg cyclosporine or placebo orally at 8 PM on day 1 and at 8 AM on day 2. At 9 AM on day 2, they ingested a single 0.25-mg dose of repaglinide. Concentrations of plasma and urine repaglinide and its metabolites (M), blood cyclosporine, and blood glucose were measured for 12 hours. The subjects were genotyped for single-nucleotide polymorphisms in *CYP2C8, CYP3A5, SLCO1B1* (encoding OATP1B1), and *ABCB1* (P-glycoprotein). The effect of cyclosporine on repaglinide metabolism was studied in human liver microsomes in vitro.

**Results:** During the cyclosporine phase, the mean peak repaglinide plasma concentration was 175% (range, 56%-365%; \(P = .013\)) and the total area under the plasma concentration–time curve [AUC(0-\(\infty\))] was 244% (range, 119%-533%; \(P < .001\)) of that in the placebo phase. The amount of unchanged repaglinide and its metabolites M2 and M4 excreted in urine were raised 2.7-fold, 7.5-fold, and 5.0-fold, respectively, by cyclosporine (\(P < .001\)). The amount of M1 excreted in urine remained unchanged, but cyclosporine reduced the ratio of M1 to repaglinide by 62% (\(P < .001\)). Cyclosporine had no significant effect on the elimination half-life or renal clearance of repaglinide. Although the mean blood glucose–lowering effect of repaglinide was unaffected in this low-dose study with frequent carbohydrate intake, the subject with the greatest pharmacokinetic interaction had the greatest increase in blood glucose–lowering effect. The effect of cyclosporine on repaglinide AUC(0-\(\infty\)) was 42% lower in subjects with the *SLCO1B1* 521TC genotype than in subjects with the 521TT (reference) genotype (\(P = .047\)). In vitro, cyclosporine inhibited the formation of M1 (IC\(_{50}\) [concentration of inhibitor to cause 50% inhibition of original enzyme activity], 0.2 \(\mu\)mol/L) and M2 (IC\(_{50}\), 4.3 \(\mu\)mol/L) but had no effect on M4.

**Conclusions:** Cyclosporine raised the plasma concentrations of repaglinide, probably by inhibiting its CYP3A4-catalyzed biotransformation and OATP1B1-mediated hepatic uptake. Coadministration of cyclosporine may enhance the blood glucose–lowering effect of repaglinide and increase the risk of hypoglycemia.

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Repaglinide is a short-acting meglitinide analog antidiabetic drug used to reduce postprandial glucose levels in patients with type 2 diabetes.\(^1^2\) It lowers blood glucose concentrations by enhancing glucose-stimulated insulin release. Repaglinide undergoes first-pass metabolism, resulting in an oral bioavailability of about 60%.\(^3\) Cytochrome P450 (CYP) 3A4 and CYP2C8 are the main enzymes that participate in its oxidative biotransformation.\(^4^5\) Repaglinide is not a substrate of P-glycoprotein, but it is extensively metabolized to inactive metabolites, which are excreted primarily into feces.\(^6^8\) The area under the concentration-time curve (AUC) of repaglinide is markedly increased
in homozygous carriers of the SLCO1B1 521T/C (Val174Ala) single-nucleotide polymorphism (SNP), strongly suggesting that repaglinide is a substrate of the SLCO1B1-encoded hepatic uptake transporter organic anion transporting polypeptide 1B1 (OATP1B1; previously known as LST-1, OATP2, and OATP-C).7

The plasma concentrations of repaglinide are moderately raised by drugs that inhibit either CYP2C8 or CYP3A4.8-11 However, gemfibrozil, a lipid-lowering drug of the fibrate class, has caused, on average, an 8-fold increase in the AUC from time 0 to infinity [AUC(0-\infty)] of repaglinide, greatly increasing its glucose-lowering effect.9 Parent gemfibrozil does not inhibit CYP3A412 and is only a moderate inhibitor of CYP2C8.13 It was recently shown that a glucuronide conjugate of gemfibrozil is a relatively potent inhibitor of CYP2C8 and OATP1B1 but does not inhibit CYP3A4.14 Combined inhibition of the hepatic uptake and CYP2C8-mediated biotransformation of repaglinide may explain the observed in vivo interaction between gemfibrozil and repaglinide.9

Cyclosporine (INN, ciclosporin) is an immunosuppressive drug used in organ transplant patients and in the treatment of chronic inflammatory diseases. Cyclosporine is metabolized in the intestine and liver, and the metabolites are excreted mainly via bile into feces. The peak concentration of cyclosporine in blood is reached approximately 2 hours after its oral administration, and its elimination half-life (t½) is about 12 hours.15 In vitro, cyclosporine potently inhibits the transporter proteins P-glycoprotein and OATP1B1, as well as CYP3A4.16-19 Cyclosporine raises the plasma concentrations of several statins, at least partially by inhibiting their OATP1B1-mediated hepatic uptake.16,20,21 For example, the AUC of pravastatin, a statin with no significant CYP-mediated biotransformation, is raised more than 10-fold by cyclosporine.22,23

Because cyclosporine markedly increases the plasma concentrations of several OATP1B1 substrates16,20-22,24 and because polymorphism in the SLCO1B1 gene encoding for OATP1B1 is a major determinant of the pharmacokinetics of repaglinide,7 we hypothesized that cyclosporine might interact with repaglinide. Therefore we have investigated the effects of cyclosporine on the pharmacokinetics and pharmacodynamics of repaglinide in healthy subjects.

**METHODS**

**Subjects.** Twelve healthy nonsmoking male volunteers (age range, 19-25 years; weight range, 56-100 kg) participated in the study after giving written informed consent (Table I). They were ascertained to be healthy by a medical history, physical examination, and routine laboratory tests. None of the subjects used any continuous medication, and use of grapefruit juice or any pharmaceuticals was not allowed for 2 weeks before the study days. The subjects had previously been genotyped for the −11187G>A SNP in the promoter region and the 521T>C SNP (Val174Ala) in exon 5 of the SLCO1B1 gene, for the 2677G>T/A SNP (Ala893Ser/
Th) in exon 21 and the 3435C>T SNP (synonymous) in exon 26 of the ABCB1 gene, for the CYP2C8*3 (416G>A, 1196A>G [Arg139Lys, Lys399Arg]) and CYP2C8*4 (792C>G [Ile264Met]) alleles, and for the CYP3A5*3 (6986A>G, nonexpressor) allele (Table I).7

**Study design.** The study protocol was approved by the Ethics Committee for Studies in Healthy Subjects and Primary Care of the Hospital District of Helsinki and Uusimaa and the National Agency for Medicines. A randomized crossover study with 2 phases and a washout period of 4 weeks was carried out. The subjects took 100 mg cyclosporine (1 Sandimmun Neoral 100-mg capsule; Novartis Pharma SA, Huningue, France) or placebo orally at 8 PM on day 1 and at 8 AM on day 2. At 9 AM on day 2, after an overnight fast and 1 hour after the second pretreatment dose, they ingested a single 0.25-mg dose of repaglinide (one half of a NovoNorm 0.5-mg tablet; Novo Nordisk A/S, Bagsvaerd, Denmark) with 150 mL water. The volunteers remained seated for 3 hours after the administration of repaglinide. The timing of repaglinide administration was chosen to ensure adequate absorption of cyclosporine before repaglinide ingestion to maximize the extent of possible interaction. For safety reasons, a subtherapeutic repaglinide dose and short-term cyclosporine pretreatment were used.

Food intake on day 2 was identical in both phases. The volunteers received a standardized light breakfast precisely 15 minutes after repaglinide administration, a standardized snack rich in carbohydrates at precisely 1 hour after repaglinide, a standardized warm meal and the snacks within 5 minutes after repaglinide. The breakfast was eaten within 10 minutes and the snacks within 5 minutes. The breakfast contained approximately 1550 kJ energy, 70 g carbohydrates, 8 g protein, and 6 g fat. The snacks were identical and contained about 840 kJ energy, 45 g carbohydrates, 2 g protein, and 1 g fat each. During the days of repaglinide administration, the subjects were under direct medical supervision and blood glucose levels were monitored throughout the day. Additional carbohydrates, glucose solution for intravenous use, and glucagon for intramuscular use were available, but they were not needed. For safety reasons, the blood pressure and heart rate of the subjects were also measured before and at 3, 6, and 12 hours after repaglinide administration. The measurement was done in a sitting position with an automatic oscillometric blood pressure monitor (HEM-711; Omron Healthcare, Hamburg, Germany).

**Sampling and determination of blood glucose concentrations.** On the days of repaglinide administration, timed blood samples (5 mL each) were drawn from a cannulated forearm vein before and at 20, 40, 60, 80, and 100 minutes and 2, 2.5, 3, 4, 5, 7, 9, and 12 hours after the administration of repaglinide. Blood samples were collected into tubes containing ethylenediaminetetraacetic acid. Blood glucose concentrations were measured immediately after each blood sampling by the glucose oxidase method (Precision G Blood Glucose Testing System; Medisense, Bedford, Mass). Plasma was separated within 30 minutes after blood sampling and stored at −70°C until analysis. Whole-blood cyclosporine concentrations were measured from additional blood samples (3 mL each), drawn before and at 20 and 60 minutes and 2, 3, 5, 7, and 12 hours after the administration of repaglinide. Urine was collected from 0 to 12 hours after the administration of repaglinide.

**Determination of drug concentrations.** Concentrations of plasma repaglinide and urine repaglinide and its metabolites M1, M2, and M4 (Fig 1) were measured by use of an API 3000 liquid chromatography–tandem mass spectrometry system (Sciex Division of MDS Inc, Toronto, Ontario, Canada). Reversed-phase chromatographic separation was achieved on a Symmetry C8 column (150 × 2.1 mm internal diameter, 3.5 μm particle size) (Waters, Milford, Mass) by use of gradient elution. The mobile phase consisted of 10-mmol/L ammonium formate (pH 3.5, adjusted with 99% formic acid) and acetonitrile. An aliquot (15 μL) was injected at a flow rate of 180 μL/min to give a total chromatographic run time of 24 minutes. Clopidogrel served as an internal standard. The mass spectrometer was operated in positive TurboIonSpray mode, and the samples were analyzed via selected reaction monitoring by use of the transition of the [M+H]+ precursor ion to product ion for each analyte and internal standard. The selected reaction monitoring ion transitions were mass-to-charge ratio (m/z) 453 to m/z 230 for repaglinide, m/z 385 to m/z 162 for M1, m/z 485 to m/z 230 for M2, m/z 469 to m/z 246 for M4, and m/z 421 to m/z 212 for clopidogrel. The limit of quantification for repaglinide was 0.02 ng/mL, and the day-to-day coefficients of variation were 13.7% at 0.05 ng/mL, 8.7% at 0.1 ng/mL, and 6.9% at 2.0 ng/mL (n = 20). Because authentic reference compounds were not available, repaglinide metabolite concentrations are given in arbitrary units (units per milliliter) relative to the ratio of the peak height of each metabolite to that of the internal standard in the chromatogram.

Whole-blood cyclosporine concentrations were measured with a commercially available radioimmunoassay method (CYCLO-Trac; DiaSorin, Stillwater, Minn). The quantification limit was 30 ng/mL. The day-to-day
coefficients of variation were 4.6% at 100 ng/mL and 3.0% at 350 ng/mL.

**Pharmacokinetics.** The pharmacokinetics of repaglinide was characterized by the peak concentration (C\text{max}) in plasma, time to C\text{max} (t\text{max}), AUC(0-\infty), and t\text{1/2}. The C\text{max} and t\text{max} values were taken directly from original data. The terminal log-linear part of each concentration-time curve was identified visually, and the elimination rate constant (k\text{e}) was determined from log-transformed data by use of linear regression analysis. The t\text{1/2} was calculated by the following equation: t\text{1/2} = ln2/k\text{e}. The AUC values were calculated by use of the linear trapezoidal rule for the rising phase of the plasma repaglinide concentration–time curve and the log-linear trapezoidal rule for the descending phase, with extrapolation to infinity, when appropriate, by dividing the last measured concentration by k\text{e}. The renal clearance of repaglinide was calculated by dividing the amount excreted into urine within 12 hours by the repaglinide AUC from 0 to 12 hours. The pharmacokinetics of cyclosporine was characterized by C\text{max} in blood and AUC(0-\infty). All pharmacokinetic calculations were performed with the program MK-Model, version 5.0 (Biosoft, Cambridge, United Kingdom).

**Pharmacodynamics.** The pharmacodynamics of repaglinide was characterized by mean change, maximum increase, and maximum decrease in blood glucose concentration. The mean change was calculated by dividing the net area under the blood glucose concentration–time curve from 0 to 3 hours and 0 to 12 hours by the corresponding time interval. The mean blood pressure and mean heart rate were calculated by dividing the area under the blood pressure– or heart rate–time curve from 0 to 12 hours by the corresponding time interval.

**Statistical analysis.** Results are expressed as mean values ± SD in the text and tables. The pharmacokinetic and pharmacodynamic variables between the placebo and cyclosporine phases were compared by use of repeated-measures ANOVA. The t\text{max} values were compared with the Wilcoxon signed rank test. The geometric mean ratio and its 95% confidence interval were calculated for all pharmacokinetic variables, except t\text{max}. The Pearson correlation coefficient was used to investigate possible relationships between cyclosporine pharmacokinetic variables, repaglinide blood glucose–lowering response, and the extent of interaction between cyclosporine and repaglinide. Possible associations of SLCO1B1, ABCB1, CYP2C8, and CYP3A5 SNPs with the degree of interaction between cyclosporine and repaglinide were investigated by use of ANOVA, followed by a posteriori testing with the Tukey test. The analysis was performed with the statistical programs Systat for Windows, version 6.0.1, and SPSS 11.0 for Windows (SPSS, Chicago, Ill). Differences were considered statistically significant at P < .05.

**In vitro study.** Pooled human liver microsomes (HLMs) (new catalog No. 452161, lot 26) and human recombinant (Supersomes) CYP2C8 + b\text{4} (new catalog No. 456252, lot 15) and CYP3A4 + b\text{4} (new catalog No. 456202, lot 55) were purchased from Gentest (Woburn, Mass). Repaglinide (Boehringer Ingelheim, Ingelheim, Germany), β-nicotinamide adenine dinucle-
otide phosphate reduced (β-NADPH), and cyclosporine (Sigma-Aldrich, St Louis, Mo) were used in this study. Methanol, acetonitrile, and 2-propanol were obtained from Rathburn Chemicals (Walkerburn, Scotland); other chemicals were from Merck (Darmstadt, Germany).

All incubations were conducted in duplicate in a shaking water bath at 37°C, and the incubation times and microsomal protein concentrations were within the linear range for reaction velocity. The incubations were carried out in 0.1-mol/L sodium phosphate buffer (pH 7.4), containing 5.0-mmol/L magnesium chloride. The stock solutions of repaglinide and cyclosporine were prepared in methanol (final concentration, 1% [vol/vol] in the incubation mixture). The drug(s), buffer, and HLMs or recombinant enzymes were premixed, and incubations were commenced by the addition of β-NADPH (final concentration, 1.0 mmol/L). The reaction was stopped at 15 minutes from the start of the incubation by the addition of phosphoric acid, and metabolite (M1, M2, and M4) concentrations were measured by liquid chromatography–tandem mass spectrometry.

The formation rates of the M1, M2, and M4 metabolites of repaglinide (2 μmol/L) were compared in recombinant CYP2C8 and CYP3A4. The effect of cyclosporine on the metabolism of repaglinide was studied by coincubating repaglinide (2 μmol/L) with cyclosporine (0-30 μmol/L) in HLMs. The concentrations of HLMs, recombinant CYP2C8, and recombinant CYP3A4 were 0.1 mg/mL, 5 pmol/mL, and 5 pmol/mL, respectively. The conversion factors are as follows: repaglinide, 1 μmol/L equals 453 ng/mL; cyclosporine, 1 μmol/L equals 1203 ng/mL.

Reaction velocity was determined for each incubation by dividing the amount of metabolite formed (arbitrary units) by the respective time interval and enzyme concentration. IC50 values (concentration of inhibitor to cause 50% inhibition of original enzyme activity) were estimated from reaction velocity data by use of nonlinear regression analysis with the FigP program (version 6.0; Biosoft).

RESULTS

Repaglinide pharmacokinetics. The plasma concentrations of repaglinide were significantly raised by cyclosporine (Fig 2 and Table II). Cyclosporine raised the mean Cmax and AUC(0-∞) of repaglinide to 175% (P = .013) and 244% (P < .001) of control values, respectively. An increase in the AUC(0-∞) was seen in every subject (range, 119%-533% of control). There was no statistically significant change in the tmax or t½ of repaglinide.
Interaction between cyclosporine and repaglinide

Table II. Pharmacokinetic variables of single oral dose of repaglinide (0.25 mg) in 12 healthy volunteers after oral administration of placebo or 100 mg cyclosporine at 13 hours and 1 hour before repaglinide administration

<table>
<thead>
<tr>
<th>Variable</th>
<th>Placebo phase (control)</th>
<th>Cyclosporine phase</th>
<th>Cyclosporine phase: Percentage of control and range</th>
<th>Geometric mean ratio and 95% CI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Repaglinide C&lt;sub&gt;max&lt;/sub&gt; (ng/mL)</td>
<td>3.9 ± 1.9</td>
<td>6.7 ± 2.1</td>
<td>175% (56%-365%)</td>
<td>1.82 (1.28-2.58)</td>
<td>.003</td>
</tr>
<tr>
<td>t&lt;sub&gt;max&lt;/sub&gt; (min)</td>
<td>40 (20-40)</td>
<td>40 (20-40)</td>
<td></td>
<td></td>
<td>.180</td>
</tr>
<tr>
<td>t&lt;sub&gt;1/2&lt;/sub&gt; (h)</td>
<td>1.3 ± 0.3</td>
<td>1.3 ± 0.3</td>
<td>97% (69%-133%)</td>
<td>0.98 (0.86-1.13)</td>
<td>.806</td>
</tr>
<tr>
<td>AUC(0-∞) (ng · h/mL)</td>
<td>4.44 ± 1.68</td>
<td>10.82 ± 3.28</td>
<td>244% (119%-533%)</td>
<td>2.54 (1.91-3.35)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Cl&lt;sub&gt;renal&lt;/sub&gt; (mL/h)</td>
<td>12.8 ± 4.5</td>
<td>13.8 ± 5.6</td>
<td>108% (67%-189%)</td>
<td>1.08 (0.86-1.35)</td>
<td>.462</td>
</tr>
</tbody>
</table>

Data are given as mean ± SD, except for t<sub>1/2</sub> data, which are given as median and range. CI, Confidence interval; C<sub>max</sub>, peak plasma concentration; t<sub>max</sub>, time to peak plasma concentration; t<sub>1/2</sub>, elimination half-life; AUC(0-∞), area under plasma concentration-time curve from time 0 to infinity; Cl<sub>renal</sub>, renal clearance.

