A Novel Transversion in the Intron 5 Donor Splice Junction of *CYP2C19* and a Sequence Polymorphism in Exon 3 Contribute to the Poor Metabolizer Phenotype for the Anticonvulsant Drug *S*-Mephenytoin[1]

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ABSTRACT

Cytochrome P-450 (CYP) 2C19 is responsible for the metabolism of a number of therapeutic agents such as *S*-mephenytoin, omeprazole, proguanil, certain barbiturates, diazepam, propranolol, citalopram and imipramine. Genetic polymorphisms in this enzyme are responsible for the poor metabolizers (PM) of mephenytoin, which represent ~13–23% of Asians and 3–5% of Caucasians. Several polymorphisms contribute to this phenotype. We have isolated two new allelic variants that contribute to the PM phenotype in Caucasians. *CYP2C19*7* contained a single T → A nucleotide transversion in the invariant GT at the 5' donor splice site of intron 5. The second PM allele, *CYP2C19*8,* consisted of a T358C nucleotide transition in exon 3 that results in a Trp120Arg substitution. In a bacterial expression system, CYP2C198 protein exhibited a dramatic (~90% and 70%) reduction in the metabolism of *S*-mephenytoin and tolbutamide, respectively, when compared with the wild-type CYP2C191B protein. Restriction fragment length polymerase chain reaction tests were developed to identify the new allelic variants.

A genetic polymorphism associated with impaired metabolism of the *S*-enantiomer of the anticonvulsant drug mephenytoin has been studied extensively in humans (Wilkinson et al., 1989). The enzyme responsible for this pathway is cytochrome P-450 (CYP) 2C19 (Wrighton et al., 1993; Goldstein et al., 1994). Population studies show that individuals can be divided into two phenotypes, extensive metabolizer (EMs) and poor metabolizers (PMs), with respect to *S*-mephenytoin 4'-hydroxylation (Wilkinson et al., 1989). The frequency of the PM phenotype is greater in Asian populations (13–23%) than in Caucasian populations (3–5%). The *CYP2C19* polymorphism is of clinical importance because CYP2C19 catalyzes the metabolism not only of *S*-mephenytoin but also the oxidation of diverse pharmacologically important therapeutic agents including omperazole, a proton pump inhibitor that

binds to the H^+/K^+ ATPase (Andersson et al., 1992), certain tricyclic antidepressants (Baumann et al., 1986; Skjelbo et al., 1991; Sindrup et al., 1993; Nielsen et al., 1994), some barbiturates (Küpfer and Branch, 1985; Adedoyin et al., 1994), the activation of antimalarial drugs such as proguanil (Ward et al., 1991), and is partially responsible for metabolism of certain β-blockers such as propranolol (Ward et al., 1989). It also metabolizes the HIV protease inhibitor nelfinavir to its major circulating metabolite, which has an antiviral activity similar to that of nelfinavir itself (Lillibridge et al., 1998).

Previous studies in our laboratory have described five mutations in the *CYP2C19* gene that affect the expression or metabolic activity of CYP2C19 with regard to the hydroxylation of *S*-mephenytoin. The two most common defects are two null alleles, including a mutation in exon 5 (*CYP2C19*2*) that introduces a cryptic splice site 40 bases into the exon and the second is a single base transition in exon 4 (*CYP2C19*3*) that produces a premature stop codon. These two defects account for >99% of the defective alleles in the Oriental populations

ABBREVIATIONS: CYP, cytochrome P-450; EM, extensive metabolizer; HI, hydroxylation index; PCR, polymerase chain reaction; PM, poor metabolizer; RFLP, restriction fragment length polymorphism.

but only ~87% of Caucasian defective alleles (de Morais et al., 1994a,b; Brøsen et al., 1995). In more recent studies, we have reported the presence of three additional defects resulting in amino acid substitutions in various domains of the enzyme. A transition in the initiation codon (*CYP2C19*4*) accounted for an additional 3% of the defective alleles in Caucasian PMs (Ferguson et al., 1998). A rare mutation (~1.5% of Caucasian PM alleles) that produced an amino acid change in the heme binding region (*CYP2C19*5*) resulted in an enzyme that had negligible catalytic activity toward CYP2C19 substrates (Ibeanu et al., 1998a). *CYP2C19*6,* which contains a single amino acid change in exon 3, also had minimal catalytic activity toward CYP2C19 substrates in a recombinant expression system (Ibeanu et al., 1998b).

In the present study, we identified two new Caucasian PM alleles for *S*-mephenytoin. *CYP2C19*7* contained a single T → A base change at the donor site in intron 5. This is the first reported PM allele of *S*-mephenytoin hydroxylase attributed to a base transversion. A second mutation (T358C) found in exon 3, now termed *CYP2C19*8,* was confirmed by protein expression and an in vitro activity assay to represent a functionally defective allele of *CYP2C19*.

