HISTORICAL PERSPECTIVE

Leading the Way: Multi-Drug Resistance Protein (MDR1) and Clinical Pharmacology—Commentary on Kim *et al*.

Kathleen M. Giacomini[1,*]

Over the last three decades, transporters have become increasingly recognized for their important roles in clinical pharmacology. As gatekeepers of drug absorption, disposition and targeting, transporters in the intestine, liver, kidney and blood brain barrier have been the subject of many clinical pharmacology studies. A seminal work published in 2001 was among the first studies to shift the focus of pharmacogenomic research from drug metabolizing enzymes to drug transporters, demonstrating that pharmacogenomic factors in genes in addition to drug metabolizing enzymes, and in particular, in transporter genes, could play an important role in interindividual variation in pharmacokinetics of drugs.

Over the last three decades, transporters have become increasingly recognized for their important roles in clinical pharmacology. As gatekeepers of drug absorption, disposition and targeting, transporters in the intestine, liver, kidney and blood brain barrier have been the subject of many clinical pharmacology studies. The focus has been on specific transporters in two major superfamilies: the ATP Binding Cassette (ABC) superfamily and the SoLute Carrier (SLC) superfamily rather than on all the genes classified as transporters in the human genome.[1] Of the transporters in these two major superfamilies, which consist of about 49 ABC transporters and >400 SLC transporters, about 10 are highly studied for their role in drug absorption, distribution and elimination, and are described in regulatory guidances.[2] These include two ABC transporters, P-glycoprotein encoded by *ABCB1*, which was previously known as the MDR1 gene and Breast Cancer Resistance Protein (BCRP, ABCG2) and eight SLC transporters, Multi-drug Toxin and Extrusion Transporters (MATE1, SLC47A1 and MATE2, SLC47A2), Organic Anion Transporters (OAT1, SLC22A6, and OAT3, SLC22A8), Organic Anion Transporting Polypeptides (OATP1B1, SLCO1B1, and OATP1B3, SLCO1B3), and Organic Cation Transporters (OCT1, SLC22A1, and OCT2, SLC22A2). Of these transporters, P-gp was one of the earliest recognized for its roles in pharmacokinetics and pharmacodynamics, initially identified for its role in causing pleiotropic drug resistance in cancer cells.[3–5] As the transporter was found to be expressed in epithelial cells of the intestine, liver, and kidney and in the endothelia cells of the blood–brain barrier,[5] its significant role in both drug absorption and distribution became evident. Notably, during the early 2000's when the Human Genome Project was underway, and questions about genetic variation in all genes and their impact on interindividual variation in drug response began to be explored, *ABCB1* became the subject of numerous pharmacogenomic studies.

Prior to the Human Genome Project, pharmacogenetic research had centered almost exclusively on candidate gene studies of genes encoding drug-metabolizing enzymes.[6] In general, the focus of these studies was on individuals of European ancestry who carried common reduced-function alleles of these enzymes. However, with the publication of the Human Genome Project[7,8] understanding genetic variation in all human genes became possible. For clinical pharmacology, understanding the spectrum of human genetic variation in drug-metabolizing enzymes and transporters that played roles in drug absorption, distribution, and elimination was of major importance. For the first time, a plethora of studies that focused on identifying and characterizing genetic variation in racial and ethnic groups beyond individuals of European ancestry were published. In the wake of the Human Genome Project, the National Institutes of Health funded the Pharmacogenomics Research Network (PGRN), a network of investigators who conducted research on many genes and

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drug response phenotypes in ethnically diverse populations.[9] In the area of drug transporters, discovering and characterizing genetic variation in *ABCB1* (or *MDR1*) led the way for pharmacogenomic studies of transporters.[10–13]

In the current issue of *Clinical Pharmacology and Therapeutics*, a manuscript co-authored by Richard Kim, Grant Wilkinson and colleagues is re-published, and represents the most highly cited manuscript in the journal in the decade between 2000 and 2010, cited over 800 times.[13] The manuscript entitled "Identification of Functionally Variant MDR1 Alleles Among European Americans and African Americans," describes a comprehensive, multi-tiered pharmacogenomic study focused on MDR1 in these populations.[13] In brief, sequencing the coding regions of DNA samples collected from 60 individuals in these populations, the investigators identified 10 genetic polymorphisms, 6 of which encode non-synonymous variations. Common linkage among the genetic variants and associated haplotypes were identified, and a nomenclature for the haplotypes was proposed. Notably, two synonymous SNPs (C1236T and C3435T) and a non-synonymous SNP (G2677T, Ala893Ser) were found to be linked (MDR1*2) and to occur at a frequency of 62% in European Americans and 13% in African Americans in the population genotyped in the study. The investigators went on to functionally characterize the Ala893Ser variant in cells and discovered that digoxin was transported at a faster rate in cells recombinantly expressing the Ser893 polymorphism of P-gp compared with the Ala893. These studies led to their third tier of studies, which was to characterize the pharmacokinetics of a model P-gp substrate in healthy volunteers who carried the various haplotypes of MDR1 identified in the study.

Fexofenadine was selected as the model substrate of P-gp because the drug was a P-gp substrate and could be safely administered to healthy volunteers.[13] The plasma concentrations of the drug were determined following the administration of a single oral dose to 37 European Americans and 23 African Americans, and the pharmacokinetic characteristics were associated with MDR1 haplotypes. The major finding was that fexofenadine plasma concentrations were significantly higher in individuals carrying MDR1*1, a haplotype which includes C3435 in linkage with G2677 (Ala893) in comparison to individuals carrying MDR1*2, a haplotype which includes T3435 in linkage with T2677 (Ser893). In fact, the area under the plasma level–time curve was almost 40% greater in individuals who carried the homozygous MDR1*1/MDR1*1 alleles compared with individuals who were homozygous MDR1*2/MDR1*2, and the heterozygotes MDR1*1/MDR1*2 had an intermediate area under the plasma level–time curve. These data were consistent with the *in vitro* data demonstrating that the Ser893 polymorphism of P-gp had a greater rate of transport of its substrates in comparison to the Ala893 polymorphism and suggested that, consistent with their in vitro findings, the MDR1*2 allele had enhanced *in vivo* activity, which was associated with a lower extent of absorption of fexofenadine.