Table III. Pharmacodynamic variables of single oral dose of repaglinide (0.25 mg) in 12 healthy volunteers after administration of placebo or 100 mg cyclosporine at 13 hours and 1 hour before repaglinide administration

<table>
<thead>
<tr>
<th>Variable</th>
<th>Placebo phase (control)</th>
<th>Cyclosporine phase</th>
<th>Mean difference between phases and 95% CI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean change, 0-3 h (mmol/L)</td>
<td>-0.3 ± 0.7</td>
<td>-0.2 ± 0.5</td>
<td>0.1 (-0.4 to 0.7)</td>
<td>.644</td>
</tr>
<tr>
<td>Mean change, 0-12 h (mmol/L)</td>
<td>-0.2 ± 0.5</td>
<td>-0.2 ± 0.5</td>
<td>0.1 (-0.3 to 0.4)</td>
<td>.718</td>
</tr>
<tr>
<td>Maximum increase (mmol/L)</td>
<td>1.6 ± 0.9</td>
<td>1.6 ± 0.6</td>
<td>0.0 (-0.7 to 0.7)</td>
<td>.981</td>
</tr>
<tr>
<td>Maximum decrease (mmol/L)</td>
<td>1.5 ± 0.7</td>
<td>1.5 ± 0.6</td>
<td>0.0 (-0.6 to 0.6)</td>
<td>&gt;.999</td>
</tr>
</tbody>
</table>

Data are given as mean ± SD.

**Repaglinide pharmacodynamics and blood pressure.** Although no statistically significant differences were observed in the mean blood glucose response between the placebo and cyclosporine phases (Table III), the blood glucose response of individual subjects correlated with the degree of pharmacokinetic interaction between cyclosporine and repaglinide. The ratio of repaglinide C<sub>max</sub> values between the cyclosporine and placebo phases correlated with the difference in the mean blood glucose change from 0 to 3 hours (r = -0.586, P = .045) and with the difference in maximum blood glucose increase (r = -0.623, P = .031). The ratio of repaglinide AUC(0-∞) values between the phases correlated with the difference in the mean blood glucose change from 0 to 3 hours (r = -0.602, P = .038). The subject with the greatest (5-fold) increase in repaglinide AUC(0-∞) also had the greatest enhancement of the blood glucose-lowering effect of repaglinide (Fig 3). However, none of the subjects had symptomatic hypoglycemia in this low-dose repaglinide study with frequent carbohydrate intake. The mean blood pressure (systolic/diastolic) was 130 ± 10 mm Hg/74 ± 5 mm Hg and 133 ± 7 mm Hg/75 ± 5 mm Hg and the mean heart rate was 62 ± 11 beats/min and 62 ± 7 beats/min in the placebo and cyclosporine phases, respectively.

**Excretion of repaglinide and its metabolites into urine.** Compared with the corresponding control values, cyclosporine increased the urinary excretion of unchanged repaglinide (to 265%, P < .001) and its metabolites M2 (to 748%, P < .001) and M4 (to 499%, P < .001) but had no significant effect on the excretion of M1 (Table II). However, cyclosporine significantly reduced the ratio of M1 to repaglinide.
(−62%, \(P < .001\)) and increased the ratio of M2 to repaglinide (+158%, \(P < .001\)) and M4 to repaglinide (+81%, \(P < .001\)) in urine. The renal clearance of repaglinide remained unchanged by cyclosporine (\(P = .521\)).

**Cyclosporine pharmacokinetics.** The mean \(C_{\text{max}}\) and AUC(0–\(\infty\)) and median \(t_{\text{max}}\) values of cyclosporine were 664 ± 158 ng/mL, 1998 ± 636 ng/mL, and 80 minutes (range, 80-120 minutes), respectively (Fig 3 and Table I). There was a significant correlation between the AUC(0–\(\infty\)) of cyclosporine and the increase in the AUC(0–\(\infty\)) of repaglinide caused by cyclosporine (Pearson \(r = 0.61\), \(P = .034\)) (Fig 4).

**Genotypes and repaglinide pharmacokinetics.** The SLC01B1, ABCB1, CYP2C8, and CYP3A5 genotypes of the subjects are shown in Table I. In subjects with the SLC01B1 521TC genotype, the increase in the AUC(0–\(\infty\)) of repaglinide by cyclosporine (4.3 ± 3.8 ng · h/mL) was 42% smaller than in subjects with the 521TT (reference) genotype (7.4 ± 2.8 ng · h/mL) (\(P = .047\)) (Fig 5). No other statistically significant associations between the investigated SNPs or haplotypes and the extent of interaction or repaglinide baseline pharmacokinetics were found.

**In vitro study.** Cyclosporine inhibited the formation of the repaglinide metabolites M1 (IC\(_{50}\), 0.2 μmol/L)
and M2 (IC\textsubscript{50}, 4.3 \textmu mol/L) in HLMs, with no effect on the formation of M4 (Fig 6, A). The maximum inhibition values of M1 and M2 formation were approximately 90% and 60%, respectively, and the IC\textsubscript{50} values for the inhibitory processes were 0.13 \textmu mol/L and 0.47 \textmu mol/L, respectively.

All metabolites were formed in incubations with recombinant CYP2C8 and CYP3A4. M1 and M2 were predominantly formed by recombinant CYP3A4, whereas M4 was formed mainly by recombinant CYP2C8 (Fig 6, B).

**DISCUSSION**

This study shows that even short-term use of cyclosporine markedly increases the plasma concentrations of repaglinide. There was large variation in the extent of the interaction, with the increase in the AUC of repaglinide ranging from 1.2-fold to over 5-fold even in this homogeneous group of young healthy volunteers. This variation can be partly explained by genetic factors and variability in cyclosporine concentrations. However, cyclosporine had no significant effect on the blood glucose-lowering effect of repaglinide, which can be explained, at least partially, by the use of a subtherapeutic dose of repaglinide and the frequent food intake after repaglinide administration.

Both CYP2C8 and CYP3A4 participate in the metabolism of repaglinide.\textsuperscript{4,5} There is evidence suggesting that the role of CYP2C8 might be more important than that of CYP3A4; inhibition of CYP3A4 by itraconazole or clarithromycin has resulted in an increase of about 40% in the AUC of repaglinide;\textsuperscript{9,11} inhibition of CYP2C8 by trimethoprim has resulted in a 61% increase.\textsuperscript{10} In addition, genetic variation in CYP2C8 is associated with altered repaglinide pharmacokinetics.\textsuperscript{7,25} Gemfibrozil, an inhibitor of CYP2C8,\textsuperscript{26} has
raised the AUC of repaglinide about 8-fold, greatly enhancing its blood glucose-lowering effect. The glucuronide conjugate of gemfibrozil inhibits CYP2C8 more strongly than parent gemfibrozil does, without inhibiting CYP3A4, and gemfibrozil and this conjugate metabolite also inhibit OATP1B1. In a recent study the AUC of repaglinide was 3-fold higher in subjects homozygous for the functionally significant SLCO1B1 521T>C SNP compared with subjects with the reference genotype, consistent with a major role for OATP1B1 in repaglinide pharmacokinetics. Thus combined inhibition of the hepatic uptake of repaglinide and its CYP2C8-mediated biotransformation could explain the observed in vivo interaction between gemfibrozil and repaglinide.

Inhibition of a single CYP enzyme by specific inhibitors, without an additional mechanism, has produced only moderate increases in the AUC of repaglinide. In the current study the mean AUC of repaglinide was raised about 2.5-fold by cyclosporine. Inhibition of CYP-mediated biotransformation of repaglinide by cyclosporine is unlikely to solely explain this cyclosporine-repaglinide interaction, because cyclosporine is weaker than itraconazole or clarithromycin as an inhibitor of CYP3A4 in vivo, and cyclosporine does not significantly inhibit CYP2C8. Our in vitro results demonstrated that cyclosporine potently inhibited the CYP3A4-mediated repaglinide metabolism to M1, without an effect on M4 formed by CYP2C8. In this study the peak concentrations of cyclosporine (range, 423-956 ng/mL) were higher than the in vitro IC50 for repaglinide metabolism to M1 but not to M2.

In our study the ratio of M1 (formed primarily by CYP3A4) to repaglinide in urine was significantly decreased by cyclosporine, suggesting that cyclosporine also inhibited the formation of M1 in vivo, similar to that which occurred in vitro. On the other hand, the ratios of M2 (formed only partially by CYP3A4) to repaglinide and M4 (formed primarily by CYP2C8) to repaglinide in urine were increased, suggesting that cyclosporine did not inhibit the formation of these major metabolites in vivo, although it caused a partial inhibition of M2 formation by HLMs in vitro. The increases in the ratios of M2 and M4 to repaglinide in urine by cyclosporine may be explained by inhibition of the hepatic (or biliary) elimination of M2 and M4. For example, cyclosporine inhibits the P-glycoprotein and multidrug resistance–associated protein efflux transporters, expressed in the canalicular membrane of the hepatocyte, and can thus affect the biliary elimination of drugs and their metabolites.

Although cyclosporine also inhibited the formation of M1 in vivo, this inhibition alone is unlikely to explain the effect of cyclosporine on the pharmacokinetics of parent repaglinide, because M1 is quantitatively only a minor metabolite of repaglinide. Furthermore, itraconazole, which raised the AUC of repaglinide by only 40%, reduced the plasma AUC ratio of M1 to repaglinide by 80%, whereas cyclosporine reduced the ratio of M1 to repaglinide in urine by only 62%. Given that the pharmacokinetics of repaglinide depends largely on the SLCO1B1 polymorphism and that cyclosporine is a potent inhibitor of OATP1B1, inhibition of the OATP1B1-mediated hepatic uptake of repaglinide by cyclosporine probably contributes to their interaction. This conclusion is further supported by the finding that the effect of cyclosporine on repaglinide pharmacokinetics was smaller in carriers of SLCO1B1 521T>C SNP than in noncarriers. Thus inhibition of both the CYP3A4-mediated biotransformation and the OATP1B1-mediated hepatic uptake by cyclosporine probably explains the interaction between cyclosporine and repaglinide.
Although cyclosporine increased the AUC of repaglinide by about 2.5-fold, it had no effect on the t₁/₂ of repaglinide in this study. In a previous study in children who had received heart transplants, cyclosporine increased the AUC of pravastatin by 10-fold without affecting its t₁/₂. Moreover, SLC01B1 521CC genotype did not affect repaglinide t₁/₂, despite a 3-fold increase in AUC. Increased bioavailability can only partly explain these increases in plasma drug concentrations, and further studies are needed to clarify the pharmacokinetic mechanisms involved.

In transplant recipients treated with cyclosporine, the exposure to all statins studied has been, on average, 3- to 20-fold higher than in control subjects. Gemfibrozil has raised the concentrations of statins 2- to 5-fold. OATP1B1 is involved in the hepatic uptake of pravastatin, cerivastatin, and rosuvastatin. It is likely that atorvastatin, simvastatin, and lovastatin are also substrates of this hepatic uptake transporter.

Cyclosporine inhibits CYP3A4, P-glycoprotein, whereas gemfibrozil (and its glucuronide conjugate) inhibits CYP2C8 and OATP1B1 but not CYP3A4 or P-glycoprotein. The similarities in the observed drug interactions of gemfibrozil and cyclosporine suggest that they share a common pharmacokinetic interaction mechanism, that is, inhibition of OATP1B1.

There was no statistically significant difference in the mean glucose-lowering effect between the 2 phases in our study, despite a marked difference in the exposure to repaglinide between the phases. The obvious reason for this apparent discrepancy is that only a small dose of repaglinide was given, followed by frequent carbohydrate intake to prevent hypoglycemia. The blood glucose-lowering effect of repaglinide is dose- and concentration-dependent. Accordingly, the greatest increases in the plasma repaglinide concentrations were associated with the greatest increases in the blood glucose-lowering effect of repaglinide. Thus concomitant use of cyclosporine may increase the blood glucose-lowering effect of repaglinide and the risk of hypoglycemia, particularly if higher cyclosporine and repaglinide doses are used. Of note, there was a linear relationship between cyclosporine AUC and the increase in repaglinide AUC by cyclosporine. It is, therefore, advisable to monitor blood glucose concentrations closely if cyclosporine is started in a patient taking repaglinide. In a previous study the combination of gemfibrozil and itraconazole synergistically increased the plasma concentrations (by nearly 20-fold) and effects of repaglinide. Such synergism may occur if cyclosporine is combined with an inhibitor of CYP2C8, leading to simultaneous inhibition of OATP1B1-mediated hepatic uptake and CYP-mediated biotransformation of repaglinide.

In conclusion, cyclosporine considerably raised the plasma concentrations of repaglinide. This interaction is probably caused by inhibition of both the CYP3A4-catalyzed metabolism and the OATP1B1-mediated hepatic uptake of repaglinide by cyclosporine. The possibility of an increased risk of hypoglycemia should be considered when the 2 drugs are used concomitantly.

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The authors have identified no conflicts of interest in relation to this article.

References


Oral contraceptives containing ethinyl estradiol and gestodene markedly increase plasma concentrations and effects of tizanidine by inhibiting cytochrome P450 1A2

**Background and Objective:** Oral contraceptives (OCs) can inhibit drug metabolism, but their effect on various cytochrome P450 (CYP) enzymes and drugs can be different. Our objective was to study the effect of combined OCs, containing ethinyl estradiol (INN, ethinylestradiol) and gestodene, on CYP1A2 activity, as well as their interaction potential with tizanidine.

**Methods:** In a parallel-group study, 15 healthy women using OCs and 15 healthy women without OCs (control subjects) ingested a single dose of 4 mg tizanidine. Plasma and urine concentrations of tizanidine, as well as several of its metabolites (M-3, M-4, M-5, M-9, and M-10), and pharmacodynamic variables were measured until 24 hours after dosing. As a marker of CYP1A2 activity, an oral caffeine test was performed in both groups.

**Results:** The mean area under the plasma concentration–time curve from time 0 to infinity \([\text{AUC}(0-\infty)]\) of tizanidine was 3.9 times greater \((P < .001)\) and the mean peak plasma tizanidine concentration \((C_{\text{max}})\) was 3.0 times higher \((P < .001)\) in the OC users than in the control subjects. In 1 OC user the \([\text{AUC}(0-\infty)]\) of tizanidine exceeded the mean \([\text{AUC}(0-\infty)]\) of the control subjects by nearly 20 times. There were no significant differences in the elimination half-life or time to peak concentration in plasma of tizanidine between the groups. Tizanidine/metabolite ratios in plasma (M-3 and M-4) and urine (M-3, M-4, M-5, M-9, and M-10) were 2 to 10 times higher in the users of OCs than in the control subjects. In the OC group the excretion of unchanged tizanidine into urine was, on average, 3.8 times greater \((P = .008)\) than in the control subjects.

The plasma caffeine/paraxanthine ratio was 2.8 times higher \((P < .001)\) in the OC users than in the control subjects. The caffeine/paraxanthine ratio correlated significantly with the \([\text{AUC}(0-\infty)]\) and peak concentration of tizanidine in plasma, with its excretion into urine, and with, for example, the tizanidine/M-3 and tizanidine/M-4 area under the plasma concentration–time curve ratios. Both the systolic and diastolic blood pressures were lowered by tizanidine more in the OC users \((-29 \pm 10 \text{ mm Hg} \text{ and } -21 \pm 8 \text{ mm Hg}, \text{ respectively})\) than in the control subjects \((-17 \pm 9 \text{ mm Hg} \text{ and } -13 \pm 5 \text{ mm Hg}, \text{ respectively})\) \((P < .01)\).

**Conclusions:** OCs containing ethinyl estradiol and gestodene increase, to a clinically significant extent, the plasma concentrations and effects of tizanidine, probably mainly by inhibiting its CYP1A2-mediated presystemic metabolism. Care should be exercised when tizanidine is prescribed to OC users. (Clin Pharmacol Ther 2005;78:400-11.)

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Ethinyl estradiol (INN, ethinylestradiol) and gestodene are exogenous female sex steroids commonly used in combined oral contraceptives (OCs). In vitro studies have shown that ethinyl estradiol and some progesto-
**Interaction of oral contraceptives with tizanidine**

Tizanidine, an α2-adrenergic agonist, is used as a centrally acting skeletal muscle relaxant. In addition to its use in patients with chronic spasticity conditions, such as multiple sclerosis, tizanidine is commonly used, for example, in patients with tension-type headache and musculoskeletal pain. These conditions are not uncommon in fertile-age women who are likely to use OCs. Tizanidine is eliminated principally by metabolism and has an extensive first-pass metabolism, mediated by CYP1A2. Its main metabolites in plasma and urine are M-3 and M-4, which lack pharmacologic activity (Fig 1). Recently, the CYP1A2 inhibitors fluvoxamine and ciprofloxacin were found to strongly increase the plasma concentrations and effects of tizanidine in vivo. The caffeine/paraxanthine ratio is a validated index for systemic CYP1A2 activity, and OCs have been found to reduce the metabolism of caffeine in humans. Tizanidine differs pharmacokinetically from caffeine, for example, by its extensive presystemic metabolism and shorter elimination half-life.

The product information for tizanidine states that OCs decrease the clearance of tizanidine by 50%, but this information is based on unpublished data. We
aimed to investigate whether OCs containing ethinyl estradiol and gestodene affect tizanidine pharmacokinetics and whether these effects parallel changes in the caffeine/paraxanthine ratio. Furthermore, by recording the pharmacodynamic effects of tizanidine, we wanted to characterize the clinical significance of the possible interaction. To this end, an OC-tizanidine interaction study, including a caffeine test, was conducted in healthy female volunteers who were either users or nonusers of OCs.

METHODS

Subjects and study design. The study protocol was approved by the Ethics Committee for Studies in Healthy Subjects of the Hospital District of Helsinki and Uusimaa, Finland, and the Finnish National Agency for Medicines, Helsinki, Finland. This was an open, parallel-group study with 15 healthy female volunteers (mean age, 22 ± 2 years [range, 18-25 years]; mean weight, 57 ± 6 kg [range, 48-63 kg]) using OCs and 15 healthy female volunteers (mean age, 22 ± 2 years [range, 19-26 years]; mean weight, 62 ± 10 kg [range, 52-74 kg]) without any concomitant medication. There were no statistically significant differences in age or weight between the groups.

The combined OCs used by the women in this study contained 75 µg gestodene and either 20 µg ethinyl estradiol (Harmonet tablet [Wyeth, Newbridge, Ireland], 7 subjects; Meliane tablet [Schering, Berlin, Germany], 5 subjects) or 30 µg ethinyl estradiol (Femoden tablet [Schering], 2 subjects; Minulet tablet [Wyeth], 1 subject). The OC users had been using these OC preparations for at least 1 menstrual cycle before the study. Before entering the study, all subjects provided written informed consent and were ascertained to be healthy by medical history, physical examination, and routine laboratory tests. For safety reasons, subjects with a systolic blood pressure lower than 110 mm Hg were excluded from the study. None of the subjects were tobacco smokers, and none used any continuous medication except OCs.