Materials and Methods

Amplification and Sequencing of *CYP2C19* Gene. Genomic DNA from a Danish PM outlier (subject 19; Brøsen et al., 1995) and a French PM outlier (subject 11204), whose genotypes were nonconcordant with their PM phenotype for the metabolism of *S*-mephenytoin, were amplified across all exons using *CYP2C19* intron-specific primers. Sequencing was performed on polymerase chain reaction (PCR) products using an Applied Biosystems (Foster City, CA) automated sequencer with a PRISM Dye Terminator Cycle Sequencing Kit and sequence comparisons made using the University of Wisconsin Genetics Computer Group (Madison, WI) software package.

Genotyping Tests. Genomic DNA was isolated from blood using QIAamp blood kits (Qiagen, Chatsworth, CA) according to the manufacturer's protocol. PCR-based restriction fragment length polymorphism (RFLP) tests for defective *CYP2C19* alleles have been previously described (Goldstein and Blaisdell, 1996; Xiao et al. 1997; Ferguson et al., 1998; Ibeanu et al. 1998a,b). In the present study, a mismatch PCR-RFLP genotyping test was developed to detect the presence of a new base change in the donor splice site in intron 5 of Danish subject 19 (*CYP2C19*7*). This test used a *CYP2C19* exon 5-specific forward primer (5′-AAACCTTGCTTTTATGGAAAGTG-3′) and a *CYP2C19* intron 5 reverse primer with a 1-base pair (bp) mismatch (underlined) (5′-ATAACTAAGCTTTTGTTAACAT<u>G</u>TT-3′), in a method similar to that described for *CYP2C19*3* (Ferguson et al., 1998). The mismatched primer introduced a *Mae*III restriction site in *CYP2C19* PCR products from DNA with the normal donor splice sites but not in PCR products exhibiting the intron change. Amplification was similar to that described for *CYP2C19*2* and *CYP2C19*3* (Goldstein and Blaisdell, 1996), except that the number of cycles was 40. The resulting 142-bp products were digested with 1 unit of *Mae*III at 55°C for 4 h or longer, and the fragments were separated on 4% agarose gels. PCR products from alleles with an intact splice site were cut into 115-bp and 27-bp fragments. Products from the new *CYP2C19*7* allele resulted in an undigested 142-bp product.

Another PCR-RFLP genotyping test was developed to detect the presence of a new base pair change found in exon 3 of Swiss subject 11204 (*CYP2C19*8*). The amplification procedure was similar to that described for *CYP2C19*2* and *CYP2C19*3* (Goldstein and Blaisdell, 1996), except that *CYP2C19* intron-specific primers (5′-GAGGA-TGGAAAACAGACTAG-3′, 5′-CAGGACTCCAAATAAAAGATC-3′)

flanking exon 3 were used. The resulting 381-bp products were digested overnight with 8–10 units of *Bsm*BI (55°C) and analyzed on 4% agarose gels. *Bsm*BI cut the PCR products containing the exon 3 mutation into 239- and 142-bp fragments, whereas the PCR products generated from other *CYP2C19* alleles remained undigested.

Construction and Site-Directed Mutagenesis of Expression Plasmids. The cloning and modification of CYP2C19*1 cDNA for expression in a pCW *ori* [+] bacterial expression system has been previously described (Goldstein et al. 1994; Ibeanu et al., 1996, 1998a). The Trp120Arg change of CYP2C19*8 was introduced directly in pCW2C19*1B using the mutagenic primer (5′-GTTTTCAG-CAATGGAAAGAGA*C*GGAAGGAGATCC-3′) and a second vector specific primer (5′-CCCACTGCCGCGGTGCGCGAGAAG-3′). The mutagenesis procedure was described by Ibeanu et al. *(*1996). Fidelity of newly generated mutants plasmids was confirmed by sequencing on an Applied Biosystems automated sequencer model 377 with Applied Biosystems Prism reagent.

Bacterial Expression of CYP2C Enzymes and Membrane Isolation. Heterologous expression of CYP2C native and mutant enzymes in *Escherichia coli* DH5-α, membrane preparation, solubilization, and partial purification were done using previously described methods (Richardson et al., 1995; Ibeanu et al., 1998a,b). Basically, a 50-fold dilution of overnight cell culture was grown for ~3 h at 37°C in terrific broth (Sigma Chemical Co., St. Louis, MO) supplemented with 200 μg/ml ampicillin and 0.5 mM D-aminolevulinic acid. The temperature was reduced to 25°C before induction with 1 mM isopropyl β-D-thiogalactoside, and cells were harvested 48 h postinduction. Harvested cells were disrupted in 0.3 volume of ice-cold sonication buffer (20 mM K_2HPO_4/KH_2PO_4, pH 7.2, 100 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride), using a Branson 200 sonicator (Danbury, CT) at 40% power and centrifuged at 150,000*g* for 1 h. Membrane pellets were homogenized in 10 mM phosphate buffer, pH 7.4, containing 20% glycerol, 0.1 mM EDTA and 1 mM dithiothreitol, diluted to <2 mg/ml protein and solubilized in 0.3% Nonidet P40 (Sigma Chemical Co.) with continuous stirring at 4°C for 30 min. The supernatant was clarified at 150,000*g* and loaded onto a hydroxyapatite column preequilibrated with membrane homogenization buffer. After extensive washes with at least 15 column volumes of buffer, the proteins were eluted in phosphate/glycerol bufffer (0.5 M phosphate, pH 7.4, 20% glycerol, containing 1 mM EDTA and 1% cholate) and dialyzed for 48 h against detergent-free 0.1 M phosphate-glycerol buffer. CYP content was determined as described by Omura and Sato (1964).