This multi-tiered elegant study, which has been highly cited since its publication, caused a ripple in the field of pharmacogenomics. Namely, the study demonstrated that pharmacogenomic factors in genes in addition to drug metabolizing enzymes, and in particular, in transporter genes, could play an important role in interindividual variation in pharmacokinetics of drugs.[13] What was most interesting at the time was that the study contradicted a study published just a year earlier by Hoffmeyer *et al*.[14] The Hoffmeyer study demonstrated a contrary result: namely, individuals who were homozygous for T3435, a synonymous variant, had higher plasma concentrations of digoxin in comparison to homozygotes for C3435. The Hoffmeyer manuscript, which has been cited over 2,000 times, was a highly influential and widely discussed paper in the field of clinical pharmacology at the time. Thus, Kim *et al*. attempted to rationalize their findings in the context of the contradictory findings of the Hoffmeyer study. Kim *et al*. had found that individuals who were homozygous for the haplotype that most commonly carried the T3435 allele (MDR1*2) had lower plasma concentrations of fexofenadine than individuals who were homozygous for the C3435 allele, in contrast to the higher digoxin concentrations found in individuals with this allele in the Hoffmeyer study. Reasons cited by Kim *et al* for the lack of agreement between the two studies were that (i) Hoffmeyer *et al*. had failed to identify the non-synonymous variant (C2677T, Ala893T) that was in almost complete linkage disequilibrium with C3435T; (ii) different conditions were used in the two studies, for example, different P-gp substrates were studied; (iii) other variants which affect P-gp expression levels had not been genotyped by either group; and (iv) the effects of C3435T, a synonymous variant, on RNA processing had not been characterized, leaving a gap in our understanding of the mechanism by which a synonymous variant may affect clinical phenotypes. They also suggested that there may be other transporters as well as dietary factors that could affect drug absorption. Later, intriguing results from Gottesmann *et al*. suggested that the haplotype with T3435 changes a preferred codon for serine to a less preferred codon, changes the rate of translation of the protein, and consequently its folding and substrate specificity,[15] thus lending credence to the idea that synonymous variants may indeed affect protein structure and function. Today, manuscripts continue to be published, which focus on the effect of the C3435T variant and the haplotypes of MDR1 on clinical drug response described by Kim *et al*.[13,16]

Thus, the study by Kim *et al*. was a leading study that preceded the plethora of studies focused on genetic variants in membrane transporters and drug response.[2,13] From 2005 onward, many pharmacogenomic studies transitioned from candidate gene studies to genomewide association studies (GWAS), which take an agnostic approach in identifying genetic variants responsible for various phenotypes. For example, in GWAS and candidate gene studies, genetic variants in SLCO1B1 encoding OATP1B1 have been found to be critical determinants of statin-induced myopathies and are the subject of several dosing guidelines from the Clinical Pharmacogenetics Implementation Consortium (CPIC).[17–19] GWAS led to the interesting discovery that genetic variants in ABCG2 encoding BCRP are determinants of response to the anti-gout medication, allopurinol,[20] and determinants of response to rosuvastatin.[21,22] Candidate gene pharmacogenomic studies focused on multiple reduced function alleles have also shown that missense

variants in the organic cation transporter, OCT1, are determinants of the pharmacokinetics and pharmacodynamics of many basic drugs.[23,24] Importantly, genetic variants in various liver transporters are associated with drug-induced hepatotoxicities and notably, cholestasis.[25–27]

For MDR1, many associations with genetic variants have been found and are cataloged in the Human GWAS Catalog.[28] These include body height, as well as gallstones and hepatic dysfunction, which suggest important roles of the transporter in the portal system. Though not strictly pharmacogenomic phenotypes, these associations underscore the critical importance and multiple roles of P-gp in human biology and pathophysiology.

In summary, the highly cited and seminal manuscript by Kim *et al*.[13] was among the first studies to shift the focus of pharmacogenomic research from drug metabolizing enzymes to drug transporters. Published in 2001 and focused on the most recognized ABC transporter, P-gp, the research resulted in new findings for the clinical pharmacology community. First and foremost, genetic variants and haplotypes exhibit widely different frequencies depending on race and ethnicity. For example, the most common haplotype in MDR1 in individuals of European ancestry, MDR1*1, has a frequency of about 10% in populations of African ancestry but over 50% in European populations. While this may seem like common knowledge, at the time it was a significant finding, which stimulated calls for pharmacogenomic studies in non-European populations. Unfortunately, even today, the focus of pharmacogenomic research remains predominantly European, although some studies have been conducted in other races and ethnic groups.[29] A second finding was the importance of haplotypes. In pharmacogenomic studies, it is crucial to consider the genetic variants that are in linkage with variants of interest. Kim *et al*. identified several important haplotypes that were not recognized in earlier studies and may have contributed to incomplete findings. A third finding was that non-synonymous variants in P-gp, in this case, Ala893Ser, may have increased function in cells, potentially translating to increased function *in vivo* affecting drug response. Importantly, both haplotype and synonymous variants should not be disregarded, as they may have mechanistic importance for the function of the transporter. The significance of understanding the mechanisms responsible for clinical observations was underscored in the multi-tiered approach by Kim *et al*, where they not only identified genetic variants, several previously unreported genetic variants, but also determined the functional consequences of these variants to transporter expression and function, linking in vitro mechanisms to clinical drug response. This study has had a profound impact on the clinical pharmacology community, leading to new ideas in pharmacogenomics and fostering a new understanding of the factors responsible for interindividual variation in drug concentrations and response.

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CONFLICT OF INTEREST

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Identification of functionally variant *MDR1* alleles among European Americans and African Americans

MDR1 (P-glycoprotein) is an important factor in the disposition of many drugs, and the involved processes often exhibit considerable interindividual variability that may be genetically determined. Single-strand conformational polymorphism analysis and direct sequencing of exonic *MDR1* deoxyribonucleic acid from 37 healthy European American and 23 healthy African American subjects identified 10 single nucleotide polymorphisms (SNPs), including 6 nonsynonymous variants, occurring in various allelic combinations. Population frequencies of the 15 identified alleles varied according to racial background. Two synonymous SNPs (C1236T in exon 12 and C3435T in exon 26) and a nonsynonymous SNP (G2677T, Ala893Ser) in exon 21 were found to be linked (*MDR1*2*) and occurred in 62% of European Americans and 13% of African Americans. In vitro expression of MDR1 encoding Ala893 (*MDR1*1*) or a site-directed Ser893 mutation (*MDR1*2*) indicated enhanced efflux of digoxin by cells expressing the MDR1-Ser893 variant. In vivo functional relevance of this SNP was assessed with the known P-glycoprotein drug substrate fexofenadine as a probe of the transporter's activity. In humans, *MDR1*1* and *MDR1*2* variants were associated with differences in fexofenadine levels, consistent with the in vitro data, with the area under the plasma level–time curve being almost 40% greater in the **1/*1* genotype compared with the **2/*2* and the **1/*2* heterozygotes having an intermediate value, suggesting enhanced in vivo P-glycoprotein activity among subjects with the *MDR1*2* allele. Thus allelic variation in *MDR1* is more common than previously recognized and involves multiple SNPs whose allelic frequencies vary between populations, and some of these SNPs are associated with altered P-glycoprotein function. (Clin Pharmacol Ther 2001;70:189-99.)