In this study each volunteer received a single dose of tizanidine. On the day of tizanidine administration, the OC users had been taking the OC preparation for 6 to 21 days during that cycle and the control subjects were in a corresponding phase of the menstrual cycle. The users of OCs were instructed to ingest the OC preparation at 8 AM for 6 days before and on the day of tizanidine administration. On the study day, after an overnight fast, a single oral dose of 4 mg tizanidine (one 4-mg Sirdalud tablet; Novartis Pharma, Wehr, Germany) was ingested with 150 mL water at 9 AM. A standard meal was served at 3 and 7 hours after tizanidine ingestion. Drinking of grapefruit juice and tobacco smoking were not allowed for 1 week before the study day. Alcohol and drinks containing caffeine were not permitted on the study day. The subjects were under direct, close medical supervision during the day of administration of tizanidine. Fluids for intravenous infusion were available for immediate use, but they were not needed.

An oral caffeine test was performed on the day before the tizanidine study day. The subjects ingested 100 mg caffeine (one 100-mg Cofi-Tabs tablet; Vitabalans, Hameenlinna, Finland) at 9 AM, after having abstained from caffeine intake for at least 12 hours, and a blood sample for analysis of plasma caffeine and paraxanthine (1,7-dimethylxanthine) concentrations was taken 6 hours after caffeine intake.

Sampling. On the days of administration of tizanidine, a forearm vein of each subject was cannulated with a plastic cannula and kept patent with an obturator. Timed blood samples were drawn before the administration of tizanidine and at 20, 40, 60, and 90 minutes and 2, 3, 4, 5, 7, 9, 12, and 24 hours later. Blood samples (10 mL each) were taken into ethylenediaminetetraacetic acid–containing tubes. Plasma was separated within 30 minutes. The urine was collected cumulatively in 2 fractions: 0 to 12 hours and 12 to 24 hours. Plasma and urine aliquots were stored at −70°C until analysis.

Drug concentrations in plasma and urine. Plasma and urine tizanidine and metabolite concentrations were quantified by use of an API 2000 liquid chromatography–tandem mass spectrometry system (MDS Sciex, Ontario, Canada). Chromatography was performed on an XTerra RP C18 column (3.9 × 100 mm; Waters, Milford, Mass) by use of gradient elution. The mobile phase consisted of 10-mmol/L ammonium acetate (pH 9.5, adjusted with 25% ammonia solution) and acetonitrile. The mass spectrometer was operated in the atmospheric pressure chemical ionization mode with positive ion detection. The ion transitions monitored were mass-to-charge ratio (m/z) 254 to m/z 44 for tizanidine, m/z 268 to m/z 211 for M-3, m/z 228 to m/z 211 for M-4, m/z 252 to m/z 216 for M-5, m/z 415 to m/z 286 for M-9, m/z 288 to m/z 188 for M-10, and m/z 230 to m/z 44 for the internal standard, clonidine. These transitions represent the product ion of the [M+H]⁺ ion. The limit of quantification for tizanidine was 0.05 ng/mL, and the day-to-day coefficient of variation was 17.4% at 0.1 ng/mL, 6.4% at 1 ng/mL, and 7.5% at 10 ng/mL (n = 8). A signal-to-noise ratio of 10:1 was used as the limit of detection for tizanidine metabolites, and
the quantities are given in arbitrary units relative to the ratio of the peak height of the metabolite to the peak height of the internal standard. Ethinyl estradiol and gestodene did not interfere with the assay.

Plasma caffeine and paraxanthine concentrations were determined by HPLC, with β-hydroxyethyltheophylline as the internal standard. The day-to-day coefficient of variation of caffeine and paraxanthine was less than 6% at relevant concentrations.

**Pharmacokinetics.** The pharmacokinetics of tizanidine and its metabolites M-3 and M-4 were characterized by peak concentration in plasma (C_{max}), time to C_{max} (t_{max}), area under the plasma concentration–time curve (AUC) from time 0 to infinity [AUC(0-∞)], and elimination half-life (t_{1/2}) by use of noncompartmental methods as described earlier. The amount of tizanidine and its metabolites excreted into the urine within 24 hours (Ae) was calculated. The renal clearance (CL_{renal}) of tizanidine was calculated as CL_{renal} = Ae/AUC from 0 to 24 hours.

**Pharmacodynamics.** Systolic and diastolic blood pressures, heart rate, and 3 psychomotor tests were assessed before administration of tizanidine and immediately after each blood sampling, as described earlier. In short, in the Digit Symbol Substitution Test, the number of digits correctly substituted in 2 minutes was recorded. Subjective drowsiness and subjective drug effect were measured with a 100-mm-long horizontal visual analog scale. For each pharmacodynamic variable, the incremental or decremental area under the effect versus time curve from 0 to 12 hours was calculated by use of the linear trapezoidal rule. In addition, the maximum responses in each pharmacodynamic variable were calculated.

**Statistical analysis.** Results are expressed as mean values ± SD in the tables and text and, for clarity, as mean values ± SEM in the figures. The pharmacokinetic and pharmacodynamic variables between the groups were compared by the t test or, in the case of t_{max}, with Kruskal-Wallis 1-way ANOVA. Logarithmic transformation of pharmacokinetic variables was done before statistical analysis. For all variables except t_{max}, 95% confidence intervals were calculated on the mean differences between groups. The Pearson correlation coefficient was used to investigate possible relationships between tizanidine pharmacokinetic variables, the caffeine/paraxanthine ratio, and changes in pharmacodynamic variables. All of the data were analyzed with the statistical program Systat for Windows, version 6.0.1 (SPSS, Chicago, Ill). The differences were considered statistically significant at P < .05.

**RESULTS**

**Plasma tizanidine.** Plasma concentrations of tizanidine were considerably higher in the OC users than in the control subjects (Fig 2). The C_{max} of tizanidine was...
Fig 3. Individual and mean values for peak concentration in plasma (C<sub>max</sub>), elimination half-life (t<sub>1/2</sub>), and area under plasma concentration–time curve from time 0 to infinity (AUC) of tizanidine in 15 users of OCs (solid circles, 75 μg gestodene and 20 μg ethinyl estradiol; diamonds, 75 μg gestodene and 30 μg ethinyl estradiol) and 15 female control subjects (Ctrl) (open circles) after 4 mg tizanidine.

Fig 4. Plasma concentrations (mean ± SEM) of tizanidine metabolites M-3 (A) and M-4 (B), tizanidine/M-3 ratio (C), and tizanidine/M-4 ratio (D) in 15 users of OCs (solid circles) and 15 female control subjects (open circles) after 4 mg tizanidine.
3.0 times higher ($P < .001$) and the AUC(0–∞) was 3.9 times greater ($P < .001$), on average, in the OC users than in the control subjects, but there was no statistically significant difference in the $t_{1/2}$ or $t_{max}$ of tizanidine between the groups (Table I and Figs 2 and 3). The greatest individual value of tizanidine AUC(0–∞) in the OC group exceeded the mean AUC(0–∞) of tizanidine in the control group by nearly 20-fold and the greatest individual control tizanidine AUC(0–∞) value by nearly 10-fold (Fig 3). In the 3 subjects with a higher ethinyl estradiol dose (30 μg) in the OCs, the $C_{max}$ and AUC(0–∞) of tizanidine were similar to those in the other OC users (Fig 3).

**Tizanidine metabolites M-3 and M-4 in plasma.** In the OC group the mean $t_{1/2}$ ($P < .001$) and $t_{max}$ ($P = .006$) of M-3 were longer and the AUC(0–∞) ($P = .017$) was greater than in the control group (Table I and Fig 4). The AUC(0–∞) ($P = .066$) and $C_{max}$ ($P = .005$) of M-4 were smaller in the OC users than in the control subjects. Furthermore, in the users of OCs, the mean plasma tizanidine/M-3 AUC(0–∞) ratio was 2.2 times higher ($P < .001$) and the mean plasma tizanidine/M-4 AUC(0–∞) ratio was 5.2 times higher ($P < .001$) than in the control subjects.

**Excretion of tizanidine and its metabolites into urine.** The amount of unchanged tizanidine excreted in urine (Ae) was 3.8 times higher in the OC users than in the control subjects ($P = .008$), but there was no difference in its renal clearance between the groups (Table I and Fig 5). The Ae values for M-3 ($P = .006$) and M-10 ($P = .01$) were larger whereas the Ae of M-4 was smaller ($P < .001$) in the OC users than in the control subjects (Fig 5). However, all tizanidine/metabolite (M-3, M-4, M-5, M-9, and M-10) excretion ratios in urine were higher in the OC users than in the control subjects ($P < .05$).

**Pharmacodynamic variables.** There were no statistically significant differences in pharmacodynamic variables between the groups at baseline. In the control subjects, tizanidine reduced the systolic and diastolic blood pressures from baseline values (mean of the maximal reductions) by $-17$ mm Hg and $-13$ mm Hg, respectively (Fig 6 and Table II). The corresponding reductions were significantly greater in the OC users; that is, $-29$ mm Hg ($P = .002$) and $-21$ mm Hg ($P = .009$), respectively. In addition, the subjective drug effect was stronger in the OC users ($P = .01$) than in the control subjects. The effects of tizanidine on heart
the AUC(0-\infty) ratio correlated significantly with, for example, and caffeine/paraxanthine ratio.

The caffeine/paraxanthine concentration ratio was 2.8 times higher in users under plasma concentration–time curve from time 0 to infinity; CL renal 0-24 , renal clearance. contrasts a similar overlap in both the pharmacodynamic and pharmacokinetic data. In any case the majorit of the OC users had Cmax of tizanidine (Fig 6, B), demonstrating a similar overlap in both the pharmacodynamic and pharmacokinetic data. In any case the majority of the OC users had Cmax and AUC(0-\infty) values that were higher than the highest values of the control subjects. It is noteworthy that there was no significant difference between the OC and control groups in the t½ of tizanidine (Table I and Fig 3). The pharmacodynamic effects of tizanidine were greatly dependent on its plasma concentrations. Thus, for example, the maximal decrease in systolic blood pressure correlated well with the Cmax of tizanidine (Fig 6, B), demonstrating a similar overlap in both the pharmacodynamic and pharmacokinetic data. In any case the majority of the OC users had Cmax and AUC(0-\infty) values that were higher than the highest values of the control subjects. It is noteworthy that there was no significant difference between the OC and control groups in the t½ of tizanidine (Table I and Fig 3).

In vitro, gestodene is a potent inhibitor of CYP3A4,\textsuperscript{2,6} but even at 100 \mu mol/L (ie, 10,000 times higher than the therapeutic concentration in plasma), it has had no effect on CYP1A2, as measured by phenacetin O-deethylation.\textsuperscript{2} The effect of ethinyl estradiol on CYP1A2 activity has only been poorly investigated in vitro. However, CYP1A2 catalyzes the biotransfor-

**Table I. Pharmacokinetic variables of 4 mg tizanidine and its metabolites M-3 and M-4 in 15 users of oral contraceptives and 15 female control subjects**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control subjects</th>
<th>OC users</th>
<th>OC/control ratio and 95% CI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cmax (U)</td>
<td>0.85 ± 0.30</td>
<td>0.72 ± 0.19</td>
<td>0.84 (0.68-1.09)</td>
<td>.21</td>
</tr>
<tr>
<td>tmax (min)</td>
<td>90 (40-240)</td>
<td>120 (60-720)</td>
<td>.034</td>
<td></td>
</tr>
<tr>
<td>t½ (h)</td>
<td>4.18 ± 1.24</td>
<td>8.82 ± 6.07</td>
<td>2.11 (1.42-2.60)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>AUC(0-\infty) (U · h/mL)</td>
<td>5.32 ± 3.11</td>
<td>9.26 ± 7.20</td>
<td>1.74 (1.11-2.67)</td>
<td>.017</td>
</tr>
<tr>
<td>AUC(0-\infty) ratio (tizanidine/M-3)</td>
<td>1.09 ± 0.39</td>
<td>2.42 ± 0.76</td>
<td>2.22 (1.68-3.00)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>M-4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cmax (U)</td>
<td>1.55 ± 0.31</td>
<td>1.19 ± 0.33</td>
<td>0.77 (0.62-0.92)</td>
<td>.005</td>
</tr>
<tr>
<td>tmax (min)</td>
<td>180 (60-240)</td>
<td>180 (90-300)</td>
<td>.25</td>
<td></td>
</tr>
<tr>
<td>t½ (h)</td>
<td>4.52 ± 0.53</td>
<td>4.95 ± 1.23</td>
<td>1.10 (0.95-1.22)</td>
<td>.21</td>
</tr>
<tr>
<td>AUC(0-\infty) (U · h/mL)</td>
<td>12.65 ± 3.09</td>
<td>10.54 ± 2.43</td>
<td>0.83 (0.69-1.01)</td>
<td>.066</td>
</tr>
<tr>
<td>AUC(0-\infty) ratio (tizanidine/M-4)</td>
<td>0.52 ± 0.28</td>
<td>2.68 ± 2.62</td>
<td>5.15 (2.59-8.25)</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>

Data are given as mean ± SD or mean and 95% CI, except for tmax data, which are given as median and range. OC, Oral contraceptives; CI, confidence interval; Cmax, peak concentration in plasma; tmax, time to reach peak concentration in plasma; t½, half-life; AUC(0-\infty), area under plasma concentration–time curve from time 0 to infinity; CLrenal 0-24 , renal clearance.

rate, subjective drowsiness, and Digit Symbol Substitution Test were not significantly different between the groups. However, the effect of tizanidine on all pharmacodynamic variables, except heart rate, correlated significantly with the plasma concentration of tizanidine (data not shown). For example, there was a significant correlation (P < .001) between the Cmax of tizanidine and the maximal decrease in systolic blood pressure (Fig 6, B).

**Caffeine test.** The mean plasma caffeine/paraxanthine concentration ratio was 2.8 times higher in users of OCs (4.60 ± 2.56) than in the control subjects (1.62 ± 0.71) (P < .001).

**Correlations between tizanidine pharmacokinetics and caffeine/paraxanthine ratio.** The caffeine/paraxanthine ratio correlated significantly with, for example, the AUC(0-\infty), Cmax, and Ae of tizanidine, as well as with the tizanidine/M-3 and tizanidine/M-4 AUC(0-\infty) ratios (Table III and Fig 7). The subject who had the highest caffeine/paraxanthine ratio had, by far, the highest Cmax and AUC(0-\infty) of tizanidine (Fig 3). The caffeine/paraxanthine ratio and the tizanidine AUC(0-\infty) correlated strongly with the urinary tizanidine/metabolite (M-3, M-4, M-5, and M-9) Ae ratios, whereas the correlations with the tizanidine/M-10 Ae ratio were weaker (Table III).
mation of ethinyl estradiol to its main metabolite, 2-hydroxy–ethinyl estradiol. In previous studies, OCs have reduced the clearance of theophylline, caffeine, and antipyrine by 30% to 55% in humans, but the C_max of caffeine or theophylline has remained unaffected. The use of ethinyl estradiol (50 μg/d) has reduced caffeine clearance by 38% and the combination of ethinyl estradiol (50 μg/d) and levonorgestrel (125 μg/d) by 34%. In another study a combination of ethinyl estradiol (30 μg/d) and levonorgestrel (125 μg/d) or gestodene (75 μg) has reduced caffeine clearance by 54% and 55%, respectively.

On the other hand, the serum concentrations of selegiline have been 10 to 20 times higher in women using OCs containing ethinyl estradiol and gestodene or levonorgestrel compared with women without concomitant medications. CYP1A2 metabolizes selegiline, at least partly, in humans.

In our study the OC users had used the OC preparation for at least 1 complete cycle before the OC study cycle, and they had used OCs daily for 6 to 21 days immediately before the study day. Given that the half-lives of ethinyl estradiol and gestodene are on the order of 24 hours, the plasma concentrations of ethinyl estradiol and gestodene were close to the steady state on the study day. However, because ethinyl estradiol induces the formation of sex hormone–binding globulin, to which gestodene binds, concentrations of total plasma gestodene can continue to increase for the whole cycle. In any case there was no significant correlation (data not shown) between the day of OC use (ranging from 6 to 21 days) and the caffeine/paraxanthine ratio or the pharmacokinetic variables of tizanidine.

In the 3 women who used a higher dose of ethinyl estradiol (30 μg compared with 20 μg in the other 12 OC users), the caffeine/paraxanthine ratios and tizanidine concentrations were not higher than in the other OC users (Fig 3). Furthermore, there were no differences in the extent of interaction between women using different brands of OCs (data not shown). There can be considerable interindividual differences in the plasma concentrations of both gestodene and ethinyl estradiol in OC users, governed mainly by genetic and acquired factors. In addition, the time interval between the ingestion of OCs and tizanidine might have an effect on the extent of interaction. In our study, however, all OC users ingested tizanidine exactly 1 hour after the OC.

The caffeine/paraxanthine ratio reflects mainly systemic CYP1A2 activity, because caffeine, in contrast to tizanidine, lacks significant presystemic metabolism. The mean caffeine/paraxanthine ratio was nearly 3 times higher in the users of OCs than in the control subjects, indicating a moderately strong inhibition of systemic CYP1A2 activity by OCs. For comparison, ciprofloxacin (500 mg twice daily) and fluvoxamine (100 mg/d) have increased caffeine/paraxanthine ratios by 2.1-fold and 12.5-fold, respectively. Ciprofloxacin and fluvoxamine have increased the C_max and AUC of tizanidine even more than did OCs in our study, and only fluvoxamine has markedly increased the t½ of tizanidine. In the OC users the C_max and AUC of tizanidine were higher than in the control subjects, but there was no significant difference between the groups in the t½ of tizanidine, indicating that mainly the presystemic metabolism of tizanidine was affected by the OCs, with a minimal effect on its systemic elimination. It should be noted that the small, statistically nonsignificant (19%) increase in the mean t½ was a result of 1 OC user with a long t½ (Fig 3).
Tizanidine is biotransformed to several metabolites (eg, M-3, M-4, M-5, M-9, and M-10), of which M-3 and M-4 are the main metabolites in plasma (Fig 1).24 The excretion of M-3 into urine was greater whereas M-4 was reduced by the OCs. The t½ of M-4 was smaller in the OC users than in control subjects (Fig 5), consistent with inhibition of the formation of these metabolites by the OCs.