In Vitro Analysis of Enzyme Activity. The 4′-hydroxylation of mephenytoin and methyl hydroxylation of tolbutamide were performed with partially purified recombinant proteins using previously established procedures (Goldstein et al., 1994; Sullivan-Klose et al., 1996).

Results

Two new *CYP2C19* PM alleles were discovered in this study. Genomic DNA from a single Danish PM outlier (subject 19) from our previous studies of 37 Caucasian putative PMs (de Morais et al. 1994a,b; Brøsen et al. 1995; Balian et al. 1995; Goldstein et al. 1997; Sarich et al. 1997; Ferguson et al. 1998; Ibeanu et al., 1998a) was amplified across all exons using *CYP2C19* intron-specific primers and sequenced. The individual was found to be heterozygous for a T → A transversion in the conserved GT splice junction donor site in intron 5. This subject was one of 11 Danish probands previously followed in family studies (Brøsen et al. 1995). This individual (subject 19) had been phenotyped in the previous study after administration of 100 mg mephenytoin followed by a urine collection for 12 h. The subject was initially classified as a putative PM of mephenytoin based on a 0- to 12-h

urinary mephenytoin *S/R* ratio (ratio of the *S*- to the *R*-enantiomers of mephenytoin in urine) of 0.87. In a subsequent independent phenotype test, the urinary *S/R* ratios were identical after 0–12 h and 24–36 h collection periods (1.1 and 1.1). *S/R* ratios of PMs have been reported to remain about unity in PMs but diminish in EMs during this period (Sanz et al., 1989). In a third independent phenotyping test, the urinary *S/R* ratios were essentially identical before and after acidification (1.05 and 1.08), indicating that subject 19 was a PM (Wedlund et al., 1987; Zhang et al., 1992). The *S/R* ratio was done before and after acidification to circumvent misclassification due to the presence of acid labile metabolite in the urine of some EMs. Therefore, this subject is considered to be a true PM of mephenytoin based on both acidified and nonacidified *S/R* ratios and 1–12 h and 24–36 h urinary ratios.

A RFLP-PCR genotype test was developed to differentiate the mutant GA donor site allele now termed *CYP2C19*7* from the naturally occurring GT variant. The procedure employed a mismatch primer to incorporate a *Mae*III restriction site in PCR products of *CYP2C19* gene sequences containing the native GT donor site in intron 5, which produced two fragments of 115 and 42 bp when digested with *Mae*III. In the case of the mutant gene, the 142-bp PCR fragment generated remains undigested by the *Mae*III restriction enzyme. To demonstrate the functionality of the test, genotyping was performed on DNA collected during a previous family study (Brøsen et al. 1995) of subject 19 (Fig. 1). This figure clearly demonstrates the inheritance of the defective *CYP2C19*7* gene in the family. The digested PCR product from subject 19 showed two fragments of 142 bp and 115 bp, a clear indication of heterozygosity for *CYP2C19*7* (lane 3, lower panel) as well as heterozygosity for *CYP2C19*2*. A 42-bp fragment was present but migrated from the gel in the process of resolving the larger 142- and 115-bp fragments. Another member of the family was also heterozygous for the *CYP2C19*7* defective allele (lane 1, lower panel). The new *CYP2C19*7* allele accounted for ~1.3% of the 40 defective alleles in 37 putative Caucasian PMs previously studied in our laboratory (de Morais et al. 1994a,b; Brøsen et al. 1995; Balian et al. 1995; Goldstein et al. 1997; Sarich et al. 1997; Ferguson et al. 1998; Ibeanu et al., 1998a,b) and 3 additional PMs.