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P-glycoprotein, the product of the human *MDR1* gene, was first identified by its overexpression in human tumor cells, where it was found to be associated with pleiotropic multidrug resistance to cancer chemotherapeutic agents.[1] However, this apically expressed membrane efflux transporter is also found in normal tissues; for example, the intestinal epithelium, the canalicular membrane of the hepatocyte, the brush border of the renal tubule, and the brain capillary endothelium.[2,3] In addition to anticancer agents, P-glycoprotein also transports a wide range of relatively hydrophobic, amphipathic compounds, including many clinically used drugs.[4,5] Knockout mice in which either the *mdr1a* gene or both the *mdr1a* and *mdr1b* genes are disrupted have been especially useful in determining the importance of P-glycoprotein in a particular drug's disposition, since in these animals the transporter is essentially or completely absent, respectively. Thus it has been shown that P-glycoprotein reduces intestinal absorp-

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Table I. Primers and themocycling conditions for PCR of *MDRI* exonic regions

Exon	*Region*	*Primer set*	*PCR cycling time (s)*
2	IC	5′-GACTAGAGGTTAGTCTCACCTC-3′ 5′-TCGCAACTATGTAAACTATG-3′	30, 30, 15
3	IC	5′-GTCATTAAATATACATTCTATG-3′ 5′-CTATACGAAAATCTTACATCAG-3′	30, 30, 15
4	IC,TM,EC	5′-GCAAATCTTCCATGAAACTG-3′ 5′-GCTTGTTTTTGCTGCAAGTTTC-3′	30, 30, 15
5	EC	5′-GAGACTAAAGAGACATAAATGGTATG-3′ 5′-AACTATCAAGAGTATTGTTCTC-3′	30, 30, 30
6	IC,TM	5′-TCAGGTATGCCTATTATTAC-3′ 5′-ACGACACCCAAGTTCAAC-3′	30, 30, 15
7	IC,TM,EC	5′-AAGTTGATGTTTACAATTCTCAC-3′ 5′-TTCACCTACCTTTGCCCAGAC-3′	30, 30, 30
8	IC	5′-ATCTTCATTTACTGATAAAGAACTCTTAGCGT-3′ 5′-ATGCAATCACAGTTCTAATTGCTGCCAAGACC-3′	30, 30, 30
9	IC,TM,EC	5′-TCTCAGGTACAACAAAAAT-3′ 5′-CTCAATGTAAACACTTACAGTGAGTACTTGTC-3′	30, 30, 15
10	TM,IC	5′-CTTCACATTCCTCAGGTA-3′ 5′-GGCCAACTCAGACTTACA-3′	30, 30, 30
11	IC	5′-ATATACATGCACTTTTTTATAATCTC-3′ 5′-CCAGCTCTCCACAAAATATCACTA-3′	60, 60,* 60
12	ABD,IC	5′-TCCTGTGTCTGTGAATTGCCTTG-3′ 5′-GCTGATCACCGCAGTCTAGCTCGC-3′	30, 30, 15
13	IC	5′-CACTTACTTTTTATTCCAGG-3′ 5′-CAAAGGGCAAGGACAACTTAC-3′	30, 30, 15
14	ABD,IC	5′-CAAGAATTAGTAGTAGAATGTTC-3′ 5′-CTGTGGTAGAAATTTGAC-3′	30, 30, 30
15	IC	5′-ATTTCTCTCTCTTTAGGCCAG-3′ 5′-TGAAGTTAAACTATACCTGC-3′	30, 30, 15
16	IC	5′-CAGTGATTAGCTTTCATTGGTT-3′ 5′-AACTAGGGAACCACAGTTAGTGAG-3′	30, 30, 30
17	IC,TM,EC	5′-ATTTGTGTTTTCTAGGATG-3′ 5′-TGGGCATCACACTTACC-3′	30, 30,* 30
18	TM,EC	5′-GTCCAGGATGGGTTCTTCACTG-3′ 5′-CCAGTTGAATAATGATGC-3′	30, 30, 15
19	IC	5′-TATGTTCCTGCCCACAG-3′ 5′-CCCTCGATAGACATA-3′	30, 30, 15
20	IC	5′-TAAAATCATTTTCTGTGCCACAG-3′ 5′-AAAGGAGGCACGTAC-3′	30, 20, 15
21	IC,TM	5′-GTTTTGCAGGCTATAGGTTCC-3′ 5′-TTTAGTTTGACTCACCTT-3′	30, 20, 20
22	IC	5′-GGTGCTGTCTGTTATCAGA-3′ 5′-ACTCTTCAGCGGTTATTAC-3′	60, 60,** 10
23	TM,EC	5′-TGTCTTCTTTTCGAGAAAC-3′ 5′-ATAGCCCAATACTTACAAC-3′	60, 60,* 60
24	TM,IC	5′-GTGTTTGTGCTTTCCAGAG-3′ 5′-GAAACATCAAACTCACCGGC-3′	30, 25,** 20
25	ABD,IC	5′-TGTGAAAGTGTGCTCACC-3′ 5′-TTCTTCTCATTGCAGAAC-3′	30, 20,** 15
26	IC	5′-CAGCTGCTTGATGGCAAAGA-3′ 5′-CTTACATTAGGCAGTGACTCG-3′	30, 30, 15
27	ABD,IC	5′-TACTTTCTATAAGCAAGCTG-3′ 5′-GTAACTGTCAATAATCTGGCTGC-3′	30, 30, 15
28	IC	5′-TGGAAGAGGAATTAGGGAAAAGAAC-3′ 5′-AAGATCTCATACAGTCAGAG-3′	30, 30, 30

PCR was generally carried out at 94°C, 53°C, and 72°C for 30 cycles (*asterisk,* annealing temperature of 51°C; *2 asterisks,* annealing temperature of 55°C). Exonic regions in MDR1 are outlined as intracellular (IC), transmembrane spanning (TM), extracellular (EC), and ATP-binding (ABD) domains.