About 100 million women are using OCs for contraception. Thus concomitant use of tizanidine and OCs may not be uncommon in countries where tizanidine is in general use. The effects of OCs on tizanidine pharmacokinetics and pharmacodynamics are clinically significant as observed, for example, in the effects on blood pressure (Fig 6, A). Although the mean effects of OCs on tizanidine are not as strong as those of ciprofloxacin and fluvoxamine, which have increased tizanidine AUC by 10-fold and 33-fold and Cmax by 7-fold and 12-fold, respectively,22,23 the effects can be alarmingly strong in certain subjects. In 1 of 15 healthy OC users, the AUC of tizanidine was nearly 20 times larger than in the control subjects, and her blood pressure fell from 111 mm Hg/74 mm Hg at baseline to 79 mm Hg/47 mm Hg with tizanidine. Thus, in an individual woman, the effect of OCs on the pharmacokinetics and pharmacodynamics of tizanidine can be much stronger than what could be inferred from

### Table II. Pharmacodynamic variables: Maximum changes from baseline values (minimum or maximum) and incremental/decremental AUC(0-12) for blood pressures, heart rate, and psychomotor tests (subjective drowsiness, subjective drug effect, and DSST) after 4 mg tizanidine in 15 users of oral contraceptives and 15 female control subjects

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control subjects</th>
<th>OC users</th>
<th>Difference between phases and 95% CI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic blood pressure</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minimum (mm Hg)</td>
<td>−17 ± 9</td>
<td>−29 ± 10</td>
<td>−12 (−19 to −4)</td>
<td>.002</td>
</tr>
<tr>
<td>Decremental AUC(0-12) (mm Hg · h)</td>
<td>−55 ± 90</td>
<td>−111 ± 85</td>
<td>−56 (−121 to 10)</td>
<td>.093</td>
</tr>
<tr>
<td>Diastolic blood pressure</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minimum (mm Hg)</td>
<td>−13 ± 5</td>
<td>−21 ± 8</td>
<td>−7 (−12 to −29)</td>
<td>.009</td>
</tr>
<tr>
<td>Decremental AUC(0-12) (mm Hg · h)</td>
<td>−73 ± 58</td>
<td>−118 ± 85</td>
<td>−46 (−100 to 9)</td>
<td>.1</td>
</tr>
<tr>
<td>Heart rate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minimum (beats/min)</td>
<td>−8 ± 5</td>
<td>−9 ± 4</td>
<td>−27 (−93 to 39)</td>
<td>.41</td>
</tr>
<tr>
<td>Incremental AUC(0-12) (beats/min · h)</td>
<td>59 ± 97</td>
<td>32 ± 78</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Drowsiness (VAS)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maximum (symbols/2 min)</td>
<td>−58 ± 104</td>
<td>−14 ± 9</td>
<td>44 (−11 to 99)</td>
<td>.11</td>
</tr>
<tr>
<td>Incremental AUC(0-12) (symbols/2 min · h)</td>
<td>−36 ± 45</td>
<td>−61 ± 61</td>
<td>−25 (−65 to 15)</td>
<td>.22</td>
</tr>
</tbody>
</table>

Data are given as mean ± SD or mean and 95% CI.

AUC(0-12), Area under effect versus time curve from time 0 to 12 hours; VAS, visual analog scale; DSST, Digit Symbol Substitution Test.
If a 50% reduction in tizanidine clearance by OCs is assumed. On the other hand, there was a significant variability in tizanidine pharmacokinetics within the 2 groups, as well as a fair amount of overlap between them, which makes prediction of individual tizanidine response difficult. Furthermore, it should be noted that the OC preparations used in this study contained ethinyl estradiol and gestodene; the findings cannot be directly generalized to OC products containing progestogens or estrogens other than those used in our study.

In conclusion, OCs containing gestodene and ethinyl estradiol can markedly increase the plasma concentration and effects of tizanidine, probably mainly by inhibiting its CYP1A2-mediated presystemic metabolism. Because the therapeutic range of tizanidine is narrow, care should be exercised when tizanidine is prescribed to OC users.

None of the authors has any financial or personal relationships that could be perceived as influencing the research described.

Table III. Correlations of caffeine/paraxanthine ratio and AUC(0-\(\infty\)) of tizanidine with AUC(0-\(\infty\)), \(C_{\text{max}}\), and \(t_{1/2}\) of tizanidine, tizanidine/M-3 and tizanidine/M-4 AUC(0-\(\infty\)) ratios, amount of tizanidine excreted into urine, and tizanidine/metabolite (M-3, M-4, M-5, M-9, and M-10) ratios in urine after 4 mg tizanidine in 15 users of oral contraceptives and 15 female control subjects.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Caffeine/paraxanthine ratio</th>
<th>Tizanidine AUC(0-(\infty))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tizanidine in plasma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AUC(0-(\infty))</td>
<td>0.87</td>
<td>0.94</td>
</tr>
<tr>
<td>(C_{\text{max}})</td>
<td>0.91</td>
<td>0.86</td>
</tr>
<tr>
<td>(t_{1/2})</td>
<td>0.73</td>
<td>0.67</td>
</tr>
<tr>
<td>AUC(0-(\infty)) ratio</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tizanidine/M-3</td>
<td>0.78</td>
<td>0.92</td>
</tr>
<tr>
<td>Tizanidine/M-4</td>
<td>0.92</td>
<td>0.95</td>
</tr>
<tr>
<td>Ae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tizanidine</td>
<td>0.87</td>
<td>0.96</td>
</tr>
<tr>
<td>Ae ratio</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tizanidine/M-3</td>
<td>0.80</td>
<td>0.94</td>
</tr>
<tr>
<td>Tizanidine/M-4</td>
<td>0.83</td>
<td>0.97</td>
</tr>
<tr>
<td>Tizanidine/M-5</td>
<td>0.86</td>
<td>0.93</td>
</tr>
<tr>
<td>Tizanidine/M-9</td>
<td>0.69</td>
<td>0.71</td>
</tr>
</tbody>
</table>

* \(r\) Value, Correlation coefficient; Ae, amount excreted in urine within 24 hours.

\(\* P < .001\) for all correlations except for tizanidine/M-10 Ae ratios \((P < .01)\).

Fig 7. Relationships between plasma caffeine/paraxanthine ratio and tizanidine AUC(0-\(\infty\)) (A), tizanidine/M-3 AUC(0-\(\infty\)) ratio (B), and tizanidine/M-4 AUC(0-\(\infty\)) ratio (C) in 15 users of OCs (solid circles) and 15 female control subjects (open circles) after 4 mg tizanidine.

References


27. Spigset O, Hägg S, Söderström E, Dahlqvist R. The paraxanthine:caffeine ratio in serum or in saliva as a measure of CYP1A2 activity: when should the sample be obtained? Pharmacogenetics 1999;9:409-12.


PHARMACODYNAMICS AND DRUG ACTION

Safety, pharmacodynamics, and pharmacokinetics of single doses of BAY 59-7939, an oral, direct factor Xa inhibitor

Background and Objective: There is a clinical need for new oral anticoagulants to prevent and treat thromboembolic diseases. Given its integral role in the coagulation cascade, factor Xa is a particularly promising target for new anticoagulation therapies. The aim of this study was to investigate the safety, pharmacodynamics, and pharmacokinetics of BAY 59-7939, an oral, direct factor Xa inhibitor.

Methods: This single-center, randomized, single-blinded, placebo-controlled, dose-escalation study included 108 healthy white male subjects aged 19 to 45 years. Subjects received single oral doses of either BAY 59-7939 (1.25-80 mg) or placebo; in addition, 1 group received 2 doses of BAY 59-7939 (5-mg tablet and oral solution) or placebo in a crossover design.

Results: Oral BAY 59-7939 in single doses up to 80 mg was safe and well tolerated and was not associated with an increased risk of bleeding compared with placebo. Pharmacodynamic effects (inhibition of factor Xa activity, prothrombin time, activated partial thromboplastin time, and HepTest) and plasma concentration profiles were dose-dependent. Maximum inhibition of factor Xa activity was achieved 1 to 4 hours after administration of BAY 59-7939 and ranged from 20% to 61% for the 5- to 80-mg doses. BAY 59-7939 selectively inhibited factor Xa activity; thrombin (factor IIa) and antithrombin were unaffected. Inhibition of factor Xa activity and prolongation of prothrombin time correlated well with BAY 59-7939 plasma concentrations ($r = 0.949$ and 0.935, respectively).

Conclusions: BAY 59-7939 was well tolerated with predictable pharmacodynamics and pharmacokinetics across a wide range of doses in healthy male subjects. BAY 59-7939 was shown to be an effective and specific factor Xa inhibitor. (Clin Pharmacol Ther 2005;78:412-21.)

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Thromboembolic disorders are a major cause of morbidity and death. Orthopedic surgery, orthopedic trauma, acute coronary syndromes, and atrial fibrillation are well-documented risk factors for thromboembolic events, such as deep vein thrombosis, pulmonary embolism, and stroke. For example, 50% to 80% of patients undergoing major orthopedic surgery, particularly total hip or knee replacement, are at risk of venous thromboembolism; the risk of pulmonary embolism has been reported to be as high as 10%. Prophylaxis with an anticoagulant can reduce these risks, and guidelines recommend the routine use of drugs such as low-molecular weight heparins (LMWHs), fondaparinux, and vitamin K antagonists (eg, warfarin).
Elective major orthopedic surgery, particularly total hip or knee replacement, is widely accepted to be a useful model in which to assess the safety and efficacy of new antithrombotic agents. Consequently, new anticoagulants are typically first assessed in this model before further studies are performed for other indications requiring long-term anticoagulation.

Most of the serine proteases within the coagulation cascade are potential therapeutic targets for new anticoagulants (Fig 1). Currently available anticoagulants act at various points in the coagulation cascade. Orally administered warfarin inhibits the functional synthesis of the serine protease thrombin and several clotting factors, including factors VII, IX, and X, by interfering with the vitamin K–mediated carboxylation of their precursors. Although warfarin is the mainstay of long-term anticoagulation, its pharmacologic profile and narrow therapeutic window warrant frequent coagulation monitoring; these factors result in underutilization and poor compliance.

Factor Xa is an integral component of the coagulation cascade and is generated via both the intrinsic and extrinsic pathways. It is also the rate-limiting step for the propagation of thrombin generation and, thus, is a particularly attractive target for anticoagulation. Studies of fondaparinux demonstrate that selective factor Xa inhibition, albeit indirect, provides effective thromboprophylaxis.

Unfractionated heparin (UFH) inhibits both thrombin and factor Xa, in a ratio of 1:1, via the UFH–antithrombin (AT) complex; UFH also binds to heparin cofactor II, which inhibits thrombin further. Introduced in the 1980s, the LMWHs are the established standard of care for the prevention of venous thromboembolism in hospitalized patients after orthopedic surgery. LMWHs inhibit factor Xa more strongly than thrombin, with a ratio of 1.5:1 to 4:1 depending on the particular LMWH. Both UFH and LMWHs also induce the release of tissue factor pathway inhibitor, which may enhance anticoagulation further. The synthetic pentasaccharide fondaparinux, launched in 2003, selectively inhibits factor Xa and has been shown to be superior to LMWHs in orthopedic surgery.

Parenterally administered, specific direct thrombin inhibitors, such as hirudin, are effective and have been approved for clinical use; however, they have a narrow therapeutic window, which limits their clinical use to special populations.

BAY 59-7939 is an oral, direct factor Xa inhibitor in development for the prevention and treatment of thromboembolic diseases. It is a member of a new class of small-molecule, active site–directed factor Xa inhibitors. In preclinical studies BAY 59-7939 has demonstrated dose-dependent inhibition of factor Xa activity and prolongation of global clotting tests (prothrombin time [PT] and activated partial thromboplastin time [aPTT]) in vitro and in vivo. In animal models BAY 59-7939 effectively inhibited thrombus formation without significantly affecting bleeding time. Here we report a clinical study with BAY 59-7939, which was performed to investigate its safety, tolerability, and pharmacodynamic effects over a wide range of single oral doses in healthy male subjects. The pharmacokinetics of BAY 59-7939 was also assessed.
METHODS

Subjects. A total of 108 healthy male subjects, who were aged 19 to 45 years and of normal body weight (body mass index between 18 and 32 kg/m²), were enrolled in this single-center, randomized, single-blinded, placebo-controlled, dose-escalation study. Subjects were ineligible if they had any known coagulation disorders, increased bleeding risk, or sensitivity to common causes of bleeding, such as acute gastroenteritis and nasal bleeding.

Study treatments. In the first dose step each subject received a single 1.25-mg tablet of BAY 59-7939 or placebo. In the second dose step each subject was given 2 doses of BAY 59-7939 (5-mg tablet and 5-mg oral solution) or corresponding placebo, in a crossover design. In step 3 subjects received either a single oral dose of 10-mg BAY 59-7939 solution or placebo. In steps 4 to 10 they received either single doses of BAY 59-7939 (10-, 15-, 20-, 30-, 40-, 60-, or 80-mg tablets) or placebo. At each dose step, it was planned that 8 subjects would receive BAY 59-7939 and 4 subjects would receive placebo; escalation to each new dose step was dependent on the safety of the preceding dose step. BAY 59-7939 was given in the morning after a fasting period of at least 10 hours. All study drugs were supplied by Bayer HealthCare AG (Wuppertal, Germany).

Each subject provided written informed consent before enrollment in this study, and full ethics committee approval was received from the North Rhine Medical Council (Düsseldorf, Germany) before commencement. In addition, this study was conducted in accordance with the Declaration of Helsinki, Good Clinical Practice guidelines, and German drug law.25,26

Safety and tolerability. Safety and tolerability were assessed for up to 48 hours after administration of BAY 59-7939. Tolerability was evaluated objectively by monitoring of vital signs (including evaluation of electrocardiograms) and clinical laboratory tests, such as hematologic testing, clinical chemistry testing, and urinalysis, and subjectively by questioning subjects about any adverse events, as well as by spontaneous reporting of adverse events. Adverse events were classified according to their severity. Bleeding time and Rumpel-Leede tests to assess potential bleeding risk or capillary leakage were performed.

Sample analysis. The pharmacodynamic effects of BAY 59-7939 were assessed by evaluation of factor Xa activity, PT, aPTT, and HepTest (LMWH activity assay). The direct action of BAY 59-7939 on factor Xa was evaluated by measurement of AT and thrombin activity. The pharmacokinetic parameters of BAY 59-7939 were calculated from the plasma concentration–time data by noncompartmental methods.

Blood samples for pharmacodynamic assays were centrifuged, and the resulting plasma samples were frozen and stored below −15°C until analyzed at the Biomarker and Pharmacogenetics Laboratory at Bayer HealthCare AG. After sample dilution, factor Xa, thrombin, and AT activities were determined by photometric assays (Chromogenix, Milan, Italy; method based on supplier’s instructions). In brief, factor Xa activity was determined by a 2-step process: In the first step total factor X in plasma was activated to factor Xa via Russell’s viper venom in the presence of calcium ions; subsequently, the chromogenic substrate ZD-Arg-Gly-Arg-pNA (S-2765; Chromogenix) was then hydrolyzed by the factor Xa releasing the chromogenic group pNA (p-nitroanilin). The quantity of pNA released, which is proportional to the factor Xa activity, was determined by photometer at 405 nm. The activity of factor IIa was determined after ecarin activation by use of the chromogenic substrate HD-Phe-Pip-Arg-pNA (S-2238; Chromogenix). All standards and controls were prepared from the 3rd International Standard Coagulation Factors II and X Concentrate, Human, 98/590 (National Institute for Biological Standards and Control, Potters Bar, United Kingdom).

To determine AT activity, plasma was incubated with an excess of factor Xa and hirudin, and the quantity of unbound factor Xa was then determined via cleavage of S-2765 and release of pNA, measured by photometer at 405 nm. This is inversely proportional to the AT activity of the plasma sample. All reagents used were obtained from the COAMATIC Antithrombin III kit (Chromogenix). All standards were prepared from Calibration Plasma (Haemochrom Diagnostica, Essen, Germany). All controls were prepared from the Second International Standard Antithrombin Plasma, Human, 93/768 (National Institute for Biological Standards and Control).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y, median and range)</td>
<td>33.0 (19-45)</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>81.2 (52-106)</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>24.9 (19.3-31.7)</td>
</tr>
</tbody>
</table>

**Table I.** Subject demographics (all subjects valid for safety, n = 103)
Factor Xa activities above 0.1 IU/mL (the lower limit of quantification) were determined with a precision of 9.5% to 14% and an accuracy of 99.5% to 114%. PT (assessed by use of freeze-dried thromboplastin from rabbit brain [Neoplastin Plus; Roche Diagnostics, Mannheim, Germany]), aPTT (assessed by use of a kaolin-activated test [Roche Diagnostics]), and HepTest (Haemachem, St Louis, Mo) were measured with a ball coagulometer (KC 10; Amelung, Lemgo, Germany) according to the manufacturer’s instructions.

Blood samples for pharmacokinetic analysis were centrifuged and then stored below −15°C until ana-

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**Fig 3.** Pharmacodynamic parameters after administration of BAY 59-7939 tablets. Median change from baseline in factor Xa inhibition (A), prothrombin time (PT) (B), activated partial thromboplastin time (aPTT) (C), HepTest (D), ecarin-stimulated thrombin activity (E), and antithrombin activity (F). Data are not shown for 15-, 30-, and 60-mg tablets.
lyzed at the bioanalytic laboratories at Bayer HealthCare AG. After solid/liquid extraction, the resulting plasma samples were analyzed by a fully validated HPLC method by use of tandem mass spectrometry detection techniques. Concentrations greater than 0.5 to 2 μg/L (the lower limit of quantification) were determined with a precision of less than 6.8% and an accuracy of 88.7% to 95.0%.

Statistical analyses. Statistical evaluations were performed by use of SAS software (SAS Institute, Cary, NC). Mean values were only calculated if at least two thirds of the individual data were measured and were above the limit of quantification (LOQ). For calculation of the mean value, data points below the LOQ were substituted by 50% of the LOQ.

RESULTS

Study population. Of the 108 healthy white male subjects enrolled in the study, 4 were withdrawn because of protocol violations and 1 withdrew consent;
data from 103 subjects are included in these analyses (Table I).

**Safety and tolerability.** There were no withdrawals because of adverse events. Overall, the incidence of adverse events was similar in subjects receiving BAY 59-7939 or placebo. Of the 103 healthy subjects included in the trial, 29 (9 in the placebo group and 20 in the BAY 59-7939 groups) reported 39 adverse events. Of the 39 adverse events, 6 were judged to be possibly related to BAY 59-7939: 3 cases of having a taste of blood at the 1.25-, 10-, and 80-mg doses (no traces of blood were found and these incidents resolved within 30-105 minutes); 2 cases of ecchymosis at the 80-mg dose (1 at the puncture site and 1 on the contralateral arm, both of which resolved spontaneously); and 1 headache, which responded to treatment with a proprietary analgesic. One serious adverse event occurred, which involved medically important increases in creatine kinase level. This was subsequently attributed to extreme physical exercise on the day before the follow-up visit and was not considered by the investigator to be related to study medication.