A second PM allele *CYP2C19*8* was discovered in a study of a previously nongenotyped group of four putative PMs of mephenytoin from a group of 152 individuals with lung cancer from a lung cancer case control study in French Caucasians (Benhamou et al., 1997). Two of the French subjects (11204 and 11402) were classified as potential PM outliers whose genotypes did not agree with their phenotypes, whereas one of the four subjects was reclassified as an EM on the basis of the urinary *S/R* ratios before and after acidification (Table 1). The phenotype of subject 11204 included a relatively high hydroxylation index (HI) of 58 and *S/R* ratio of 1.25, which did not increase on acidification (1.11). The phenotype was not in agreement with the EM heterozygous genotype (*CYP2C19*1/CYP2C19*2*). Sequencing of the exons and intron-exon junctions revealed that the subject was heterozygous for a T358C base change in exon 3, which resulted in the substitution of a tryptophan at position 120 for an arginine residue. This new allele, *CYP2C19*8*, is otherwise similar to the wild-type *CYP2C19*1B* allele except for a silent T99C transition in exon 1. A genetic test was developed
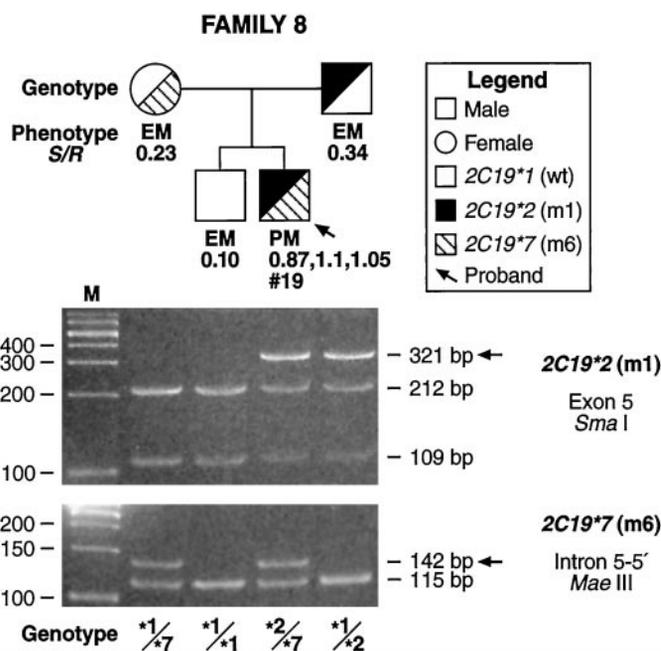

Fig. 1. Family tree of the proband (arrow) of family 8 (Brøsen et al., 1995) with gel analysis of restricted PCR products from the genomic DNA of each family member. Urinary *S/R* ratios are shown for each family member and are indicated below the phenotype, including three replicate collections for the PM. The first gel lane labeled M contains DNA size markers, and subsequent lanes correspond to the family member from the family tree directly above. Top gel shows *Sma*I-digested exon 5 PCR products for the detection of *CYP2C19*2* (Goldstein and Blaisdell, 1996). The arrow to the right points to the undigested product, indicating the presence of *CYP2C19*2* alleles. The bottom gel shows *Mae*III-digested exon 5-intron 5 PCR products (demonstrating the new *CYP2C19*7* allele). The arrow points to undigested products from the *CYP2C19*7* allele. Final genotyping assignments for family members of subject 19 are summarized at the bottom of the figure. The new allele nomenclature (Daly et al., 1996) and our previous trivial nomenclature for the *CYP2C19* alleles are both shown in the legend.

TABLE 1

Putative PMs from a lung cancer group in a French Caucasian population

Individuals with HI >40 from the 152 lung cancer cases from a French lung cancer/case control study. Putative PMs from controls but not lung cancer patients were previously reported (Ferguson et al. 1998). Unified allele nomenclature of Daly et al. (1996) was used.

Sample No.	HI	*S/R* ratio		Sample Phenotype	*CYP2C19* Genotype
		Before acidification	After acidification		
11402	117.4	1.04	1.05	PM outlier	*1/*2
11204	57.9	1.25	1.11	PM outlier	*1/*2[a]
11748	47.7	1.14	1.02	PM	*2/*2
11252	80.4	0.55	0.75	Probable EM (Acid *S/R*)	*1/*2

[a] Reclassified as *2/*8 in this study.

for the new *CYP2C19*8* allele. Two *CYP2C19* intron-specific primers flanking exon 3 amplified a 381-bp fragment that was digested by *Bsm*BI to yielded two fragments of 239 and 142 bp in genes containing the T358C transition (Fig. 2).

The frequencies of the *CYP2C19*7* and *CYP2C19*8* alleles were then determined in both the noncancer controls (172 individuals) and lung cancer patients (152 individuals) recruited for a case-control study of tobacco-related lung cancers in French Caucasian smokers using the newly developed genotyping tests (Table 2) (Benhamou et al., 1997). No *CYP2C19*7* alleles were found in either controls (frequency
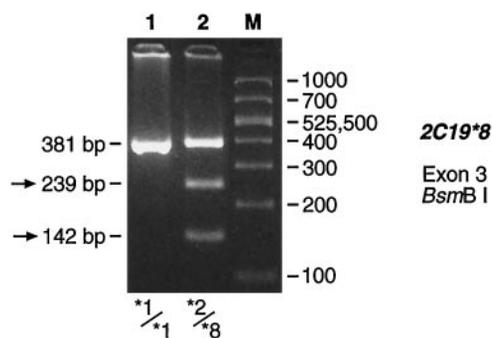
Fig. 2. Genotyping test for *CYP2C19*8* with gel analysis of *BsmB*I-restricted PCR products from genomic DNA. The arrows on the left point to digested exon 3 products, indicating the presence of the defective *CYP2C*8* allele. Lane 1 demonstrates undigested PCR products from a *CYP2C19*1/*1* individual (w/w); lane 2 shows the digested PCR products from the French PM outlier (11204); lane M contains DNA size markers. Previous genotyping studies indicate that this PM subject was also heterozygous for the *CYP2C19*2* allele. Genotypes appear at the bottom of the figure.