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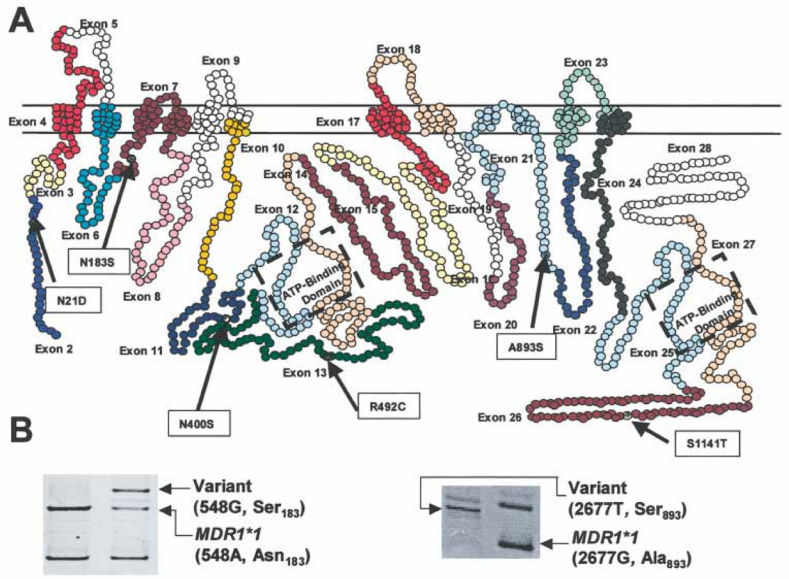

Fig 1. A, Schematic drawing of human P-glycoprotein transporter; exons encoding regions of P-glycoprotein are indicated in *different colors*, whereas nonsynonymous nucleotide substitutions identified in this study are indicated by *arrows*. **A** and **B** represent SSCP patterns of two identified *MDR1* variants. The variant bands were sequence verified.

tion, enhances biliary, intestinal, and renal excretion, and limits the brain uptake of its substrates.[6-8]

Variability in P-glycoprotein activity caused by drug selection or site-directed mutagenesis has been extensively used to investigate structure-function relationships of the transporter, and a number of variants with altered in vitro activity have been identified.[9,10] A null genetic polymorphism has also been described in vivo in a subpopulation of outbred CF-1 mice, which are physiologically normal but unusually sensitive to the toxic effects of certain drugs that are P-glycoprotein substrates.[11,12] More recently, a silent mutation in exon 26 of human *MDR1* has been found to be associated with the impaired oral bioavailability of digoxin in humans; however, the causative molecular genetic mechanism of this observation is unknown.[13]

This study was, therefore, designed to identify single nucleotide polymorphisms (SNPs) in the coding region of the *MDR1* gene with single-strand conformational polymorphism (SSCP) analysis and direct sequencing and to assess their prevalence in European American and African American populations. In addition, the possible functional significance of such polymorphism was investigated by the use of in vitro cell lines expressing the relevant allelic variants and in vivo with a probe drug of P-glycoprotein activity, fexofenadine.[14]

METHODS

Identification, additional cloning, and sequencing of MDR1 intronic regions. Although the complete intron-exon boundary data for human *MDR1* have been reported,[15] the published sequences at the intron-exon boundaries were not always found to be optimal for polymerase chain reaction (PCR) primer design. A 177-kilobase (kb) BAC clone (G2337872) was, therefore, identified from the Human Genome Project (GenBank) that had the complete 5′-flanking region and genomic sequences complete to exon 7 of *MDR1*. Long PCR was used to amplify the exonic-intronic sequences flanking exons 9, 11, 14, 16, 18, 27, and 28 (Expand Long PCR; Boehringer Mannheim, Indianapolis, Ind), with primers

Table II. *MDR1* alleles and fexofenadine disposition

Genotype	*Subjects*		*AUC(0-4)*	*AUC(0-8)*	*AUC(0-16)*
	No.	*%*	*(ng · mL^{-1} · h)*	*(ng · mL^{-1} · h)*	*(ng · mL^{-1} · h)*
Genotype **1*					
Caucasian					
**1/*1*	6	16	1316 ± 543	2218 ± 738	2573 ± 838
**1/*1a*	1	3	1356	1927	2261
**1/*1b*	1	3	1242	1585	1839
**1/*2*	4	11	1171 ± 967	1906 ± 1451	2266 ± 1687
**1/*2a*	4	11	1191 ± 816	2146 ± 943	2596 ± 962
**1/*2b*	1	3	567	1058	1263
**1/*3*	1	3	3944	5914	6742
**1/*5*	2	5	1650 ± 559	2552 ± 397	2968 ± 390
**1/*6*	1	3	1617	2511	2799
African American					
**1/*1*	9	39	1030 ± 435	1787 ± 728	2191 ± 900
**1/*1b*	1	4	2601	4098	4722
**1/*1c*	1	4	1078	1767	2128
**1/*2*	3	13	1099 ± 70	1951 ± 358	2271 ± 444
**1/*4*	3	13	1723 ± 1142	2311 ± 1371	3045 ± 1322
**1/*6*	2	9	785 ± 867	1387 ± 1220	1648 ± 1375
Other genotypes					
Caucasian					
**1a/*1a*	1	3	1417	2085	2460
**2/*2*	5	14	837 ± 311	1515 ± 745	1855 ± 898
**2/*2a*	2	5	799 ± 589	1648 ± 718	2227 ± 470
**2/*3*	1	3	1404	2141	2529
**2/*5*	1	3	1327	2117	2517
**2/*8*	1	3	1030	1875	1315
**2a/*2c*	1	3	664	1129	1345
**6/*1a*	1	3	1702	2660	2975
**6/*2*	1	3	1156	1784	2049
**6/*3*	1	3	920	1424	1665
**7/*2b*	1	3	2100	3502	4131
African American					
**1b/*1c*	1	4	1845	3035	3384
**6/*4*	1	4	1797	3356	3991
**6/*6*	1	4	491	1312	1446
**7/*7*	1	4	2599	4328	5004

Allelic combinations in *MDR1* and fexofenadine pharmacokinetic profiles are shown for all subjects (N = 60; mean ± standard deviation). ANOVA (with Tukey-Kramer multiple comparison test) was used for statistical comparison. AUC, Area under the concentration-time profile (at time zero to 4 hours, time zero to 8 hours, and time zero to 16 hours); C_{max}, maximum plasma drug concentration after single dose administration; T_{max}, time to reach maximum concentration after drug administration; $t_{½}$, elimination half-life.

5′-ATTTTCATTTACTGATAAAGAACTCTTAGCGT-3′ and 5′-TAGTAGTGCATATGTCTGTAGTATTCAACA-GTTG-3′ for intron 8/exon 9 (approximately 7 kb), 5′-CTTCACATTCCTCAGGTA-3′ and 5′-CAAAGGGC-AAGGACAACTTAC-3′ for intron 10/exon 11/intron 11 (approximately 1 kb), 5′-CACTTACTTTTTATT-CCAGG-3′ and 5′-TGAAGTTAAACTATACCTGC-3′ for intron 13/exon 14/intron 14 (approximately 3.5 kb), 5′-ATTTCTCTCTCTTTAGGCCAG-3′ and 5′-TGGGC-ATCACACTTACC-3′ for intron 15/exon 16/intron 16 (approximately 1.6 kb), 5′-ATTTGTGTTTTCTAG-GATG-3′ and 5′-CCCTCGATAGACATA-3′ for intron 17/exon 18/intron 18 (approximately 5 kb), 5′-CAGCTGCTTGATGGCAAAGA-3′ and 5′-CTCATAC-AGTCAGAGTTCAC-3′ for intron 27/exon 27/intron 27 (approximately 5 kb), and 5′-CTTTATTTACA-GAAATATAGC-3′ and 5′-TCACTGGCGCTTTGTT-CCAGCCTGGA-3′ for intron 27/exon 28 (approxi-mately 1.6 kb).