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**Fig 5.** Plasma concentration–time profiles of BAY 59-7939 after administration of BAY 59-7939 tablets (data not shown for 15-, 30-, and 60-mg tablets) (A) and 5 and 10 mg BAY 59-7939 as oral solution or tablet (B). Data are geometric means.
**Table II.** Pharmacokinetic parameters of BAY 59-7939 in plasma

<table>
<thead>
<tr>
<th>Parameter</th>
<th>1.25-mg tablet (n = 8)</th>
<th>5-mg solution (n = 6)</th>
<th>5-mg tablet (n = 6)</th>
<th>10-mg solution (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC (µg · h/L)</td>
<td>119/24.5 (94-189)</td>
<td>461/17.2 (348-587)</td>
<td>446/23.0 (348-677)</td>
<td>997/25.1 (613-1383)</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;norm&lt;/sub&gt; (g · h/L)</td>
<td>7386/26.5 (5647-11,630)</td>
<td>7734/18.9 (6626-11,040)</td>
<td>7479/21.4 (5155-9213)</td>
<td>8366/35.3 (4291-12,080)</td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt; (µg/L)</td>
<td>23/22.4 (17-33)</td>
<td>119/18.5 (97.2-158)</td>
<td>72/19.7 (55-96)</td>
<td>266/25.1 (187-412)</td>
</tr>
<tr>
<td>C&lt;sub&gt;max,norm&lt;/sub&gt; (g/L)</td>
<td>1436/29.9 (919-2229)</td>
<td>199/28.6 (1339-2967)</td>
<td>1208/20.0 (914-1484)</td>
<td>2231/31.8 (1312-3501)</td>
</tr>
<tr>
<td>t½ (h)</td>
<td>3.93/35.5 (2.94-7.53)</td>
<td>3.24/8.4 (2.89-3.70)</td>
<td>4.27/25.3 (3.29-6.14)</td>
<td>4.15/20.8 (2.87-5.13)</td>
</tr>
<tr>
<td>V&lt;sub&gt;f&lt;/sub&gt; (L/kg)</td>
<td>0.77/40.7 (0.46-1.65)</td>
<td>0.605/18.3 (0.442-0.718)</td>
<td>0.82/30.7 (0.58-1.14)</td>
<td>0.716/19.5 (0.532-0.965)</td>
</tr>
<tr>
<td>CL&lt;sub&gt;f&lt;/sub&gt; (L/h)</td>
<td>10.5/24.5 (6.6-13.4)</td>
<td>10.8/17.2 (8.51-14.4)</td>
<td>11.2/23.0 (7.4-14.4)</td>
<td>10.0/25.1 (7.23-16.3)</td>
</tr>
<tr>
<td>A&lt;sub&gt;exr&lt;/sub&gt; (%)</td>
<td>41.8/10.3* (30.5-64.9)</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>t&lt;sub&gt;max&lt;/sub&gt; (h)</td>
<td>3.00 (2.50-4.00)</td>
<td>0.63 (0.50-0.75)</td>
<td>1.88 (0.50-4.00)</td>
<td>0.50 (0.25-1.00)</td>
</tr>
</tbody>
</table>

Data are given as geometric mean values/percent geometric coefficient of variation and range, unless otherwise indicated. Data are not shown for 15-, 30-, and 60-mg tablets.

AUC, Area under plasma concentration–time curve; C<sub>max</sub>, peak plasma concentration; t<sub>½</sub>, terminal half-life; t<sub>max</sub>, time to peak plasma concentration; AUC<sub>norm</sub>, area under concentration-time curve divided by dose per kilogram of body weight; C<sub>max,norm</sub>, maximum drug concentration in plasma divided by dose per kilogram of body weight; V<sub>f</sub>/f, apparent volume of distribution during terminal phase after oral administration; CL/f, total plasma clearance calculated after oral administration (apparent oral clearance); A<sub>exr</sub>, amount of drug excreted via urine.

*Arithmetic mean ± SD and range, 0- to 48-hour sampling interval.
†Arithmetic mean ± SD and range, 0- to 72-hour sampling interval.
‡Median and range.

**Fig 6.** Correlation between plasma concentration of BAY 59-7939 and factor Xa (FXa) inhibition (A) and PT (B).
No clinically relevant signs or symptoms of bleeding were observed. Isolated prolongation of the bleeding time of more than twice the baseline value occurred after placebo and low doses of BAY 59-7939 but not after high doses. Vessel leakage was not detected by the Rumpel-Leede test, performed when plasma concentrations of BAY 59-7939 were at or near maximum.

**Pharmacodynamics.** Median inhibitions of factor Xa activities ranged from 20% to 61% for the 5- to 80-mg tablet doses, and factor Xa activity was inhibited up to 75% in some subjects at the highest dose tested (80 mg) (Fig 3, A; only the most relevant data are shown [15-, 30-, and 60-mg dose steps are not included]). No significant inhibition of factor Xa activity was observed for the 1.25-mg dose compared with placebo. Inhibition data of factor Xa activities for the oral solution (5 and 10 mg) are presented in Fig 4.

Maximum inhibition of factor Xa activity occurred 45 minutes after administration of the oral solution and 1 to 4 hours after administration of BAY 59-7939 tablets. The half-life of the biologic effect was 4 hours for the solution and 6 to 7 hours for the tablet. Factor Xa activities had not completely returned to baseline at 24 hours for doses above 5 mg. PT prolongation followed a similar profile to inhibition of factor Xa activities. At the 80-mg dose, the response peaked at twice the baseline value by 2 hours and had not completely reverted to baseline after 24 hours (Fig 3, B).

BAY 59-7939 prolonged aPTT and HepTest (Fig 3, C and D); the profiles were similar in shape to those for PT prolongation and inhibition of factor Xa activity and demonstrated a change of 1.5- and 2.3-fold from baseline, respectively, at the 80-mg dose level. BAY 59-7939 had no effect on thrombin and AT activity (Fig 3, E and F).

**Pharmacokinetics.** After administration of the oral solution, BAY 59-7939 was absorbed rapidly and reached peak plasma concentrations after approximately 30 minutes (Fig 5, A). These plasma concentrations decreased rapidly, resulting in a terminal half-life of 3 to 4 hours. After administration of oral tablets, peak plasma concentrations of BAY 59-7939 were reached after 2 hours (Fig 5, B) and were 50% lower compared with solution. However, the area under the plasma concentration–time curve (AUC) values were similar for both formulations. At BAY 59-7939 doses greater than 10 mg, increases in peak plasma concentration and AUC were less than dose-proportional. The terminal half-lives ranged from 7 to 17 hours (Fig 5, A, and Table II).

Urinalysis for unchanged BAY 59-7939 showed that approximately 40% of the administered dose was excreted renally in the unchanged form after administration of the 1.25-mg dose. However, at the 60- and 80-mg doses, only approximately 10% of the administered dose was present in urine as the parent drug. The pharmacokinetic parameters are summarized in Table II (only the most relevant data are shown [15-, 30-, and 60-mg dose steps are not included]).

**Pharmacokinetic and pharmacodynamic correlation.** Inhibition of factor Xa activity (Fig 6, A) and PT prolongation (Fig 6, B) both correlated strongly with BAY 59-7939 plasma concentrations ($r = 0.949$ and $0.935$, respectively).

**DISCUSSION**

There is a medical need for new oral anticoagulants that offer effective and predictable anticoagulation without increased risk of adverse events, such as bleeding or drug interactions. In this study oral BAY 59-7939 was shown to have predictable anticoagulant activity without any clinically relevant signs or symptoms of bleeding. Single doses of BAY 59-7939 (5-80 mg) resulted in sustained, dose-dependent inhibition of factor Xa activity and prolongation of PT, without a significant effect on thrombin or AT activity. The phar-
macokinetics of BAY 59-7939 was predictable and dose-dependent over a wide dose range. Furthermore, pharmacokinetic parameters correlated closely with inhibition of factor Xa activity and PT prolongation.

The results of this study confirm that BAY 59-7939 does not enhance AT activity (Fig 3, F) and does not exhibit anti-IIa activity; this supports in vitro findings. Conversely, indirect factor Xa inhibitors, such as fondaparinux, LMWHs, and UFH, all require AT to be effective. The resulting AT-drug combination is too large to inhibit factor Xa within the prothrombinase complex or within a pre-existing clot, thereby limiting their effects to free factor Xa only. In contrast, the direct factor Xa inhibitor BAY 59-7939 not only inhibits free factor Xa but also inhibits factor Xa within the prothrombinase complex. Hypothetically, this inhibition should also extend to activity within the clot, if the molecule is small enough and does not require cofactors, which may bring additional benefits when treating patients with a pre-existing clot. This is the subject of ongoing research.

The ideal serine protease to inhibit within the coagulation cascade is the subject of continuing debate. UFH inhibits factor Xa and thrombin and also binds to platelet factor 4 (PF4), which may cause heparin-induced thrombocytopenia (HIT), an autoimmune response. The LMWHs also bind to PF4; therefore HIT may still occur with these drugs. As an indirect factor Xa inhibitor, fondaparinux does not bind to either platelets or PF4, and HIT has not been reported. Although extremely rare, cross-reactivity with HIT sera has been reported; therefore the risk of inducing HIT cannot be completely disregarded. Although parenteral direct thrombin inhibitors have been developed and are approved for clinical use, they are limited by a narrow therapeutic window and, therefore, cannot be used widely. Ximelagatran, an oral, direct thrombin inhibitor, has been approved for limited use in orthopedic surgery; however, concerns remain over its long-term safety. BAY 59-7939 is selective for factor Xa and shows no direct effect on thrombin (Fig 3, E); this also confirms in vitro findings.

Data from studies of families with factor Xa deficiency showed that spontaneous bleeding only occurred when factor Xa levels were lowered to 5% of normal levels. Although these data suggest that factor Xa can be markedly suppressed without affecting hemostasis, any clinical implication of such findings on the efficacy or safety of factor Xa inhibitors, or how this compares with direct thrombin inhibition, has yet to be determined.

In addition to the pharmacologic effects, the biopharmaceutical properties of a drug are important to ensure predictable pharmacokinetics. An oral solution of BAY 59-7939 was used to allow flexible dosing in the first steps of this study and also for the determination of the relative bioavailability, which was found to be approximately 80%. The solution was absorbed rapidly, achieving higher plasma concentrations, and also had a shorter time to peak plasma concentration; however, it resulted in greater differences in the peak/trough ratio (Fig 5).

The pharmacokinetics of BAY 59-7939 tablets was dose-dependent; however, at doses above 10 mg, less than dose-proportional increases in peak plasma concentration and AUC were observed. This suggests that limited solubility may be responsible for incomplete absorption of higher doses of the tablet formulation of BAY 59-7939. This is consistent with the apparent lower proportion of BAY 59-7939 excreted unchanged in the urine at the highest doses (60 and 80 mg) compared with the lowest dose (1.25 mg) in this study (10% versus 40%, respectively). A benefit of the decreased bioavailability of the highest doses of BAY 59-7939 may be to reduce the risk of unintentional overdosing.

The availability of both pharmacodynamic and pharmacokinetic data for such a large number of patients enabled us to show a good correlation between these parameters, particularly between BAY 59-7939 concentrations and PT and factor Xa activity tests. Therefore tests such as PT may be used in future trials for monitoring, if necessary.

BAY 59-7939 demonstrated sustained dose-dependent inhibition of factor Xa and prolongation of global clotting tests across a wide range of doses, without significantly affecting AT or thrombin activity. The pharmacokinetics of BAY 59-7939 was predictable and dose-dependent and correlated well with its pharmacodynamic effects. All doses were well tolerated, with no evidence of an increased risk of bleeding. The favorable pharmacodynamic, pharmacokinetic, and safety profiles of BAY 59-7939 provide the foundation for future studies to determine the clinical benefits of BAY 59-7939 in greater detail.

All authors are employees of Bayer HealthCare AG, Wuppertal, Germany, the sponsors of this study.

References

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Glutathione peroxidase, thioredoxin, and membrane protein changes in erythrocytes predict ribavirin-induced anemia

Objective: Low erythrocyte membrane protein sulfhydryl concentrations are a risk factor for ribavirin-induced anemia. We further studied the role of oxidative stress and erythrocyte membrane alterations in ribavirin-induced anemia.

Methods: The levels of thioredoxin, glutathione peroxidase, protein sulfhydrils, and protein-mixed disulfides, as well as the electrophoretic membrane protein pattern, were determined in freshly isolated erythrocytes from healthy control subjects, patients without severe anemia during previous ribavirin treatment (still hepatitis C virus [HCV]–positive), and patients who had had severe anemia with ribavirin (still HCV-positive or HCV-negative), 6 months after full recovery. Erythrocytes were also incubated with buffer, ribavirin, phenylhydrazine, or dehydroepiandrosterone, and concentrations of protein sulfhydrils, protein-mixed disulfides, thiobarbituric acid–reactive substances, and total and oxidized glutathione, as well as osmotic resistance, were determined.

Results: Patients with previous severe ribavirin-induced anemia had lower levels of protein sulfhydrils (30.9 nmol/mg protein versus 43.2 nmol/mg protein, \( P < .001 \)) and thioredoxin (0.6 nmol/g hemoglobin versus 1.2 nmol/g hemoglobin, \( P < .001 \)), higher levels of protein-mixed disulfides (1.5 nmol/g hemoglobin versus 0.5 nmol/g hemoglobin, \( P < .001 \)) and glutathione peroxidase (618 mU/mg protein versus 393 mU/mg protein, \( P < .001 \)), and a membrane protein pattern consistent with band 4 dimer disaggregation. These differences were independent of HCV seropositivity. There were negative correlations between levels of glutathione peroxidase and thioredoxin (\( r = -0.87 \)) and between levels of protein sulfhydrils and protein-mixed disulfides (\( r = -0.93 \)). In vitro studies showed that erythrocytes of patients who had had hemolysis during treatment of HCV are more susceptible to oxidative stress.

Conclusions: Pronounced differences in markers of oxidative stress and membrane proteins exist between patients with and without a history of ribavirin-induced anemia. Our findings suggest that there are erythrocyte-related risk factors for ribavirin-induced severe anemia. (Clin Pharmacol Ther 2005;78:422-32.)

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Severe anemia has been associated with a large number of drugs, and may occur as a rare idiosyncratic adverse reaction that is mediated by autoimmune mechanisms. In contrast, few drugs have a known intrinsic erythrocyte toxicity that affects most if not all patients. These mechanisms of toxicity include oxidative damage to erythrocyte constituents and disruption of membrane structures. In the first case, iron-mediated free radicals and reactive compounds lead to oxidative changes in membrane thiols and lipids, as well as formation of methemoglobin. In these cases (menadione, dapsone, phenacetin, or phenylhydrazine), iron-
chelating agents have shown protective potential against lipid peroxidation and hemolysis.\(^6\)\(^-\)\(^8\) In contrast, nonoxidative erythrotoxic damages involve neither hemoglobin oxidation nor lipid peroxidation (tamoxifen citrate [INN, tamoxifen] and dehydroepiandrosterone [DHEA]), and intercalation into membranes is the supposed injuring mechanism.\(^4\)\(^,\)\(^9\) Oxidative and nonoxidative alterations may subsequently cause anemia by hemolysis of circulating erythrocytes or an increased splenic removal of “presenile” or damaged erythrocytes from the circulation. However, in many cases the exact mechanism of drug-induced anemia remains unknown.

Ribavirin is an antiviral synthetic nucleoside analog used in the treatment of hepatitis C in combination with interferon.\(^10\) Ribavirin enters cells via the equilibrative sensitive-type (es) nucleoside membrane transporter and undergoes irreversible phosphorylation at the expense of adenosine triphosphate.\(^11\)\(^,\)\(^12\) Because phosphorylated ribavirin cannot be exported outside cells, it accumulates in the erythrocytes to concentrations that are about 50- to 70-fold higher than in plasma and reduces the efficiency of anaerobic glycolysis.\(^13\) Ribavirin causes severe anemia in about 10% of treated patients,\(^14\) and oxidative membrane damage has been suggested as a mechanism.\(^15\)\(^,\)\(^16\) Furthermore, extracellular oxidative stress related to hepatitis C virus (HCV) infection itself also may play a contributory role in ribavirin-induced erythrocyte membrane changes.\(^15\)\(^,\)\(^17\)

From the clinical point of view, not only the mechanism but also the predictability of drug-induced anemia are important issues. Previous studies identified some factors with a predictive value for ribavirin-induced anemia including haptoglobin phenotype,\(^18\) age greater than 60 years, and female sex.\(^19\) However, the predictive value of erythrocyte factors was not studied. In our previous prospective study we had found that erythrocyte low-protein sulphydrils before treatment predicted the development of severe anemia during subsequent ribavirin treatment.\(^15\) This observation suggests changes in the regulation of the erythrocytes’ redox system and may lead to further insight into the mechanisms and prediction of ribavirin-induced anemia in clinical practice. The aims of this study, therefore, were to further investigate associations between oxidative stress markers, erythrocyte membrane alterations, and ribavirin-induced anemia by reproducing both the pretreatment phase and the stage of maximal hemolysis (week 4 and later), when ribavirin concentrations are at steady state,\(^1\) and to study the effect of exposure of erythrocytes from these patients to known pro-oxidant and nonoxidant hemolytic compounds.

**METHODS**

**Study design**

Isolated erythroocytes were obtained from 4 different groups of 5 age- and sex-matched subjects each, as follows: (1) healthy control subjects without HCV infection, (2) patients who were nonresponsive to a previous treatment with ribavirin and interferon (ie, still HCV ribonucleic acid [RNA]–seropositive) and did not show severe anemia during this treatment, (3) patients who were responsive to a previous treatment with ribavirin and interferon (ie, HCV RNA–seronegative) and who had severe anemia during this treatment, and (4) patients who were nonresponsive to a previous treatment with ribavirin and interferon in whom severe anemia developed during this treatment (ie, still HCV RNA–seropositive). Six of the 15 patients and 2 of the 5 control subjects participated in our earlier study.\(^15\)

Severe ribavirin-induced anemia was defined as previously reported.\(^20\) In our study all patients who had severe anemia had a hemoglobin decrease below 10 g/dL during ribavirin therapy. Indeed, each of them showed a change in hemoglobin level of more than 6 g/dL from the start of ribavirin treatment.

All patients were recruited from the Department of Internal Medicine of the Bari University Hospital, Bari, Italy. All subjects joined the study by providing written consent, and the protocol was approved by the Ethical Committee of the University Medical School of Bari, Bari, Italy.