TABLE 2

Allele frequencies of *CYP2C19*7* and *CYP2C19*8* in a lung cancer case-control study in French Caucasians

Samples were obtained from a lung cancer case control study in French Caucasians [324 individuals including 172 case controls (344 alleles) and 152 lung cancer cases (304 alleles)].

Alleles	Allele Frequencies (confidence limits)	
	Case controls	Lung cancer cases
*CYP2C19*7*	0 (0–0.011)	0 (0–0.012)
*CYP2C19*8*	0 (0–0.011)	0.007 (0.001–0.024)

0, 95% confidence limits 0–0.11) or cancer patients (frequency 0, 95% confidence limits 0–0.12). *CYP2C19*8* was not found in the controls (frequency 0, 95% confidence limits 0–0.011), but two *CYP2C19*8* alleles were found in the lung cancer group, which included a total of 304 alleles (frequency 0.007, 95% confidence limits of 0.001–0.024) (Table 2). The frequency of this allele in the control group versus lung cancer group was not significantly different ($p = .22$). This is not surprising, because PMs of mephenytoin were not found to be significantly different in these two groups (Benhamou et al., 1997).

To verify whether the *CYP2C19*8* represented a defective allele, the coding change (T358C, Trp120Arg) was introduced in the native CYP2C19*1B cDNA and the recombinant protein expressed in *E. coli* DH5-α. Expression of CYP2C198 in DH5-α was similar to that of the native CYP2C191B enzyme when measured by the carbon monoxide binding spectra. However, when recombinant proteins were tested in an in vitro reconstituted system for their ability to hydroxylate the specific CYP2C19 substrate *S*-mephenytoin, the mutant CYP2C198 showed 11-fold lower activity than the wild-type CYP2C191B protein enzyme (Table 3). CYP2C198 also ex-

TABLE 3

Catalytic activity of recombinant CYP2C19 proteins purified from bacteria

Expressed Protein	*S*-Mephenytoin Hydroxylase Activity	Tolbutamide Hydroxylase Activity
	nmol/min/nmol P-450	*nmol/min/nmol P-450*
CYP2C191B	20.67 ± 0.22	4.19 ± 0.07
CYP2C198[a]	1.83 ± 0.06	0.54 ± 0.05

[a] CYP2C198 differs from wild-type CYP2C191B by a Trp120Arg change in exon 3.

hibited 7-fold lower activity for a universal CYP2C substrate tolbutamide when compared with wild-type CYP2C191B (Table 3), confirming that *CYP2C19*8* is a defective allele of *CYP2C19*.

Discussion

Impaired metabolism of mephenytoin and a number of clinically important therapeutic drugs result from genetic defects in *CYP2C19*. Previous studies in our laboratory have identified five different defects associated with this isoform that may be segregated into two distinct classes. The first group are those inactivating polymorphisms that result in the premature termination of protein synthesis. The principal defect in *CYP2C19*2* is a point mutation generating an alternate splice site consensus sequence within exon 5, which alters the reading frame and results in the premature termination of protein synthesis (de Morais et al., 1994a). The second defect (*CYP2C19*3*) consists of a single base transition that produces an in-frame termination codon in exon 4 and a truncated protein (de Morais et al.,1994b, 1995). In population studies, these two mutations have been estimated to account for >99% and ~88% of Oriental and Caucasian PM alleles, respectively.

A second group of defective alleles consists of point mutations that result in single amino acid substitutions that prevent protein translation or substantially reduce their biological activity. Three different mutations have been reported in this category. *CYP2C19*4* is an allelic variant of the gene in which a transition in the +1 adenine to guanine nucleotide ($A_1 \rightarrow G$) results in the substitution of the initiator methionine (ATG) codon for valine (GTG) codon and leads to inhibition of protein translation (Ferguson et al., 1998). In *CYP2C19*5*, a C1297T transition in the heme binding domain resulting in Arg $\rightarrow$ Trp change alters the ability of the apoprotein to efficiently incorporate the heme moiety leading to a dramatic reduction in enzyme activity (Ibeanu et al., 1998a). Another mutant (*CYP2C19*6*) has a G395A transition in exon 3 that reduces recombinant CYP2C19 enzyme activity by ~98% (Ibeanu et al., 1998b). In the current study, we have identified two additional alleles of the poor metabolizer phenotype for *S*-mephenytoin 4-hydroxylation, a single T $\rightarrow$ A base transversion at the donor site of intron 5 (*CYP2C19*7*) and a T358C transition in exon 3 (*CYP2C19*8*) resulting in Trp120Arg substitution.