Identification of variants in MDR1 gene. A sample of 10 mL blood was obtained from each subject, and deoxyribonucleic acid (DNA) was isolated with the Qiamp system (Qiagen Inc, Valencia, Calif). PCR was carried out with approximately 200 ng of human

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C_{max} ($ng \cdot mL^{-1}$)	T_{max} (h)	$t_{1/2}$ (h)
508 ± 205	2.7 ± 0.8	2.8 ± 0.4
511	1	2.9
334	2	2.9
400 ± 282	3.3 ± 1	3.1 ± 0.7
530 ± 293	3.3 ± 1.5	2.9 ± 0.5
206	4	2.6
1572	2	2.8
1655 ± 223	1.5 ± 0.7	3.2 ± 0.03
596	3	2.3
386 ± 163	1.9 ± 0.9	3.3 ± 0.3
802	3	3.1
315	1	3.0
405 ± 29	2.3 ± 1.2	2.7 ± 0.4
587 ± 327	1.3 ± 0.6	3.2 ± 0.5
302 ± 255	4 ± 1.4	2.5 ± 0.2
452	1	3.4
317 ± 185	2.4 ± 1.7	3.5 ± 0.9
327 ± 129	5 ± 1.4	2.9 ± 0.1
487	1	3.2
449	2	3.6
401	1	3.0
221	4	3.2
619	2	2.4
388	1	3.1
298	1	3.2
927	3	2.9
734	2	2.2
636	2	2.5
260	2	2.7
1012	3	2.9

genomic DNA, consisting of each of the deoxynucleoside transaminases (0.25 mmol/L each) and the specific primer pair (4 µmol/L each), in PCR buffer II (Perkin-Elmer, Branchburg, NJ) with 2.5-mmol/L magnesium chloride and 2.5 units of AmpliTaq DNA polymerase (Perkin-Elmer), in a final reaction volume of 50 µL. The complete oligonucleotide primer sequences and the PCR cycling conditions are outlined in Table I. After the PCR reaction, nonisotopic SSCP analysis was used to identify the presence of *MDR1* allelic variants.[16] The running time of the gel was varied to yield optimal separation of the single strands; for the most part running times of 4 to 6 hours were sufficient (Table I). Variations in the single-strand mobility patterns were clearly visualized. Exonic regions in *MDR1* in which mutations were identified by SSCP analysis were fully sequenced in all subjects with an ABI 3700 DNA Analyzer (Applied Biosystems, Foster City, Calif), and the mutation frequencies were verified.

Haplotype assignment. Haplotypes could be defined when subjects who were homozygous at a single polymorphic site or multiple sites were identified. Additionally, when subjects who were homozygous for an SNP at one site were found to be heterozygous for another SNP at a different site, an allelic assignment was possible. Furthermore, in the case of polymorphic sites located close to each other, individual alleles were separated by SSCP and haplotypes were determined by direct sequencing. Because of their low frequency, certain SNPs could not be definitively assigned an allele; in such cases a putative predicted allele was suggested. These haplotype assignments were necessary for comparison of genotype versus phenotype.

Retroviral expression of variant MDR1 alleles. Ala893-containing *MDR1sc* complementary DNA[17] was subjected to site-directed mutagenesis with a mutant primer and the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, Calif) to generate MDR1-Ser893, which was confirmed by DNA sequencing. HEK293T cells were plated on 100 mm dishes at 4×10^6 cells, and 20 hours later 5 µg of each MDR1 plasmid was triple transfected along with 3.3 µg of packaging plasmid (VSV-G) and 3.3 µg of gal-Pol expression plasmid (pEQPAM$_3$-E) with GenePorter (Gene Therapy Systems, San Diego, Calif) into the HEK293T cells. Supernatants from the cells were collected at 24, 48, and 72 hours, pooled, filtered, supplemented with 6 µg/mL Polybrene (Sigma Chemical Company, St Louis, Mo), and used to infect the murine NIH-3T3 ecotropic packaging cell line GP+E86. After 3 days, cells were trypsinized, washed with phosphate-buffered saline solution (PBS), and resuspended in this buffer, which contained monoclonal antibody to an external MDR1 epitope (4E3; DAKO Corporation, Carpinteria, Calif) for 30 minutes at 22°C. Cells were washed with PBS and incubated with secondary RPE *(Rhodophyta phycoerythrin)*–conjugated antimouse antibody (DAKO Corporation) in the dark for 30 minutes. The cells were washed once more with PBS and analyzed by fluorescence-activated cell sorting (FACS) analysis on a Vantage flow cytometer (Becton Dickinson, San Jose, Calif). The MDR1 antibody–positive population of cells was collected, expanded, and repetitively reanalyzed, as described,

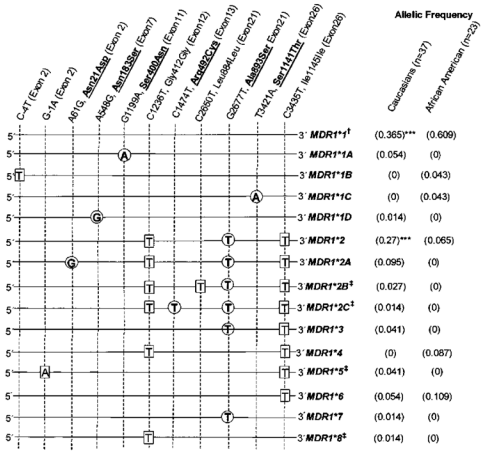

Fig 2. Schematic of *MDR1* allelic variants. Mutations resulting in nonconservative amino acid changes are shown in *bold* and *circled*, whereas conservative mutations are *boxed*. The first published *MDR1* sequence is shown as the *MDR1*1* allele. *Dagger,* First published *MDR1* sequence and represents predicted potential allele. Allelic frequencies of the variant *MDR1* among European American and African American subjects are also shown. Interethnic differences in *MDR1*1* and *MDR1*2* allelic frequencies were statistically significant (*3 asterisks,* Fisher exact test, $P < .001$).

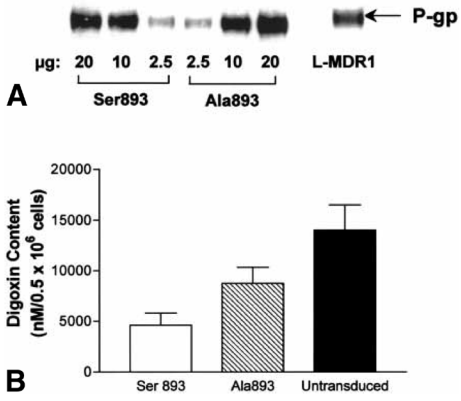

Fig 3. A, Immunoblot of lysates from NIH-3T3 GP+E86 cells stably transduced with MDR1-Ser893 or MDR1-Ala893 alleles and from L-MDR1 cells in which P-glycoprotein is overexpressed. **B,** Accumulation of digoxin in untransduced cells and those expressing *MDR1* with either Ser893 or Ala893 substitutions (mean ± standard deviation of two independent experiments; $P < .002$ between the two variants).

until 100% of the population displayed an MDR1 antibody–positive phenotype. These cells were used for subsequent experiments.