Before entering the study, patients had discontinued ribavirin therapy at least 6 months earlier and those with previous ribavirin-induced anemia had fully recovered. All subjects with known forms of chronic or hemolytic anemia, glucose-6-phosphate dehydrogenase deficiency, or hemoglobin abnormalities were excluded on the basis of pretreatment hemoglobin levels, glucose-6-phosphate dehydrogenase enzyme activity, and hemoglobin electrophoresis.

Venous blood samples were collected at 8 AM into heparinized tubes after overnight fasting and were centrifuged at 4000g for 5 minutes. Plasma and buffy coat were carefully removed. Hematocrit and hemoglobin concentrations were determined by routine methods in the hospital’s central laboratory. Levels of thioredoxin, glutathione peroxidase, membrane protein sulphydryl, and protein-glutathione–mixed disulfide, as well as the electrophoretic membrane protein pattern, were subsequently determined in freshly isolated erythrocytes.
In parallel experiments, erythrocytes were also incubated with buffer, ribavirin, phenylhydrazine, or DHEA. Isolated erythrocytes (1.3 mL) were added to 2.7 mL of plasma and 9.3 mL of the following buffer (pH 7.4): 120-mmol/L sodium chloride, 5-mmol/L potassium chloride, 1.2-mmol/L magnesium sulfate, 1.2-mmol/L potassium phosphate (monobasic), 24-mmol/L sodium bicarbonate, and 10-mmol/L glucose with and without ribavirin, phenylhydrazine, or DHEA dissolved in 20-mmol/L dimethyl sulfoxide (final concentrations of 1 mmol/L). Equivalent amounts of dimethyl sulfoxide were also added to control vials. Round-bottom glass vials were incubated for 18 hours at 37°C in a heated shaking water bath. Subsequently, levels of thiobarbituric acid–reactive substances (TBARs), total and oxidized glutathione, protein sulfhydrils, and osmotic resistance were determined in the incubated erythrocytes. Triple incubations were performed for each subject, and their mean values were used for further analysis.

To investigate the time-course relationship between membrane protein–mixed disulfide formation and the appearance of hemolysis, erythrocytes (0.2 mL) were removed from each vial and processed before incubation was started and thereafter every 15 minutes for 1 hour.

**Analytic methods**

**Thioredoxin.** Levels of thioredoxin, a thiol-containing protein with a variety of biologic activities including the regulation of redox-sensitive molecules and protein sulfhydryls, were measured on freshly isolated erythrocytes according to the method described by Holmgren and Bjornstedt and the preparation procedure described by Bindoli and Rigobello.

**Glutathione peroxidase activity.** The activity of glutathione peroxidase, a glutathione-dependent enzyme that actively scavenges oxygen free radicals and hydrogen peroxide in particular and delivers oxidized glutathione, was assessed by use of the method described by Flohé and Gunzler. Calculations were made with 1 unit enzyme considered as the amount consuming 1.15 μmol of nicotinamide adenine dinucleotide phosphate reduced (NADPH) in 1 minute at 37°C (pH 7.0).

**Preparation of erythrocyte membrane proteins (“erythrocyte ghosts”).** Erythrocyte membranes were prepared by lysing 1 mL of packed erythrocytes in 40 volumes of 5-mmol/L sodium phosphate buffer (pH 8.0) under vigorous agitation and subsequent centrifugation at 50,000g for 30 minutes. The pellet (ghosts) was washed 4 times with 40 mL of the same buffer and centrifuged again at the same speed. Ghosts were finally suspended in phosphate-buffered saline solution buffer containing 0.2 mmol/L of the protease inhibitor phenylmethylsulfonyl fluoride.

**Protein sulfhydryls.** Protein sulfhydryls represent a category of proteins with mainly structural and enzymatic functions that are very rich in sulfhydryl residues. Levels of protein sulfhydryls were measured as described previously. Erythrocyte membrane proteins were precipitated with 4% sulfosalicylic acid (SSA) and centrifuged at 10,000g for 5 minutes. The resulting pellet was washed twice with 2% SSA and then resuspended in 800 μL of 6-mol/L guanidine, pH 6.0. Absorbance was measured at 412 and 530 nm before and 30 minutes after incubation with 50 μL of 10-mmol/L 5,5′-dithiobis-(2-nitrobenzoic) acid (DTNB).

**Protein-glutathione mixed disulfides.** Protein-glutathione mixed disulfides are proteins in which the sulfhydryl residues are blocked to disulfides by glutathione and are currently indicative of protein sulfhydryls’ oxidative alteration. Protein-mixed disulfides were measured as previously described. Erythrocyte membrane proteins were precipitated with 15% SSA containing 0.02-mol/L ethylenediaminetetraacetic acid. Precipitated proteins were then dissolved in 300 μL of 0.2-mol/L ammonium bicarbonate containing 8-mol/L urea and mixed with 5 mg of sodium borohydride solution (Na₂BH₄). Pentanol (50 μL) was added to avoid frothing. After 20 minutes, proteins were precipitated with 100 μL of 15% SSA. The amount of glutathione in the supernatant obtained after centrifugation at 45,000g for 15 minutes was measured according to the method of Tietze. Results are expressed as nanomoles of glutathione per milligram of protein.

**Sodium dodecyl sulfate–polyacrylamide gel electrophoresis.** Electrophoresis was performed according to the procedure of Laemmli. Gels (0.75 mm of thickness) consisted of 5% polyacrylamide stacking gel and 13% polyacrylamide separating gel. Erythrocyte membrane protein samples were diluted with Laemmli sample buffer (Bio-Rad, Munich, Germany) and heated at 65°C for 20 minutes. Molecular weight marker (5 μL) and proteins (30 μg) of each sample were applied onto the same gel. Proteins were separated by applying 25 mA during the course through stacking gel and 50 mA every 1 to 2 hours during separating gel, by use of Tris-glycine–sodium dodecyl sulfate running buffer. After the course, gels were stained with Coomassie brilliant blue R-250 and destained with a solution of 50% ethanol, 40% acetic acid, and 10% deionized ultrapure water. The staining intensity of the bands was densitometrically...
Risk factors for ribavirin-induced anemia

Quantified. Band nomenclature was given according to the classification of Fairbanks et al. 33

**Glutathione.** Glutathione, one of the major intracellular antioxidant molecules, was measured as follows. One hundred microliters of packed erythrocytes was lysed by addition of 900 μL of ice-cold bidistilled water. Total glutathione concentration was measured enzymatically at 412 nm by addition of 15% SSA to lysed erythrocytes. After centrifugation, 20 μL of supernatant was processed by the glutathione reductase recycling procedure. 31 Oxidized glutathione was spectrophotometrically quantified after incubation of the acidic SSA precipitate with 2-vinylpyridine and triethanolamine. 34

**Osmotic resistance.** Packed erythrocytes (100 μL) were carefully added to 13 tubes containing 1 mL each of 0.85% to 0% saline solution in distilled water. After gentle mixing, the tubes were kept for 20 minutes at room temperature and then centrifuged at 3000g for 5 minutes. The absorbance of the supernatant was measured at 540 nm. Total hemolysis was assumed to occur in tubes containing only water. Hemolysis was then calculated by use of the following formula: a = (b · 100)/c, where b is the absorbance of each sample and c is the absorbance of totally hemolysed sample.

**TBARs.** TBARs are one of the most used indexes of lipid peroxidation. TBAR levels were determined in 200 μL of lysed erythrocytes (100 μL of packed erythrocytes in 900 μL of ice-cold bidistilled water) added to 2 mL of 0.67% thiobarbituric acid dissolved in 10% trichloroacetic acid and 0.25 mol/L hydrochloric acid. 35 After heating at 100°C for 5 minutes, the tubes were cooled and centrifuged at 3000g for 15 minutes. The supernatant was spectrophotometrically read at 535 nm, and calculations were made with 1.56 · 10^5 mol/L · cm^−1 considered as the molar extinction coefficient.

**Protein concentrations.** Protein concentrations were measured with a commercially available kit (Bio-Rad).

**Chemicals**

Acrylamide, bisacrylamide, tetramethylethylenediamine, ammonium persulfate, and protein markers for molecular weight were obtained from Amersham Biosciences (Cologno Monzese, Italy). Coomassie brilliant blue R-250, Laemmli sample buffer, and the kit for protein assay were from Bio-Rad. All other reagents and substances were purchased from Sigma-Aldrich (Milan, Italy).

Statistics

Data are expressed as mean ± SD unless stated otherwise. Correlations between 2 parameters are described by the Pearson correlation coefficient (r). Effects of previous ribavirin-induced anemia and HCV status on each parameter determined in freshly isolated erythrocytes were calculated as coefficients in a multiple linear regression model with subject groups (absent versus present history of ribavirin-induced hemolysis and negative versus positive HCV status) as independent factors. For the incubation studies, subject groups and incubation conditions were included as independent factors. ANOVA statistics (F test) were used for model evaluation, and coefficients were tested by t test. All analyses were performed in STATA version 8.2 for MacOS X (StataCorp, College Station, Tex).

RESULTS

Demographic information on the study subjects together with their hematocrit and hemoglobin values are shown in Table I. Age and sex distributions of all 4 groups were comparable, and all patients with a history of ribavirin-induced severe anemia had normal hemoglobin values, yet the mean value was 1.2 g/dL lower compared with the value in subjects without previous ribavirin-induced anemia.

**Experiments with freshly isolated erythrocytes**

Membrane protein sulfhydryl and protein-mixed disulfide concentrations and thioredoxin and glutathione peroxidase determinations on freshly isolated erythrocytes from the 4 groups of subjects are presented in Table II and Fig 1, respectively. As shown in Fig 1, there were strong negative correlations between levels of protein sulfhydryls and protein-mixed disulfides (r = −0.93) and between log glutathione peroxidase and thioredoxin levels (r = −0.87). Protein sulfhydryl and thioredoxin concentrations were lower and protein-mixed disulfide concentrations were higher in all patients with a history of ribavirin-induced severe anemia compared with subjects without such a history. The level of glutathione peroxidase was higher in all HCV RNA–seropositive patients with a history of ribavirin-induced anemia compared with all other subjects. Accordingly, regression analysis showed a history of ribavirin-induced anemia as a highly significant factor for the observed differences in levels of protein sulfhydryls, protein-mixed disulfides, thioredoxin, and glutathione peroxidase (P < .001 for all parameters). In addition, there was also a strong independent association of positive HCV status with higher log glutathione peroxidase level (P < .001). Although we also observed significant independent associations of positive HCV status with lower levels...
of protein sulfhydrils \( (P = .01) \) and thioredoxin \( (P = .04) \) and higher levels of protein-mixed disulfides \( (P = .001) \), there was considerable overlap of the values between the 2 groups as shown in Fig 1.

The gel electrophoresis of erythrocyte membrane proteins showed differences in the electrophoretic band of 60 to 65 kd, and this alteration occurred in the weight range of band 4, appearing as a dimer disaggregation. This finding was independent of the subjects’ HCV status. Healthy control subjects did not show a band disaggregation. As densitometrically quantified, protein disaggregation was practically absent (4% ± 3%) in subjects without previous ribavirin-induced anemia, whereas the new bands appearing in patients who were positive for a history for severe anemia during previous ribavirin treatment ranged from 40% to 60% of the total.

**In vitro incubations of erythrocytes**

Erythrocyte total and oxidized glutathione and TBAR concentrations, membrane protein sulfhydryl levels, and osmotic hemolysis determined after 18 hours’ incubation of erythrocytes with buffer, ribavirin, phenylhydrazine, and DHEA are presented in Fig 2.

Compared with incubation with buffer alone, the addition of phenylhydrazine to the incubation medium expectedly produced changes that were compatible with oxidative stress (ie, lower total glutathione and protein sulfhydryl levels and higher oxidized glutathione and TBAR levels) and osmotic fragility also increased. Regression analysis accordingly indicated that these differences were highly significant \( (P < .001 \) for all parameters, as determined by \( t \) test). When erythrocytes were incubated with ribavirin, similar and also statistically significant changes \( (P < .001) \) could be observed, although they were less pronounced. DHEA also caused changes, except with regard to oxidized glutathione level \( (P = .5) \) for oxidized glutathione, \( P < .001 \) for all other parameters).

Comparable with the determinations in freshly isolated erythrocytes, in vitro incubations also showed significant differences between patients with and without a history of ribavirin-induced anemia. Previous ribavirin-induced anemia was significantly associated

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### Table I. Demographic data and hematocrit and erythrocyte hemoglobin levels in the 4 study groups (n = 5 per group)

<table>
<thead>
<tr>
<th></th>
<th>Healthy subjects</th>
<th>HCV+ patients with no severe anemia and with RBV</th>
<th>HCV− patients with severe anemia and with RBV</th>
<th>HCV+ patients with severe anemia and with RBV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age range (y)</td>
<td>27-42</td>
<td>29-44</td>
<td>27-42</td>
<td>34-46</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>2/3</td>
<td>2/3</td>
<td>2/3</td>
<td>2/3</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>45.2 ± 1.5</td>
<td>45.0 ± 2.2</td>
<td>41.8 ± 1.6</td>
<td>42.6 ± 2.1</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>15.2 ± 0.5</td>
<td>15.0 ± 0.3</td>
<td>13.9 ± 0.5</td>
<td>13.9 ± 0.4</td>
</tr>
</tbody>
</table>

Hematocrit and hemoglobin values are reported as mean ± SD.

HCV, Hepatitis C virus; RBV, ribavirin.

### Table II. Determinations of thioredoxin, glutathione peroxidase activity, membrane content of protein sulfhydrils, and protein-glutathione mixed disulfides on freshly isolated erythrocytes in the 4 study groups (n = 5 per group)

<table>
<thead>
<tr>
<th></th>
<th>Healthy subjects</th>
<th>HCV+ patients with no severe anemia and with RBV</th>
<th>HCV− patients with severe anemia and with RBV</th>
<th>HCV+ patients with severe anemia and with RBV</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRX (nmol/mg protein)</td>
<td>1.29 ± 0.15</td>
<td>1.07 ± 0.16</td>
<td>0.64 ± 0.12*</td>
<td>0.58 ± 0.10*</td>
</tr>
<tr>
<td>GSH-Px (mU/mg protein)</td>
<td>351 ± 30</td>
<td>435 ± 38*</td>
<td>508 ± 20*</td>
<td>728 ± 45*†</td>
</tr>
<tr>
<td>PSH (nmol/mg protein)</td>
<td>44.9 ± 1.8</td>
<td>42.6 ± 1.6</td>
<td>32.3 ± 1.6*</td>
<td>29.5 ± 1.5*</td>
</tr>
<tr>
<td>PSSG (nmol/mg protein)</td>
<td>0.49 ± 0.10</td>
<td>0.59 ± 0.11</td>
<td>1.27 ± 0.11*</td>
<td>1.74 ± 0.14*†</td>
</tr>
</tbody>
</table>

Data are reported as mean ± SD.

TRX, Thioredoxin; GSH-Px, glutathione peroxidase activity; PSH, membrane content of protein sulfhydrils; PSSG, protein-glutathione mixed disulfides.

*Significantly different \( (P < .05) \) compared with healthy subjects and patients showing no severe anemia.

†Significantly different \( (P < .05) \) compared with the other groups.
with lower total glutathione and protein sulfhydryl levels and higher oxidized glutathione and TBAR levels and osmotic fragility ($P < .001$ for all parameters). Furthermore, although current HCV infection was significantly associated with lower protein sulfhydryl levels and higher oxidized glutathione and TBAR levels and osmotic fragility on incubation with drugs ($P < .001$), its effect was much smaller than that of previous ribavirin-induced anemia.

The 60-minute time-course studies of protein-mixed disulfides and osmotic hemolysis under different incubation conditions are presented in Fig 3. As shown, no marked changes over time were observed for protein-mixed disulfides during incubation under buffer conditions, but under all other conditions, there was an increase in erythrocyte membrane protein-mixed disulfide levels, which was accompanied by an increase in the osmotic fragility of eryth-

**Fig 1.** Individual results of determinations in freshly isolated erythrocytes and presentation of Pearson correlation coefficients between membrane protein sulfhydrys (PSH) and protein-glutathione mixed disulfides (PSSG) and between glutathione peroxidase (GSH-peroxidase) and thioredoxin. The figures also demonstrate retrospective differentiation regarding previous ribavirin (RBV)–induced anemia. HCV, Hepatitis C virus.
Erythrocytes. The increase in protein-mixed disulfide levels and osmotic hemolysis was most pronounced with incubation with phenylhydrazine. Erythrocytes from patients with previous ribavirin-induced anemia had higher protein-mixed disulfide levels at baseline. Protein-mixed disulfide levels then showed a similar absolute increase over time in both groups under all incubation conditions. Osmotic hemolysis was similar in both groups at baseline and showed a much more pronounced increase over time in patients with previous ribavirin-induced severe anemia with phenylhydrazine and ribavirin (+23.3% versus +13.8% and +15.5% versus +7.5%, respectively), whereas no pronounced between-group differences were observed with buffer and DHEA (+5.3% versus +3.1% and +11.7% versus +9.7%, respectively). P values of ANOVA statistics were < .001 for protein-mixed disulfides and osmotic fragility, and R² values (coefficient of determination of regression model) were 0.96 (protein-mixed disulfides) and 0.88 (osmotic fragility).

**DISCUSSION**

Recent reports have suggested that ribavirin causes oxidative damage to erythrocytes and that this may explain, at least in part, the clinical problem of ribavirin-induced anemia. Our previous study prospectively identified low-protein sulfhydrils as a predictor of severe ribavirin-induced anemia in vivo and found that erythrocytes from patients with previous ribavirin-induced anemia were apparently more susceptible to oxidative stress and that glutathione supplementation as ester conjugated had a protective effect in vitro. The current research extended these investigations by studying additional parameters in freshly isolated erythrocytes and different incubation conditions.

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**Fig 2.** Total (GSH) and oxidized (GSSG) glutathione, PSH, and thiobarbituric acid-reactive substance (TBAR) concentrations and osmotic hemolysis (mean ± SD) assessed in erythrocytes after incubation of erythrocytes for 18 hours under different conditions (buffer, RBV, phenylhydrazine [PHZ], and DHEA; final concentrations of 1 mmol/L). Different **bar shadings** indicate erythrocytes obtained from 4 different groups of 5 subjects each. Significant differences (*P* < .001) for all parameters in RBV versus buffer, PHZ versus buffer, DHEA versus buffer (except for GSSG), patients with versus without previous ribavirin-induced severe anemia, and between HCV-positive (HCV+) versus HCV-negative (HCV-) patients (except for GSH). Hb, Hemoglobin; NaCl, sodium chloride.
conditions and used a retrospective study design that allowed us to analyze the effects of previous ribavirin-induced anemia and active HCV infection independently in a regression model.