There is ample evidence to support the concept that splice site junctions conform to a well-defined consensus sequence. For most vertebrates, this consensus sequence in the intron 5′ splice site donor sequence begins with a GT, whereas the 3′ splice site acceptor sequence terminates with an AG (Mount, 1982). This pattern is known as the canonical GT/AG

***CYP2C19*8* POLYMORPHISM**

EXON 3							
	G	K	R	W	K	E	I	R
*CYP2C19*1*	GGA	AAG	AGA	TGG	AAG	GAG	ATC	CGG
*CYP2C19*8*	GGA	AAG	AGA	CGG	AAG	GAG	ATC	CGG
				R				
Proprionate binding motif			W	X	X	X	R

Fig. 3. Partial nucleotide and amino acid sequence in exon 3 of *CYP2C19* showing the conserved WXXXR binding motif for the D-ring proprionate of heme and the Trp → Arg residue change in *CYP2C19*8* allele. This motif is thought to be important for structure and catalytic activity in CYP.

rule. In a compilation of 1893 exon-intron boundaries, only 9 donor site sequences did not follow the GT rule. Among those were three G → C substitutions at the +1 position and six T → G or C substitutions at the +2 position. T → A transversion was not found in this position in this study. However, a T → A transversion was later reported at position two of the donor splice site of intron 1 in the DNA of a patient with β-thalassemia (Bouhass et al. 1990). Mutations in these bases or those within the defined consensus splice region have been shown to cause splicing errors such as exon skipping (Krawczak et al., 1992; Suzuki et al., 1998). In some hereditary disorders such as hemophilia, β-thalassemia, and Lesch-Nyhan syndrome, a single base change at the consensus +5 position causes the spliceosome to skip the preceding exon to produce a truncated message from which the synthesis of a biologically inactive molecule is directed (Krawczak et al., 1992). Exon skipping has also been implicated in genetic disorders of CYP including the steroid 17α-hydroxylase (*CYP17*) gene (Suzuki et al., 1998). Extensive phenotyping of the Danish PM indicated that the individual with *CYP2C19*7* represents a true PM allele. Therefore, exon skipping may be the relevant mechanism underlying the *S*-mephenytoin 4′-hydroxylase-poor metabolizer phenotype associated with this allele.

Using the bacterial expression system and in vitro metabolic reconstitution studies of recombinant proteins, we verified that a single amino acid change (Trp120Arg) in exon 3 caused a substantial reduction in the catalytic activity of CYP2C198 protein toward *S*-mephenytoin and tolbutamide when compared with the native CYP2C19 protein. The ~91% reduction in activity observed for this enzyme is consistent with the borderline HI value of 57.8 obtained in vivo with *S*-mephenytoin phenotyping of the patient. We recently reported another exon 3 defective allele, *CYP2C19*6,* that essentially lacked catalytic activity toward *S*-mephenytoin and tolbutamide (Ibeanu et al., 1998b). The Trp120Arg change in exon 3 of CYP2C198 enzyme, like the Arg132Gln change of CYP2C196 protein, did not occur in a putative substrate recognition site. However, the substituted tryptophan residue resides within the C-helical domain of the enzyme and occupies the amino terminus of a conserved WXXXR sequence motif (Fig. 3) that interacts with the propionate side chain of heme in bacterial CYP proteins (Hasemann et al., 1995). This residue may be involved in proprionate coordination, a process suggested to have an influence on the redox potential of heme iron (Mathews, 1985). Trp120 in concert with the Arg124 residue of the WXXXR motif could be part of a salt bridge complex to the D-ring proprionate that is important for structure and catalytic activity in CYP. Therefore, we propose that replacement of the hydrophobic uncharged Trp120 residue with a positively charged hydrophilic arginine residue may affect protein conformation and is consistent with the decrease in activity associated with the CYP2C198 enzyme.

Thus the present study reports two new rare defective *CYP2C19* alleles that contribute to the PM phenotype in Caucasians. The first is a change in the donor splice site in intron 5. The second allele, *CYP2C19*8*, results in a protein with only ~9% of the activity of the wild-type enzyme.

References

Adedoyin A, Prakash C, O'Shea D, Blair IA and Wilkinson GR (1994) Stereoselective disposition of hexobarbital and its metabolites: Relationship to the *S*-mephenytoin polymorphism in Caucasian and Chinese subjects. *Pharmacogenetics* **4:**27–38.

Andersson T, Regardh C-G, Lou Y-C, Zhang Y, Dahl ML and Bertilsson L (1992) Polymorphic hydroxylation of *S*-mephenytoin and omeprazole metabolism in Caucasian and Chinese subjects. *Pharmacogenetics* **2:**25–31.

Balian JD, Sukhova N, Harris JW, Hewett J, Pickel L, Goldstein JA, Woosley RL and Flockhart DA (1995) The hydroxylation of omeprazole correlates with *S*-mephenytoin metabolism: A population study. *Clin Pharmacol Ther* **57:**662–669.

Baumann P, Jonzier-Perez M, Kerb L, Küpfer A, Tingueley D and Schöpf J (1986) Amitriptyline pharmacokinetics and clinical response. II. Metabolic polymorphism assessed by hydroxylation of debrisoquine and mephenytoin. *Int Clin Psychopharmacol* **1:**102–112.