Digoxin drug accumulation assay. To assess digoxin drug uptake, a modification of a previously described procedure was used.[18] Briefly, cultured cells were harvested and counted, and 0.5×10^6 cells placed in a tube of medium containing digoxin labeled with tritium (50 μmol/L) at 37°C. After 30 minutes, cells were washed 4 times with ice-cold PBS, resuspended in PBS, sonicated, and analyzed for radioactivity by scintillation counting.

Immunoblots. NIH3T3-GP+E86 untransduced control cells or derivative cells stably expressing MDR1-Ser893 or MDR1-Ala893 were pelleted and lysed in storage buffer (100-mmol/L potassium phosphate, pH 7.4; 1.0-mmol/L ethylenediaminetetraacetic acid, 20% glycerol, 1-mmol/L dithiothreitol, 20-μmol/L butylated hydroxytoluene, 2-mmol/L phenylmethylsulfonyl fluoride), and 2.5 to 20 μg was resolved on 7.5% polyacrylamide gel electrophoresis, transferred to nitrocellulose, and developed with polyclonal rabbit anti-mdr (Ab-1) immunoglobulin G (Oncogene Science, Uniondale, NY) and appropriate secondary antibody with the ECL detection system (Amersham, Piscataway, NJ).

Fexofenadine disposition in healthy subjects. Studies were undertaken in 37 European Americans (20 women) who were 19 to 48 years old, weighing 50 to 94 kg, and in 23 African Americans (20 women) who were 19 to 55 years old and weighed 55 to 97 kg. The subjects were all unrelated residents of Middle Tennessee and self-identified their racial background.[19] On the basis of medical history, physical examination, and routine biochemical laboratory test results, all of the subjects were considered healthy. During the 7 days before the study, subjects did not take any drugs and did not drink any alcohol. On the study day each subject received a 180-mg oral dose of fexofenadine, after an overnight fast. Heparinized blood samples (10 mL) were obtained through an indwelling cannula at 0, 1, 2, 3, 4, 5, 6, 8, 12, 16, and 24 hours after drug administration. The protocol was approved by the Vanderbilt University Institutional Review Board–Health Services, and written informed consent was obtained.

Analysis of fexofenadine. Fexofenadine plasma levels were determined with a method that was based on HPLC.[20] Briefly, after the addition of internal standard (MDL 26,042A; Hoechst Marion Roussel, Inc, Kansas

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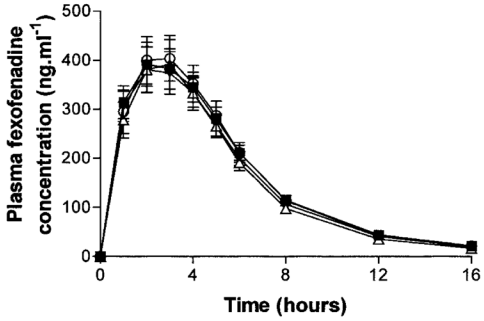

Fig 4. Fexofenadine plasma concentration-time profiles according to sex and racial background after oral administration of 180 mg fexofenadine. *Triangles,* Males (n = 20); *boxes,* females (n = 40); *circles,* African Americans (n = 23); *inverted triangles,* European Americans (n = 37).

City, Mo) to 1 mL of sample, solid-phase extraction was performed with a 500-mg C_{18} minicolumn (Bond Elut; Varian, Harbor City, Calif). The analytes were eluted from the column with methanol and evaporated at 55°C under a stream of nitrogen. The sample was then reconstituted with 100 µL of mobile phase, and 50 µL was injected onto the HPLC column (250 mm × 4.5 mm inner diameter; Sperisorb 55 CN-RP; Waters Corp, Milford, Mass). The mobile phase was acetonitrile/methanol/0.012-nmol/L ammonium acetate buffer (19:29:52 [vol/vol/vol]) at a flow rate of 0.8 mL/min. A model FP-920 Intelligent Fluorescence Detector (Jasco, Easton, Md) was used to monitor the HPLC effluent at an excitation wavelength of 230 nm and an emission wavelength of 280 nm. Under these conditions, the internal standard and fexofenadine eluted at 12.4 minutes and 14.7 minutes, respectively. Within-day coefficients of variation in quality control samples (n = 6 for each) at low (25 ng/mL), medium (100 ng/mL), and high (200 ng/mL) concentrations were 6.2%, 4.4%, and 4.1%, respectively.

Statistical analysis. Determination of the statistical differences between various group parameters were determined with either a Student *t* test, Mann-Whitney *U* test, or ANOVA (with Tukey-Kramer multiple comparison test) or the Fisher exact test, as appropriate. $P < .05$ was taken to be the minimum level of statistical significance.

RESULTS

Identification of variants in* MDR1 *gene. Abnormal SSCP conformers were directly sequenced to identify

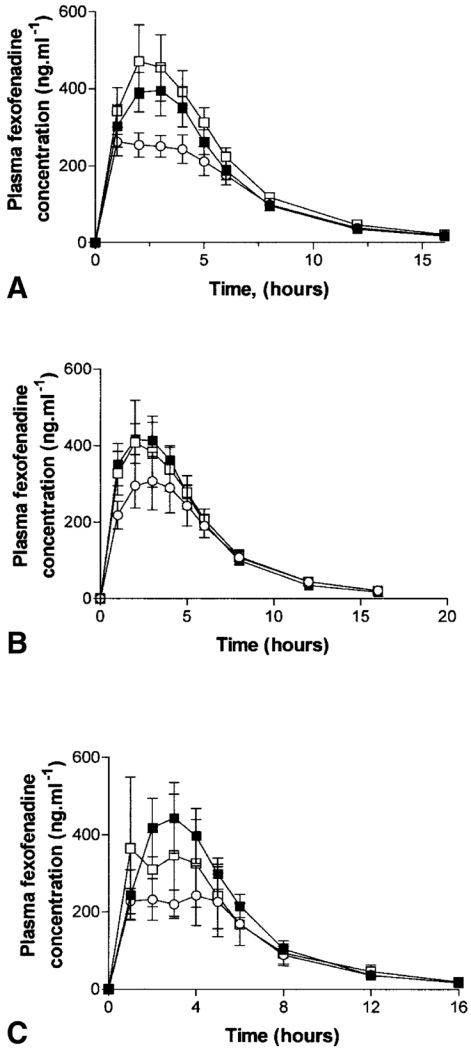

Fig 5. Plasma concentration-time profiles after administration of 180 mg fexofenadine orally according to genotype (mean ± standard error): **A,** *MDR1* exon 26, C3435T. *Solid squares,* CC (n = 9); *open squares,* CT (n = 16); *circles,* TT (n = 12). **B,** *MDR1* exon 21 G2677T. *Solid squares,* GG (n = 13); *open squares,* GT (n = 14); *circles,* TT (n = 10). **C,** *MDR1*1* or *2 alleles. *Solid squares, *1*1* (n = 6); *open squares, *1*2* (n = 4); *circles,* *2*2 (n = 5). AUC at time zero to 4 hours: $P = .054$, exon 21, GG versus TT; $P = .036$, exon 26, CC vs TT.