As previously shown, protein sulfhydryl levels were lower in patients who had had severe ribavirin-induced anemia, and this was also confirmed in the additional patients studied. In addition, we were able to demonstrate further changes in erythrocyte levels of patients with ribavirin-induced anemia in a retrospective way: Glutathione peroxidase activity was increased and correlated with lower thioredoxin levels, protein-mixed disulfide levels were increased and inversely correlated with protein sulfhydryl levels, and there were changes in the electrophoretic patterns of erythrocyte membranes. The fact that these differences were present even without recent ribavirin exposure is remarkable for 2 reasons: First, the observed differences may be used as clinically valuable indicators of an increased risk for severe anemia before and during treatment with ribavirin. Other studies have indicated some predictive factors for ribavirin-induced anemia, and these included haptoglobin phenotype, female sex, and age greater than 60 years. However, and in particular, we believe that older subjects may have other factors that may affect erythrocyte osmotic resistance. Second, our study identified differences that further support the hypothesis that there are erythrocyte-related factors which modulate the risk of ribavirin-induced anemia, as well as that these factors apparently involve the regulation of the erythrocyte redox status and their membrane structure.

Consequently, there is an up-regulation of glutathione peroxidase, which may then lead to an increased formation of protein-mixed disulfides and oxidized glutathione at the expense of total glutathione, protein sulfhydrials, and thioredoxin. Ribavirin exposure may then cause additional oxidative stress that cannot be compensated in predisposed erythrocytes that have already up-regulated their antioxidant defense. Eventually, the formation of disulfide bridges between skeletal proteins and their glutathionylation, which are known mechanisms of drug-

Fig 3. Time course of PSSG formation in erythrocyte membranes and osmotic hemolysis in erythrocytes during incubations under different conditions (buffer, RBV, PHZ, and DHEA; final concentrations of 1 mmol/L). Data points present mean ± SD (n = 3) at different time points by history of ribavirin-induced anemia. Significant differences (P < .001) were found for both parameters in RBV and PHZ incubations of erythrocytes from patients with versus without history of ribavirin-induced severe anemia.
induced erythrocyte toxicity,\textsuperscript{2,37} may then lead to hemolysis. Proteins belonging to bands 4.1 and 4.2 are part of the cytoskeleton and confer mechanical stability and deformability to erythrocytes.\textsuperscript{38} Moreover, they interact with band 3 proteins, which have ionic channel function.\textsuperscript{39} The most important functional part of these proteins is the amino terminal domain, which is rich in cysteine residues available to form disulfide bonds.\textsuperscript{38} The observed disaggregation of band 4 could thus contribute to the susceptibility of erythrocytes to ribavirin-induced hemolysis. Mutations of protein 4.1 are associated with spherocytosis and anemia,\textsuperscript{40,41} but none of our patients with protein band alterations and major ribavirin-induced anemia had morphologically abnormal erythrocytes.

Extracellular oxidative stress associated with active HCV infection\textsuperscript{15,17} may, therefore, be fully compensated as long as there is no additional oxidative charge but may make a relevant contribution to the occurrence of anemia during treatment with ribavirin. In this respect, the cell membrane redox status, which is regulated by both extracellular environment and intracellular glutathione, is believed to modulate intracellular signaling by protein glutathionylation.\textsuperscript{42,43} Thioredoxin itself is a target for glutathionylation.\textsuperscript{44}

The aim of the incubations with phenylhydrazine and DHEA, respectively, was to differentiate between increased susceptibility of erythrocytes to oxidative versus nonoxidative mechanisms of hemolysis. Quantitative differences between incubation conditions were observed after both 18 hours’ and 60 minutes’ incubation with ribavirin, phenylhydrazine, and DHEA. In particular, the time course of the 2 parameters that were determined in the incubations for only 60 minutes is of interest: Ribavirin and phenylhydrazine, but not DHEA, caused a more pronounced increase in protein-mixed disulfide formation and osmotic fragility in erythrocytes from patients with previous ribavirin-induced anemia, suggesting that the erythrocytes of these patients may indeed be particularly susceptible to oxidative stress insults.

In conclusion, these studies in freshly isolated erythrocytes suggest that patients with low protein sulfhydryl and thioredoxin levels, high protein-mixed disulfide levels, and changes in erythrocyte membrane proteins are at risk of severe ribavirin-induced anemia. These changes are associated with an up-regulation of glutathione peroxidase and are not primarily caused by HCV infection itself. Although the exact mechanism of how these events are interrelated is not clear, this study provides further evidence for a primary role of oxidative mechanisms and membrane changes in ribavirin-induced erythrocyte damage, as well as for the existence of erythrocyte-related risk factors. The predictive value of our results should be investigated in a larger prospective study with the determination of additional parameters that may also address the genetic factors of redox regulation and, therefore, provide more insight into the mechanisms of ribavirin-induced anemia.

We are grateful to Stefano Bruno for his technical assistance in preparing gel electrophoresis and to Paola De Benedictis and Rosa De Venuto for their assistance in laboratory experiments. We also thank James A. Kaye from the Boston Collaborative Drug Surveillance Program for guidance on statistical methods. The authors have no conflict of interest to declare.

References


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Imatinib induces hypothyroidism in patients receiving levothyroxine

Interactions of imatinib with other drugs have been scarcely reported. We report a previously unknown effect of imatinib on levothyroxine therapy. Eleven patients (1 with gastrointestinal stromal tumor and 10 with medullary thyroid carcinoma) received imatinib. Eight had undergone thyroidectomy and used levothyroxine, and 3 had the thyroid in situ. Thyroid function was measured before, during, and within 2 weeks after any change in either imatinib or levothyroxine dosage. We observed symptoms of hypothyroidism in all patients who had undergone thyroidectomy, whereas patients with the thyroid in situ remained clinically and biochemically euthyroid. On average, thyrotropin (INN, thyrotrophin) levels increased to 384% ± 228% of the upper limit in patients after thyroidectomy, whereas free thyroxine (fT4) and free tri-iodothyronine (fT3) values remained within the reference range (59% ± 17% of the upper limit for fT4 and 63% ± 4% of the upper limit for fT3). Clinicians should be aware that hypothyroid subjects receiving imatinib have a high likelihood for increased levothyroxine replacement and should be closely monitored for elevations in thyrotropin indicating worsening hypothyroidism. (Clin Pharmacol Ther 2005;78:433-8.)

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In 2000 the first clinical studies with the selective, small-molecule tyrosine kinase inhibitor 571 (STI571) (imatinib) (Glivec, Gleevec, CGP57148B; Novartis Pharmaceuticals, East Hanover, NJ) were started. It belongs to the 2-phenylaminopyrimidine class, and it proved to target BCR-ABL, platelet-derived growth factor receptor, and c-kit receptor tyrosine kinases. Small-molecule drugs that can selectively inhibit tyrosine kinases have proved to be useful in the systemic treatment of a number of neoplastic diseases. Beneficial effects of imatinib are evident in patients with chronic myeloid leukemia and gastrointestinal stromal tumor (GIST), and the drug is now broadly used in these patients. Adverse effects have been moderate and usually manageable without dosage reduction or discontinuation of therapy.1,2 For optimal progression-free responses, imatinib therapy has to be sustained, preferably at full therapeutic doses.3 Therefore preventing discontinuation of therapy is of the utmost importance.

Given that many patients receive thyroid hormone, it is important that clinicians are aware of the potential interactions between various drugs and thyroid hormone replacement. Here, we report the previously unknown effects of imatinib on levothyroxine therapy in patients after thyroidectomy.

METHODS

We treated 11 patients (7 men and 4 women) for 6 months on average (± 3.3 months) with imatinib at 2 major referral centers in The Netherlands. The median age was 54 years (range, 33-72 years). Eight patients
previously underwent total thyroidectomy. One patient who had a thyroidectomy because of follicular thyroid cancer was treated for GIST, and 10 patients were treated for medullary thyroid carcinoma (MTC). Those MTC patients were treated with imatinib in a study protocol (approved by the local medical ethics committee) based on the in vitro observation of imatinib as an inhibitor of a tyrosine kinase encoded by the MTC-associated mutated RET proto-oncogene. All patients were clinically euthyroid before treatment. All patients who had undergone thyroidectomy used levothyroxine and were thought to be taking their medication as recommended.

Determination of thyroid function included measuring levels of thyrotropin (INN, thyrotrophin) (TSH) in all patients, free thyroxine (fT4) in 10 of 11 patients, and free tri-iodothyronine (fT3) in 4 of 11 patients. We used an automatic immunoassay (PerkinElmer Life Sciences [Groningen, The Netherlands] or Bayer Diagnostics [Mijdrecht, The Netherlands]) to determine fT4 and TSH levels. Levels of fT3 were determined by use of radioimmunoassay (Dynotest; Brahms Diagnostica, Berlin, Germany) or chemiluminescence immunoassay (PerkinElmer Life Sciences). Total thyroxine (T4) levels were determined by use of chemiluminescence immunoassay (Bayer Diagnostics). Serum concentrations of thyroxine-binding globulin (TBG) were determined with a Delfia TBG kit (PerkinElmer Life Sciences).

RESULTS

We observed symptoms of hypothyroidism in all postthyroidectomy patients and suspected imatinib to be causing the complaints. Initially, imatinib doses in patients 1 and 2 were lowered. However, only after patients stopped taking imatinib did complaints disappear within several weeks. On routine measurement of thyroid function, we discovered that all postthyroidectomy patients had markedly elevated TSH levels and were clinically hypothyroid whereas patients with the thyroid in situ remained euthyroid after initiation of imatinib therapy (Table I). Moreover, TSH levels returned to normal in all patients who discontinued imatinib (patients 3-7). In Fig 1 and Table II, the mean TSH and fT4 measurements in all postthyroidectomy patients during imatinib treatment are shown. On average, TSH levels increased to 384% and fT4 and fT3 values decreased, on average, to 59% and 63% of the upper limit for fT4 and fT3 but remained within the reference range. Changes in thyroid function for the individual patients are given in Table I.

![Table I. Patient clinical characteristics, thyroid function, and medications](image)

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (y/sex)</th>
<th>Diagnosis</th>
<th>LT4 dose before imatinib (µg)*</th>
<th>Imatinib dose (mg)*</th>
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<th>During imatinib (mean ± SD)</th>
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<tr>
<td>1</td>
<td>50/M</td>
<td>MTC</td>
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<td>400</td>
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<td>13 ± 1.7</td>
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<tr>
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<td>600</td>
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<td>12.6 ± 1.6</td>
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<tr>
<td>3</td>
<td>53/F</td>
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<td>112.5</td>
<td>600</td>
<td>19</td>
<td>14.5 ± 0.6</td>
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<tr>
<td>4</td>
<td>72/F</td>
<td>GIST</td>
<td>200</td>
<td>400</td>
<td>20</td>
<td>14.2 ± 1.2</td>
</tr>
<tr>
<td>5</td>
<td>46/M</td>
<td>MTC</td>
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<td>600</td>
<td>22</td>
<td>10.7 ± 0.6</td>
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<tr>
<td>6</td>
<td>33/M</td>
<td>MTC</td>
<td>187.5</td>
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</tr>
<tr>
<td>7</td>
<td>44/M</td>
<td>MTC</td>
<td>225</td>
<td>800</td>
<td>23</td>
<td>11.3 ± 0.6</td>
</tr>
<tr>
<td>8</td>
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<tr>
<td>9‡</td>
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<td>16 ± 2.8</td>
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<tr>
<td>10‡</td>
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<td>600</td>
<td>19</td>
<td>19†</td>
</tr>
<tr>
<td>11‡</td>
<td>57/M</td>
<td>MTC</td>
<td>None</td>
<td>600</td>
<td>13</td>
<td>13.5 ± 2.1</td>
</tr>
</tbody>
</table>

In patients 2 through 4, reference values for fT4, fT3, and TSH are 6.3 to 18.2 pmol/L, 2.4 to 6.7 pmol/L, and 0.42 to 7.2 mU/L, respectively. In patients 1 and 5 through 11, reference values for fT4, fT3, and TSH are 9.0 to 27.0 pmol/L, 4.0 to 7.8 pmol/L, and 0.35 to 5.0 mU/L, respectively. LT4, Levothyroxine; fT4, free thyroxine; fT3, free tri-iodothyronine; TSH, thyrotropin; MTC, medullary thyroid carcinoma; GIST, gastrointestinal stromal tumor; ND, not determined.

*All doses are given per day.
†Only 1 determination was available.
‡These patients have the thyroid in situ as opposed to the other patients, who do not.
or levothyroxine dose occurred. During treatment with imatinib, thyroid function was determined at least twice in all but 1 patient (patient 10), who still had the thyroid in situ and had no complaints of hypothyroidism. TSH levels increased within 2 weeks after initiation of imatinib treatment.

Serum concentrations of TBG (reference range, 12-30 mg/L) were determined in patients 2 and 3 and increased marginally during imatinib therapy (17 and 28 mg/mL before therapy and 21 and 32 mg/L during therapy, respectively). In patients 1 and 5 total T4 levels (reference range, 50-150 nmol/L, respectively) remained within the reference range (74 and 101 nmol/L, respectively) during imatinib treatment. In addition, in patient 2, intake of levothyroxine and imatinib was separated by at least 5 hours during 4 weeks. However, TSH levels did not decrease.

Despite a stepwise rise in levothyroxine administration to a mean of 206% (range, 100%-350%) of the dose before imatinib treatment in all postthyroidectomy patients, hypothyroidism could be reversed in only 3 patients (patients 1, 2, and 8) (Table I) before imatinib was discontinued. Eventually, 5 patients ended imatinib therapy because of fatigue, anorexia, nausea, and edema. In 1 patient (patient 6) MTC progressed and imatinib was subsequently stopped.

**DISCUSSION**

We report a previously unmentioned effect of imatinib in patients with a history of thyroid cancer. Postthyroidectomy patients taking levothyroxine have strikingly elevated TSH levels and need increased doses of levothyroxine after starting imatinib. In contrast, patients with a normal functioning thyroid remain euthyroid. These findings and the decline in TSH values after treatment is ended are highly suggestive that imatinib is the causative agent. Patients did not use other drugs that have been reported to interfere with levothyroxine therapy such as iron sulfate, aluminum antacids, calcium

<table>
<thead>
<tr>
<th>Table II. Number of patients receiving imatinib, mean change in imatinib dose, and mean change in levothyroxine dose during different time points</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Before treatment</strong></td>
</tr>
<tr>
<td><strong>Patients taking imatinib</strong></td>
</tr>
<tr>
<td>Change in mean imatinib dose</td>
</tr>
<tr>
<td>Change in mean LT4 dose</td>
</tr>
</tbody>
</table>
carbonate,7 raloxifene,8 cholestyramine resin,9 sucralfate,10 or colestipol.11 In a recent phase III study in GIST patients treated with 400 to 800 mg imatinib, hypothyroidism was not reported (or measured) as a side effect of imatinib.2 However, hypothyroidism developed in all postthyroidectomy patients taking imatinib in our study. Imatinib-associated complaints led to discontinuation of treatment in 36% of patients. Whether these complaints were a result of imatinib or hypothyroidism is difficult to distinguish. It is, however, remarkable that patients appeared clinically hypothyroid despite normal fT3 and fT4 levels.

We were able to reverse hypothyroidism in 3 patients by increasing the levothyroxine dose. In the other 5 patients imatinib was discontinued before the dose could be sufficiently increased because of complaints or disease progression. In the patients who again became euthyroid, hypothyroidism was recognized relatively early, which underscores the importance of increasing the dose of levothyroxine in a timely manner.

Reasons for the increased need for exogenous thyroid hormone in hypothyroid patients are listed in Table III. The mechanism that underlies imatinib-induced hypothyroidism cannot be explained by nonthyroidal illness, because normal to low TSH levels would be expected and the clinical presentation is different. Synthesis or secretion of thyroid hormones or TSH is unaffected by imatinib because individuals with a functioning thyroid have normal TSH levels when taking imatinib. Furthermore, it is unlikely that absorption of levothyroxine from the gut is impaired by imatinib. Separating levothyroxine and imatinib administration, leading to levothyroxine absorption before imatinib, did not reduce TSH levels and increased the levothyroxine requirement. Moreover, patients did not vomit.

**Table III.** Reasons for increased need for levothyroxine in hypothyroid patients and their mechanism of action

<table>
<thead>
<tr>
<th>Decreased absorption of levothyroxine</th>
<th>Altered transport of T3 and T4 in serum</th>
<th>Altered T4 and T3 metabolism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferrous sulfate</td>
<td>Raise the level of thyroid-binding protein</td>
<td>Inhibition of T4 deiodination</td>
</tr>
<tr>
<td>Aluminum hydroxide</td>
<td>Pregnancy</td>
<td>Amiodarone</td>
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<tr>
<td>Calcium carbonate</td>
<td>Liver disease</td>
<td>Propylthiouracil</td>
</tr>
<tr>
<td>Raloxifene</td>
<td>Estrogens</td>
<td>Propranolol</td>
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<tr>
<td>Cholestyramine</td>
<td>Tamoxifen citrate</td>
<td>Glucocorticoids</td>
</tr>
<tr>
<td>Sucralfate</td>
<td>Mitotane</td>
<td>Flavonoids</td>
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<tr>
<td>Colestipol</td>
<td>Fluorouracil</td>
<td>Increased hepatic metabolism</td>
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<tr>
<td></td>
<td>Heroin</td>
<td>Phenobarbital</td>
</tr>
<tr>
<td></td>
<td>Methadone</td>
<td>Phenytion</td>
</tr>
<tr>
<td>Competing for hormone-binding sites</td>
<td></td>
<td>Carbamazepine</td>
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<tr>
<td>Nonsteroidal anti-inflammatory drugs</td>
<td></td>
<td>Rifampin</td>
</tr>
<tr>
<td>Furosemide</td>
<td></td>
<td>Nicardipine</td>
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</tbody>
</table>

T4, Thyroxine; T3, tri-iodothyronine.

Fig 1. Thyrotropin (TSH) and free thyroxine (fT4) values as a function of number of weeks of treatment with imatinib in patients with hypothyroidism due to thyroidectomy. Data are shown as mean ± SD. A mean normalized value (percent of upper limit of normal) is used to correct for the different thyroid tests.
immediately after taking levothyroxine and therapy compliance was good. Increased TBG levels (found in pregnant patients, patients with liver disease, and patients taking estrogens, tamoxifen citrate [INN, tamoxifen], mitotane, fluorouracil, heroin, and methadone) lead to elevated total T4 levels.11-15 In hypothyroid patients taking levothyroxine, this may result in lower levels of fT4, higher levels of TSH, and an increased levothyroxine requirement.12 In our study no change in TBG or total T4 levels was observed that could have explained the increased need for levothyroxine substitution. Furthermore, competition for thyroid hormone-binding sites on TBG (and other hormone-binding proteins), as observed in patients taking high doses of furosemide or some nonsteroidal anti-inflammatory drugs, is also unlikely, because rising fT4 levels, which were not observed in this study, are expected in those cases.16-18

The most likely mechanism that contributes to imatinib-induced hypothyroidism involves stimulation of T4 and tri-iodothyronine (T3) clearance. The metabolism of T4 occurs via a variety of pathways. The largest part is metabolized by sequential deiodination, which has been shown to occur in the human thyroid, kidney, liver, placenta, and muscle.11,19 Drugs that inhibit deiodination include amiodarone, propylthiouracil, β-adrenergic receptor inhibitors, glucocorticoids, and flavonoids.11,20,22 In case of inhibition of T4 deiodination through imatinib, increased T4 levels are expected. This was not seen in our patients; therefore this mechanism is unlikely to be responsible for the hypothyroidism. We, therefore, suspect that the mechanism responsible for the observed hypothyroidism is non-deiodination clearance. The fraction of T4 that is not metabolized by deiodination is mainly subject to conjugation with glucuronates and sulfates by hepatic microsomal enzymes. Although the liver primarily mediates glucuronidation and sulfation, it occurs in extrahepatic sites such as the kidney and intestine as well.