Benhamou S, Bouchardy C and Dayer P (1997) Lung cancer risk in relation to mephenytoin hydroxylation activity. *Pharmacogenetics* **7:**157–159.

Bouhass R, Augercif M, Trabuchet G and Godet J (1990) A new mutation at IVS1 nt 2 (T→A) in β-thalassemia from Algeria. *Blood* **76:**1054–1055.

Brøsen K, de Morais SMF, Meyer UA and Goldstein JA (1995) A multifamily study on the relationship between *CYP2C19* genotype and *S*-mephenytoin oxidation phenotype. *Pharmacogenetics* **5:**312–317.

Daly AK, Brockmoller J, Broly F, Eichelbaum M, Evans WE, Gonzalez FJ, Huang JD, Idle JR, Ingelman-Sundberg M, Ishizaki T, Jacqz-Aigrain E, Meyer UA, Nebert DW, Steen VM, Wolf CR and Zanger UM (1996) Nomenclature for human *CYP2D6* alleles. *Pharmacogenetics* **6:**193–201.

de Morais SMF, Wilkinson GR, Blaisdell J, Nakamura K, Meyer UA and Goldstein JA (1994a) The major genetic defect responsible for the polymorphism of *S*-mephenytoin in humans. *J Biol Chem* **269:**15419–15422.

de Morais SMF, Wilkinson GR, Blaisdell J, Meyer UA, Nakamura K and Goldstein JA (1994b) Identification of a new genetic defect responsible for the polymorphism of *S*-mephenytoin metabolism in Japanese. *Mol Pharmacol* **46:**594–958.

de Morais SMF, Goldstein JA, Xie HG, Huang SL, Lu YQ, Xia H, Xiao ZS, Ile N and Zhou HH (1995) Genetic analysis of the *S*-mephenytoin polymorphism in a Chinese population. *Clin Pharmacol Ther* **58:**404–412.

Ferguson RJ, de Morais SMF, Benhamou S, Bouchardy C, Blaisdell J, Ibeanu G, Wilkinson GR, Sarich TC, Wright JM, Dayer P and Goldstein JA (1998) A new genetic defect in human *CYP2C19*: Mutation of the initiation codon is responsible for poor metabolism of *S*-mephenytoin. *J Pharmacol Exp Ther* **284:**356–361.

Goldstein JA and Blaisdell J (1996) Genetic tests which identify the principle defects in *CYP2C19* responsible for the polymorphism in mephenytoin metabolism. *Methods Enzymol* **272:**210–217.

Goldstein JA, Faletto MB, Romkes-Sparks M, Sullivan T, Kitareewan S, Raucy JL, Lasker JM and Ghanayem BI (1994) Evidence that CYP2C19 is the major *S*-mephenytoin 4′-hydroxylase in humans. *Biochemistry* **33:**1743–1752.

Goldstein JA, Ishizaki T, Chiba K, de Morais SM, Bell D, Krahn PM and Evans DA (1997) Frequencies of the defective *CYP2C19* alleles responsible for the mephenytoin poor metabolizer phenotype in various Oriental, Caucasian, Saudi Arabian and American Black populations. *Pharmacogenetics* **7:**59–64.

Hasemann CA, Kurumbali RG, Boddupalli SS, Peterson JA and Deisenhofer J (1995) Structure and function of cytochromes P450: A comparative analysis of three structures. *Structure* **2:**41–62.

Ibeanu GC, Blaisdell J, Ghanayem BI, Beyeler C, Benhamou S, Bouchardy C, Wilkinson GR, Dayer P, Daly AK and Goldstein JA (1998a) An additional defective allele, *CYP2C19*5*, contributes to the *S*-mephenytoin poor metabolizer phenotype in Caucasians. *Pharmacogenetics* **8:**129–135.

Ibeanu GC, Ghanayem BI, Linko P, Leiping L, Pedersen LG and Goldstein JA (1996) Identification of residues 99, 220, and 221 of human cytochrome P450 2C19 as key determinants of omeprazole hydroxylase activity. *J Biol Chem* **271:**12496–12501.

Ibeanu GC, Goldstein JA, Meyer U, Benhamou S, Bouchardy C, Dayer P, Ghanayem BI and Blaisdell J (1998b) Identification of new human *CYP2C19* alleles (*CYP2C19*6* and *CYP2C19*2B*) in a Caucasian poor metabolizer of mephenytoin. *J Pharmacol Exp Ther* **286:**1490–1495.

Krawczak M, Reiss J and Cooper DN (1992) The mutational spectrum of single base-pair substitutions in mRNA splice junctions of human genes: Causes and consequences. *Hum Genet* **90:**1–41.

Küpfer A and Branch RA (1985) Stereo selective mephobarbital hydroxylation cosegregates with mephenytoin hydroxylation. *Clin Pharmacol Ther* **38:**414–418.