allelic variants (Fig 1). On the basis of the summed frequency of each SNP in the two study populations, these were termed *MDR1*2* through *MDR1*8*, according to a recently suggested approach to defining nomenclature systems,[21] with the originally published sequence (Gene Bank accession number AC002457; AC005068) being defined as *MDR1*1* (Fig 2). In the first published *MDR1* sequence, derived from a KB1 tumor cell line, two tandem mutations at positions 554 and 555, encoding Val185, were reported; however, these mutations were not observed in any of the subjects described here or in other studies.[13] Accordingly, these mutations were considered to be present only in KB1 tumor cells and not characteristic of the *MDR1*1* allele. Among the observed SNPs, 4 of the 10 were synonymous, and 6 would be expected to result in amino acid substitutions in the encoded protein. Moreover, in the case of *MDR1*2A* and *MDR1*2C*, two nonsynonymous SNPs were present simultaneously. In addition, a number of detected SNPs, such as A548G (Asn183Ser), C1472T (Arg492Cys), and T3421A (Ser1141Thr), represented previously undescribed nonsynonymous SNPs. Because of previous reports of genetic variants,[10,22] exons 7 and 24 were also sequenced even though SSCP analysis did not suggest any polymorphism. Such sequencing did not reveal any additional SNPs.

*MDR1*1*-related alleles were the most common allelic variants with an overall frequency of 43.3% in European Americans and 69.5% in African Americans. Accordingly, at least one copy of this allele was detected in 67% of European Americans and 86% of African Americans (Table II). The second most common allele among European Americans (62%) but rarer in the African American population (13%) (Fig 2) was found to contain 3 SNPs simultaneously (*MDR1*2*); C1236T in exon 12, G2677T in exon 21, and C3435T in exon 26. Direct sequencing of DNA from subjects homozygous for all 3 of these SNPs strongly suggested that they were linked. In addition, 21 of 22 subjects (9/9 European Caucasian subjects) who were homozygous for the exon 26 SNP (C/C) were G/G homozygous at base position 2677 and 9 of 14 subjects (9/12 European Americans) who were exon 26 T/T homozygotes were also T/T homozygous for the exon 21 SNP. Furthermore, 15 of 24 heterozygous exon 26 individuals (11/16 European Americans) were also heterozygous with respect to the exon 21 SNP. The C2677T transversion in exon 21 resulted in an alteration in the encoded protein (Ala893Ser). This allelic variant was frequently associated with additional nucleotide base substitutions; some were synonymous (allele *MDR1*2B*) but some also altered other encoded amino acids (alleles *MDR1*2A*, *MDR1*2C*). Indeed the SNPs in exon 12, 21, and 26 SNPs appeared to be closely linked in both populations. A number of the rarer SNPs were only detected in one of the two populations, and therefore the frequency of certain of the alleles was dependent on racial background (Fig 2).

Variant MDR1 expression and function in vitro. P-glycoprotein expression was found to be similar in cells transduced with MDR1-Ala893 compared with MDR1-Ser893 (Fig 3, *A*). When MDR1-Ser893 or MDR1-Ala893 was incubated with digoxin and compared with untransduced control, the Ser893 variant transporter resulted in a 47% lower intracellular digoxin concentration ($P < .002$) than did the Ala893 variant (Fig 3, *B*).

Phenotyping studies in humans. In humans, the fexofenadine plasma concentration-time profile after single oral dose administration did not differ by sex or racial background (Fig 4; Table II). Comparison of area under the plasma concentration time (AUC) values of European American subjects homozygous for CC versus TT at position 3435 (exon 26) revealed a statistically significant ($P = .036$) difference in the AUC values at time 0 to 4 hours (Fig 5). A similar trend was observed when genotyping was done for exon 21 (position 2677) GG versus TT subjects ($P = .054$). Moreover, substratification of the data according to the two commonest alleles, *MDR1*1* and *MDR1*2*, which include the linked SNPs in exons 12, 21, and 26, indicated genotype-phenotype associations (Fig 5; Table II). In European Americans, the population in which the variants were more numerous, subjects homozygous for thymidine (T/T, encoding Ser893) at position 2677 of exon 21 (*MDR1*2*) had lower plasma levels than those with the G/G (*MDR1*1*, Ala893) genotype. Moreover, a gene-dose effect was present, with the AUC at time 0 to 16 hours being almost 40% greater in the **1/*1* genotype compared with the **2/*2* homozygotes, and the **1/*2* heterozygotes had intermediate plasma concentrations (Fig 5).

DISCUSSION

P-glycoprotein, a member of the adenosine triphosphatase–binding cassette (ABC) superfamily of transporters, was initially identified as an efflux pump responsible, in part, for the multidrug resistance phenotype to certain cytotoxic cancer chemotherapeutic agents.[1, 23] Many ABC transporters such as MRP2, ABCR, ABC1, and BSEP exhibit genetic variability that has functional consequences,[24-27] but comparable information on P-glycoprotein is sparse. In vitro studies with either drug selection or site-directed mutagen-

esis have indicated that such manipulations can result in ribonucleic acid (RNA) misprocessing, altered substrate specificity, or reduced function[28-30]; however, the existence and consequences of such variations in vivo have not been studied. A subpopulation of CF-1 mice in which P-glycoprotein is not expressed have been described and appear to be physiologically normal although exhibiting increased sensitivity to certain drugs.[11] Whether a similar null phenotype or other genetic variants are present in humans is largely unknown. Recently, however, a number of SNPs have been described in the human *MDR1* gene and one, a conservative "wobble" polymorphism in exon 26 (C3435T), was found to be associated with reduced expression and function of P-glycoprotein located in the intestinal epithelium.[13]

This study confirms the presence of multiple SNPs in human *MDR1*, defines their frequencies, and describes several new nonconservative variants in the coding region. In particular, the previously reported[13] SNP in exon 26 (C3435T) was found to be common but at a frequency dependent on racial background, in that 62% of European Americans but only 13% of African Americans carried at least one such allele. It is important that this SNP was found to be linked to a G2677T transversion in exon 21 (Ala893Ser), as well as a synonymous C1236T SNP in exon 12. The presence or absence of these 3 SNPS was the basis of *MDR1*1* and *MDR1*2* haplotype assignments, and interethnic differences in the frequencies of *MDR1*1* and *MDR1*2* were statistically significant ($P < .001$). The G2677T SNP was previously reported in selected cell lines, refractory lymphoma tissue, and a number of healthy subjects,[10,22] with a heterozygote frequency of 43%. Given the observed linkage between the exon 12, 21, and 26 SNPs, it is not clear why the SNP in exon 21 was not detected in the recent study that identified the conservative "wobble" polymorphism in exon 26 (C3435T) among Caucasian subjects.[13] In addition, other SNPs occurred among subjects with *MDR1*1* or *MDR1*2* genotypic backgrounds, some of which would produce additional nonconservative amino acid changes in the encoded P-glycoprotein. Previously, a G2955C transversion in exon 24 (Ala999Trp) had been reported to be common in both tumor and normal tissues[10]; however, neither this study nor an earlier one,[13] collectively involving a total of 84 healthy subjects, identified this SNP.