There are 2 major enzyme systems involved in the conjugation of T4. The first system, the mixed function oxygenases (MFOs) or cytochrome P450s (CYPs), is a large, heterogeneous family of enzymes responsible for the oxidative metabolism of many endogenous and exogenous compounds.11,19 The second system conjugates the oxidized product of the MFOs, which is mainly performed by the uridine diphosphate–glucuronosyltransferases (UGTs).19 Induction of these enzymes results in enhanced clearance of T4 and T3. In subjects with a normal pituitary-thyroid function, more T4 is produced, increased generation of T3 from T4 arises, and TSH values remain within the normal range. Obviously, in hypothyroid, levothyroxine-substituted patients, TSH levels increase because thyroidal compensation does not occur.

Several hepatic enzyme-inducing drugs have been described. Phenobarbital, phenytoin, carbamazepine, rifampin (INN, rifampicin), and nicardipine induce both MFOs and UGTs and cause hypothyroidism in patients undergoing levothyroxine substitution.11,19,23,24 Moreover, rifampin induced hypothyroidism in patients receiving levothyroxine therapy after thyroidectomy and also in patients with Hashimoto’s thyroiditis within 2 weeks of treatment.11,25 Imatinib is a potent competitive inhibitor of several MFOs (CYP2C9, CYP2D6, and CYP3A4/5).26 Therefore induction of UGTs seems to be the most likely mechanism of action. Unfortunately, it is not yet known whether imatinib can actually induce other MFOs, and the effects of imatinib on UGTs need to be elucidated as well. Our data suggest that imatinib affects levothyroxine metabolism through a mechanism similar to that of rifampin and induces UGTs. However, further evidence needs to be provided.

The clinical significance of the study findings is broadened considering that, although this study evaluated subjects with hypothyroidism from thyroidectomy, subjects with hypothyroidism of a more common cause, such as Hashimoto’s thyroiditis, would be equally likely to require increased replacement with levothyroxine during imatinib treatment. Physicians should be aware that hypothyroid patients receiving imatinib need increased levothyroxine doses. We propose an at least 2-fold increase in levothyroxine substitution therapy before initiation of imatinib treatment and close monitoring of thyroid function during treatment.

All authors declare that they have no conflict of interest.

References


4. Cohen MS, Hassain HB, Moley JF. Inhibition of medullary thyroid carcinoma cell proliferation and RET phos-
Informed consent or acknowledgment of disclosure

To the Editor:

Stein1 and Miller2 have commented on the importance of the assessment of risk to benefit in evaluating whether a proposed clinical research project is ethical. The responsibility for this assessment lies with the investigators, the sponsor, the institutional review board (IRB), the regulatory authorities, and so on, but not with the subjects. Expert knowledgeable opinion must determine whether the benefit of the planned research is sufficient to justify the risk. If the benefit does justify the risk, then the potential subject must learn enough about the research to make an informed decision as to whether to volunteer to be a subject. Part of this learning process is to read the consent form.

Unfortunately, the descriptions of the risks to the patient in clinical trial consent forms are often hard to understand. On one such form I was given to review, the “risks” section was very hard to understand. Not previously aware of the sponsor, I found the sponsor’s Web site, saw a button labeled “risks,” and clicked on it. There was an easily understood statement of risks to investors on the company’s Web site.

We then evaluated the reading difficulty of 20 statements of risks to subjects from clinical research informed consent forms, some of which were from multicenter clinical trials, compared with the reading difficulty of 20 statements of risks to investors in initial public offering (IPO) documents. The Flesch Reading Ease score was determined from Microsoft Word (Microsoft, Redmond, Wash). The Flesch-Kincaid Grade levels were determined by use of WordPerfect (Corel, Ottawa, Ontario, Canada) because it goes up to grade level 16 whereas Microsoft Word is truncated at grade 12.

The results are shown in Table I. None of the informed consent forms (or IPO documents) was written at the eighth grade level, the average reading level of adult Americans.3 Six consent forms were written at a higher grade level than 2 IPO documents despite the differences in educational level of their intended readers. The reading ease (or difficulty) of 11 informed consent forms coincided with that of 9 IPO documents. Of the informed consent forms, 9 (45%) required at least part of a college education.

Paasche-Orlow et al4 surveyed all US medical schools and found that their consent form template language, even before specifics were added, was harder to read than the schools’ own readability standards. Readability of the average template language required 1 year of college (grade 13). Ancker5 described how reading difficulty alone is inadequate to evaluate actual readability. Annas6 wrote that the National Bio-

Table I. Reading levels of risk statements

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<th>IPO</th>
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The higher the grade level and the lower the reading ease level, the harder the document is to read.

IPO, Initial public offering.

The ethic Advisory Committee noted that IRBs “… have tended to focus on the disclosure found in the consent form” rather than on having an informed consent. Epstein and Lasagna7 found that long, more detailed and complete written consent forms produced less comprehension than short simple forms.

One can conclude that a research subject’s signature on a usual consent form today indicates acknowledgment of disclosure, not informed consent. This is a perversion of the whole informed consent process, which has been articulated from the time of the Nuremberg Code, which stated that the subject “… should have sufficient knowledge and comprehension of the elements of the subject matter involved as to enable him to make an understanding and enlightened decision.”8 The present practice of full disclosure appears to be less concerned with the research subject’s comprehension of the research than with the welfare and protection of other parties to the research. An improvement of present practice would be to follow the model of the Food and Drug Administration, which requires full disclosure in the official labeling for prescribers but adds a medication guide (patient package insert), a concise document understandable to the patient, for the patient. An example of the simplification of the patients’ medication guide is the list of the adverse effects to be included. These are the effects that are “…reasonably likely to be caused by the drug product that are serious or
occur frequently and not all adverse events associated with the drug. The government and IRBs should work toward replacing the present informed consent form, really an “acknowledgment of disclosure” form, with 2 different documents. One should be a full disclosure document primarily for the investigators and the IRB. These are the individuals primarily responsible for the assessment of benefit and risks of the study and making the determination that the study is ethical. This document would be analogous to the official labeling of a prescription drug for the prescribers. Although this document should be available to potential subjects, it is not written primarily for them. Another document, the informed consent document, would be modeled after the medication guide and be designed and written for the potential volunteer to provide “sufficient comprehension of the elements of the subject matter involved” to enable the potential volunteer “to make an understanding and enlightened decision.” The present language and full disclosures in our current informed consent forms appear no better at giving subjects sufficient comprehension about a study and its risks than the official labeling of a drug can give average patients a real understanding about a drug and its benefits and risks. The latter was corrected by medication guides. The former requires that we develop a new and truly informing informed consent document.

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References


Additional discussions regarding the altered metabolism and transport of omeprazole after long-term use of St John’s wort

To the Editor:

In a recent issue of the Journal, Wang et al1 reported an interesting finding: Cytochrome P450 (CYP) 2C19–genotyped extensive metabolizers had a significant reduction in omeprazole’s bioavailability compared with poor metabolizers after long-term use of St John’s wort (SJW). However, their discussion about this was limited, and additional discussions are necessary.

The major metabolic pathways of omeprazole are 5-hydroxylation (catalyzed by CYP2C19 and, to a lesser extent, by CYP3A4) and sulfoxidation (catalyzed by CYP3A4) in the human liver.2 For the formation of 5-hydroxyomeprazole, which was significantly induced by SJW in the CYP2C19 extensive metabolizers but not in the poor metabolizers,3 the following additional explanations would need to be taken into account. First, CYP2C19*2 and CYP2C19*3 were shown to encode 2 truncated nonfunctional enzymes whose catalytic activities cannot be induced by SJW.3,4 Furthermore, CYP3A4 is a low-affinity omeprazole 5-hydroxylase (with a high Michaelis-Menten constant value, 60 μmol/L)2 and thus plays a minor role in the omeprazole 5-hydroxylation at low substrate concentrations as noted in the study of Wang et al,1 despite the fact that omeprazole itself5 and SJW6 can significantly induce the expression of CYP3A4 messenger ribonucleic acid and protein, as well as activity, with greater induction expected in the CYP2C19 poor metabolizers because of higher plasma omeprazole levels than in the extensive metabolizers. On the other hand, omeprazole was shown to be a potent, competitive inhibitor of CYP2C19 and a weak, noncompetitive inhibitor of CYP3A4.7 Similar to SJW,6 the initial effect of omeprazole is expected to be its direct inhibition of CYP2C19 and, to a lesser extent, of CYP3A4, because the induction of these 2 enzymes needs to follow a certain time course that is involved in their gene transcription and translation. Therefore, for a single oral dose of omeprazole, its inhibition is expected to be greater than its induction. In addition, the magnitude of induction by SJW is expected to be greater for CYP2C19 than for CYP3A4, because constitutive CYP2C19 activity is expressed in the human liver at much lower levels.

For its second metabolic pathway, the formation of omeprazole sulfone was greater in the CYP2C19 poor metabolizers than in the extensive metabolizers before and after induction by SJW, although there was a similar extent of induction. For the placebo phase, again, higher omeprazole levels in the CYP2C19 poor metabolizers than in the extensive metabolizers may contribute to greater induction of CYP3A4, resulting in a greater amount of omeprazole sulfone being generated. For the induction phase, long-term use of SJW produced a significant synergistic induction of CYP3A4 that was integrated with the induction by omeprazole alone.5
In addition, omeprazole was shown to be a moderate substrate and inhibitor of an efflux transporter, P-glycoprotein (P-gp). Other drug transporters, such as organic anion transporting polypeptides (OATPs, uptake transporters) and multidrug resistance-associated protein 3 (MRP3, an efflux transporter), may also contribute to omeprazole’s bioavailability. In fact, omeprazole significantly induced MRP3 messenger ribonucleic acid and protein in both livers of patients treated with omeprazole and HepG2 cells pretreated with omeprazole, but no direct evidence is currently available to show whether omeprazole is a substrate of MRP3. In the study of Wang et al., the mean value of the area under the plasma concentration–time curve in the CYP2C19 poor metabolizers was 2.8 times that in the extensive metabolizers during the placebo phase and 3.3 times that after induction by SJW. This suggests that impaired CYP2C19 activity results in increased bioavailability of omeprazole, regardless of induction by SJW. However, if omeprazole was identified to be a substrate for the previously mentioned drug transporters, its bioavailability might, to some extent, depend on the relative activity of each of them, in particular, as a result of their genetic variation, inhibition, or induction. Long-term use of SJW can induce P-gp expression and function, but no data are available showing whether SJW can also induce the other efflux and uptake transporters that may be involved in the disposition of omeprazole and there is no evidence indicating the genotype-phenotype correlation of P-gp and other transporters before and after induction by SJW, because it is unclear whether induction of P-gp by SJW has any role in the disposition of omeprazole and voriconazole.

In summary, the bioavailability of omeprazole involves the integrated effects that are produced by both drug-metabolizing enzymes (CYP2C19 and CYP3A4) and drug transporters (efflux or uptake) before and after induction by SJW, and the roles of drug transporters in the disposition of affected drugs should not be underestimated or ignored.

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Dr Xie has no conflict of interest to disclose.

References


The CYP2C9 genotype does not influence sildenafil pharmacokinetics in healthy volunteers

To the Editor:

The phosphodiesterase type 5 inhibitor sildenafil is extensively metabolized to N-desmethylsildenafil by cytochrome P450 CYP3A4 (CYP) enzymes. Whereas 75% to 79% of N-desmethylsildenafil formation is attributed to CYP3A4, CYP2C9 is responsible for up to 20% of the formation of this metabolite in vitro. However, it is unclear to what extent CYP2C9 contributes to the in vivo metabolism of sildenafil.

We re-evaluated data from our study published in this Journal that revealed that the inhibition of intestinal CYP3A4 by grapefruit juice only leads to small increases in sildenafil exposure. In this crossover study 24 healthy men received 50 mg sildenafil with either twice 250 mL grapefruit juice (administered 1 hour before and together with the tablet) or water. Sildenafil and N-desmethylsildenafil concentrations were quantified in plasma up to 24 hours after administration by use of liquid chromatography–tandem mass spectrometry. To assess the role of CYP2C9 in vivo, we have now determined the CYP2C9 genotype in 23 volunteers (1 volunteer did not give his consent for genetic analysis) by direct sequencing of the CYP2C9 exons 3 and 7 to identify the alleles *2 to *5 as described previously. If no mutation was detected, the *1 allele was assigned. The groups of CYP2C9*1/*2 and CYP2C9*1/*3 heterozygotes were combined for analysis to detect differences in pharmacokinetic parameters between groups of 40% with an α of .05, a power of .6, and a variability within groups of 40%. Mann-Whitney
Fig 1. Geometric mean sildenafil (solid lines with solid symbols) and N-desmethylsildenafil plasma concentration–time curves (dashed lines with open symbols) in 15 CYP2C9*1/*1 homozygotes (circles), 3 CYP2C9*1/*2 heterozygotes (triangles), and 5 CYP2C9*1/*3 heterozygotes (squares) after intake of 50 mg sildenafil with water. Error bars are not given, because they overlap the other curves of the respective analyte.

Table I. Geometric means (geometric coefficients of variation) of pharmacokinetic characteristics of sildenafil and N-desmethylsildenafil according to CYP2C9 genotype in 23 healthy white men after intake of 50 mg sildenafil with twice 250 mL water or grapefruit juice

<table>
<thead>
<tr>
<th></th>
<th>CYP2C9*1/*1 (n = 15)</th>
<th>CYP2C9*1/<em>2 and CYP2C9</em>1/*3 (n = 8)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sildenafil with water</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AUC_{0-\infty} (ng/mL · h)</td>
<td>601.4 (41%)</td>
<td>711.9 (48%)</td>
<td>.30</td>
</tr>
<tr>
<td>C_{max} (ng/mL)</td>
<td>231.5 (53%)</td>
<td>336.8 (39%)</td>
<td>.08</td>
</tr>
<tr>
<td>t_{1/2} (h)</td>
<td>3.7 (14%)</td>
<td>4.2 (20%)</td>
<td>.11</td>
</tr>
<tr>
<td>Sildenafil with grapefruit juice</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AUC_{0-\infty} (ng/mL · h)</td>
<td>764.7 (46%)</td>
<td>779.8 (55%)</td>
<td>.89</td>
</tr>
<tr>
<td>C_{max} (ng/mL)</td>
<td>250.5 (59%)</td>
<td>243.7 (56%)</td>
<td>.85</td>
</tr>
<tr>
<td>t_{1/2} (h)</td>
<td>3.7 (18%)</td>
<td>3.9 (23%)</td>
<td>.50</td>
</tr>
<tr>
<td>N-desmethylsildenafil with water</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>AUC_{0-\infty} (ng/mL · h)</td>
<td>219.5 (45%)</td>
<td>311.5 (37%)</td>
<td>.053</td>
</tr>
<tr>
<td>C_{max} (ng/mL)</td>
<td>76.5 (62%)</td>
<td>119.4 (35%)</td>
<td>.12</td>
</tr>
<tr>
<td>t_{1/2} (h)</td>
<td>4.7 (52%)</td>
<td>4.7 (28%)</td>
<td>.80</td>
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<tr>
<td>N-desmethylsildenafil with grapefruit juice</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AUC_{0-\infty} (ng/mL · h)</td>
<td>277.7 (51%)</td>
<td>372.2 (39%)</td>
<td>.16</td>
</tr>
<tr>
<td>C_{max} (ng/mL)</td>
<td>74.4 (49%)</td>
<td>93.0 (41%)</td>
<td>.22</td>
</tr>
<tr>
<td>t_{1/2} (h)</td>
<td>5.4 (44%)</td>
<td>5.2 (26%)</td>
<td>.75</td>
</tr>
</tbody>
</table>

P values are derived from results of Mann-Whitney U tests. AUC_{0-\infty}, Area under plasma concentration–time curve from time 0 to infinity; C_{max}, maximum plasma concentration; t_{1/2}, terminal elimination half-life.
tests were used for statistical analysis (SPSS 11.0.1; SPSS, Chicago, Ill).

Of the 23 volunteers, 5 were *1/*3 heterozygotes, 3 had the *1/*2 genotype, and 15 were classified as *1/*1 homozygotes. Mean concentration-time curves of sildenafil and N-desmethylsildenafil were discretely but not significantly higher in heterozygotes compared with CYP2C9*1/*1 homozygotes (Fig 1). Although intestinal CYP3A4 was inhibited by grapefruit juice in 1 period, which might enhance possible effects of CYP2C9 genotypes on pharmacokinetics, none of the pharmacokinetic parameters of both analytes was influenced by genotype, irrespective of water or grapefruit juice intake (Table I).

A recently published review on CYP2C9 substrates revealed that, compared with CYP2C9*1/*1 homozygotes, changes in pharmacokinetics observed in CYP2C9*2/*2 or CYP2C9*3/*3 homozygotes are anticipated to a smaller extent in heterozygotes. Because sildenafil and N-desmethylsildenafil pharmacokinetics did not differ significantly between CYP2C9*1/*1 homozygotes and heterozygous carriers of *2 and *3 alleles, it seems unlikely that, in vivo, a strong reduction of CYP2C9 activity, as seen in variant homozygotes, leads to clinically relevant changes in sildenafil pharmacokinetics.

**References**


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NOTES OF THE AMERICAN SOCIETY FOR CLINICAL PHARMACOLOGY AND THERAPEUTICS

A MESSAGE FROM THE ASCPT MEMBERSHIP COMMITTEE CHAIRPERSON

On behalf of the Membership Committee, I am pleased to welcome the new members accepted into the Society in July 2005. Please take a moment to review their names and welcome those in your geographic area. Thank you also to the current ASCPT members who have sponsored these applicants.

Please continue your recruitment efforts on behalf of ASCPT and take an active role in ensuring new members can both contribute to and benefit from the Society. Again, welcome to our new members!

John T. Sullivan, MD
Chairperson, Membership Committee

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