Lillibridge JH, Lee CA, Pithavala YK, Sandoval TM, Wu EY, Zhang K, Mazabel EL, Zhang M and Kerr BM (1998) The role of *CYP2C19* in the formation of nelfinavir hydroxy-T-butylamide (M8): In vitro/in vivo correlation. 5th International ISSX Meeting, Vol 13, (Abstr. 109) Cairns, Australia.

Mathews FS (1985) The structure, function and evolution of cytochromes. *Prog Biophys Mol Biol* **45:**1–56.

Mount SM (1982) A catalog of splice junction sequences. *Nucleic Acids Res* **10:**459–472.

Nielsen K, Brøsen K, Hansen J and Gram LF (1994) Single-dose kinetics of clomipramine: Relationship to the sparteine/debrisoquine and *S*-mephenytoin oxidation polymorphisms. *Clin Pharmacol Ther* **55:**518–527.

Omura T, Sato R (1964) The carbon monoxide-binding pigment of liver microsomes. *J Biol Chem* **239:**2370–2378.

Richardson TH, Jung F, Griffin KJ, Wester M, Raucy JL, Kemper B, Bornheim LM, Hassett C, Omiecinski CJ and Johnson EF (1995) A universal approach to the expression of human and rabbit cytochrome P450 of the 2C subfamily in *Escherichia coli*. *Arch Biochem Biophys* **323:**87–96.

Sanz EJ, Villen T, Alm C and Bertillsson L (1989) *S*-mephenytoin hydroxylation

phenotypes in a Swedish population determined after coadministration with debrisoquine. *Clin Pharmacol Ther* **45:**495–499.

Sarich T, Kalhorn T, Magee S, Al-Sayegh F, Adams S, Slattery J, Goldstein J, Nelson S and Wright J (1997) The effect of omeprazole pretreatment on acetaminophen metabolism in rapid and slow metabolizers of *S*-mephenytoin. *Clin Pharmacol Ther* **62:**21–28.

Sindrup SH, Brøsen K, Hansen MGJ, Aaes-Jorgensen T, Overo KF and Gram LF (1993) Pharmacokinetics of citalopram in relation to the sparteine and the mephenytoin oxidation polymorphisms. *Ther Drug Monit* **13:**11–17.

Skjelbo E, Brøsen K, Hallas J and Gram LF (1991) The mephenytoin oxidative polymorphism is partially responsible for the *N*-demethylation of imipramine. *Clin Pharmacol Ther* **49:**18–23.

Sullivan-Klose TH, Ghanayem BI, Bell DA, Zhang ZY, Kaminsky LS, Shenfield GM, Miners JO, Birkett DJ and Goldstein JA (1996) The role of the *CYP2C9*-Leu359 allelic variant in the tolbutamide polymorphism. *Pharmacogenetics* **6:**341–349.

Suzuki Y, Nagashima T, Nomura Y, Onigata K, Nagashima K and Morikawa A (1998) A new compound heterozygous mutation (W17X, 436 + 5G→T) in the cytochrome P450c17 gene causes 17α-hydroxylase/17,20-lyase deficiency. *J Clin Endocrinol Metab* **83:**199–202.

Ward SA, Helsby NA, Skjelbo E, Brøsen K, Gram LF and Breckenridge AM (1991) The activation of the biguanide antimalarial proguanil co-segregates with the mephenytoin oxidation polymorphism: A panel study. *Br J Clin Pharmacol* **31:**689–692.

Ward SA, Walle T, Walle UK, Wilkinson GR and Branch RA (1989) Propranolol's metabolism is determined by both mephenytoin and debrisoquine hydroxylase activities. *Clin Pharmacol Ther* **45:**72–79.

Wedlund PJ, Sweetman BJ, Wilkinson GR and Branch RA (1987) Pharmacogenetic association between the formation of 4-hydroxymephenytoin and a new metabolite of *S*-mephenytoin in man. *Drug Metab Dispos* **15:**277–279.

Wilkinson GR, Guengerich FP and Branch RA (1989) Genetic polymorphism of *S*-mephenytoin hydroxylation. *Pharmacol Ther* **43:**53–76.

Wrighton SA, Stevens JC, Becker GW and VandenBranden M (1993) Isolation and characterization of human liver cytochrome P450 2C19: Correlation between 2C19 and *S*-mephenytoin 4′-hydroxylation. *Arch Biochem Biophys* **306:**240–245.

Xiao ZS, Goldstein JA, Xie HG, Blaisdell J, Wang W, Jiang CH, Yan FX, He N, Huang SL, Xu ZH and Zhou HH (1997) Differences in the incidence of the *CYP2C19* polymorphism affecting the *S*-mephenytoin phenotype in Chinese Han and Bai populations and identification of a new rare *CYP2C19* mutant allele. *J Pharmacol Exp Ther* **281:**604–609.

Zhang Y, Blouin RA, McNamara PJ, Steinmetz J and Wedlund PJ (1992) Limitation to the use of urinary *S/R*-mephenytoin ratio in pharmacogenetic studies. *Br J Clin Pharmacol* **31:**350–352.