Comparison of MDR1 transport in stably transfected cells, with similar levels of protein expression, indicated that the Ala893Ser amino acid change associated with the exon 21 SNP altered the protein's function. The results with digoxin uptake showed that Ser893 variant–containing cells exhibited enhanced efflux characteristics compared with those in which the Ala893 was expressed.

To establish the in vivo functional consequences of the exon 21 and 26 SNPs, studies were undertaken in healthy subjects with fexofenadine used as an in vivo probe of P-glycoprotein. This widely used and safe drug was chosen on the basis of in vitro transport and comparative tissue distribution in *mdr1a*(+/+) and *mdr1a*(–/–) mice, indicating that this transporter was an important determinant of fexofenadine's in vivo disposition.[14] Moreover, the drug's plasma concentration-time profile in mice reflects the *mdr1a/b* genotype in a gene-dose–related fashion (data not shown). However, it should be noted that, like digoxin (another putative in vivo probe of P-glycoprotein), other transporters such as OATP (organic anion-transporting polypeptide)[31,32] may be involved in the uptake of fexofenadine into certain tissues such as the liver[14]; thus a role for such transporters in the measured plasma concentration-time profiles of fexofenadine or digoxin cannot be excluded.

Fexofenadine's aggregate disposition and hence, by extension, P-glycoprotein function were found to be similar in men and women, and no population difference was found to be present, on average, between European Americans and African Americans (Fig 4). However, further analysis indicated that the *MDR1* genotype was associated with phenotypic differences; only the European American data were analyzed in detail, since the number of African Americans in each genotypic group was small. When all European Caucasian subjects were evaluated for the exon 26 (C3435T) SNP, both exon 21 and exon 26 SNPs were associated with altered fexofenadine disposition, especially when the two homozygous groups are compared. In fact, subjects homozygous for the exon 26 CC genotype had significantly ($P = .036$) higher fexofenadine AUC values at time zero to 4 hours than the TT subjects (1266 ± 145 ng $\cdot$ mL^{-1} $\cdot$ h versus 890 ± 95 ng $\cdot$ mL^{-1} $\cdot$ h; Fig 5, *A*). Similarly, the exon 21 G2677T polymorphism, which in a majority of the subjects is linked to the exon 26 C3435T transition, revealed a similar difference in AUC values at time zero to 4 hours ($P = .054$; Fig 5, *B*). Indeed, when these two linked SNPs were considered together (ie, *MDR1*2* compared with the *MDR1*1* genotype), a gene-dose effect appeared to be present, suggesting the Ala893Ser amino acid substitution was associated with lower oral bioavailability of fexofenadine (Fig 5, *C*). This finding is fully consistent with the in vitro expression studies demonstrating enhanced efflux transporting ability of

the Ser893 variant and the important role of P-glycoprotein in determining the absorption of its substrates from the gastrointestinal tract after oral administration.[33] The exact mechanism by which Ser893 in P-glycoprotein results in enhanced activity of the transporter is not clear. It is known that certain serine residues in P-glycoprotein are subject to phosphorylation by protein kinase C.[34] Additional studies will be needed to determine whether Ser893 can be phosphorylated and thereby affect the activity of this transporter.

The exon 26 C3435T SNP has previously been found to be associated with a change in digoxin's oral absorption; specifically, TT homozygotes were found to have higher steady-state maximum plasma concentrations compared with those in CC subjects.[13] Given the linkage of this SNP with that in exon 21 encoding a transporter with increased in vitro activity, this finding is unexpected and also contrary to results obtained with fexofenadine used as an in vivo probe. The reason for this discrepancy is currently unclear, as is the failure of the previous study[13] to identify the apparently common G2677T substitution in exon 21, although it should be noted that the linkage between exon 21 and 26 SNPs is not complete and they can occur independently. It is interesting that in a recent study in which nelfinavir disposition was compared among subjects genotyped for the exon 26 SNP, those with the TT genotype were more likely to have lower plasma nelfinavir concentrations than subjects with the CC genotype.[35] In addition to metabolism, nelfinavir disposition is highly dependent on P-glycoprotein[8]; thus the finding that the exon 26 TT genotype was associated with a lower nelfinavir level is in concordance with the data presented in this study. Genotype-related differences in fexofenadine AUC levels were most prominent during the first 4 hours after fexofenadine administration. Therefore the TT genotype may be associated with increased P-glycoprotein transport activity that reduces the extent of initial oral drug absorption. On the other hand, the silent or "wobble" polymorphism in exon 26 is linked to the exon 21 G2677T polymorphism in a majority of subjects, so it is possible that the previously reported differences with digoxin and also the expression level of P-glycoprotein in the human duodenum might reflect either linkage to an as yet undefined promoter/enhancer region polymorphism(s) or nucleotide sequences that are important for messenger RNA processing. Moreover, dietary/environmental differences, as well as the involvement of other transport system(s), for both digoxin and fexofenadine may have played a role. Clearly, randomized crossover studies for fexofenadine and digoxin disposition will be needed to clarify this issue.

In summary, the described study indicates the presence of multiple SNPs in the human *MDR1* gene and the fact that these can be present in a single allele and alter P-glycoprotein activity. Moreover, the frequencies of these genetic variants differ according to the racial background of the study population. Future studies relating *MDR1* genotype to the encoded protein's phenotype will, therefore, need to consider such haplotypes rather than single polymorphisms, as well as the likelihood of population differences. Moreover, as additional genetic variants of *MDR1* are described and their frequencies defined in larger populations of different racial/ethnic backgrounds, standardization of allelic assignment will undoubtedly become necessary. The nomenclature used in this study is the first to address this issue and may need to be modified as more information becomes available. Finally, it appears that in vivo phenotyping with fexofenadine, a safe and widely used, nonsedating antihistamine, can be used to assess the functional importance of SNPs in a complex gene such as *MDR1*.

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