Identification of New Human CYP2C19 Alleles (CYP2C19*6 and CYP2C19*2B) in a Caucasian Poor Metabolizer of Mephenytoin

GORDON C. IBEANU, JOYCE A. GOLDSTEIN, URS MEYER, SIMONE BENHAMOU, CHRISTINE BOUCHARDY, PIERRE DAYER, BURHAN I. GHANAYEM and JOYCE BLAISDELL

National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina (G.C.I., J.A.G., B.G., J.B.), Department of Pharmacology, Biozentrum University of Basel, Basel CH-4056, Switzerland (U.M.), INSERM U351, Villejuif, France (S.B.), Geneva Cancer Registry, Geneva, Switzerland (C.B.), and Geneva University Hospital, Geneva, Switzerland (P.D.)

Accepted for publication May 6, 1998. This paper is available online at http://www.jpet.org

ABSTRACT

A genetic polymorphism in the metabolism of the anticonvulsant drug S-mephenytoin has been attributed to defective CYP2C19 alleles. This genetic polymorphism displays large interracial differences with the poor metabolizer (PM) phenotype representing 2–5% of Caucasian and 13–23% of Oriental populations. In the present study, we identified two new mutations in CYP2C19 in a single Swiss Caucasian PM outlier (JOB) whose apparent genotype (CYP2C19*1/CYP2C19*2) did not agree with his PM phenotype. These mutations consisted of a single base pair mutation (G395A) in exon 3 resulting in an Arg132→Gln coding change and a (G276C) mutation in exon 2 resulting in a coding change Glu92→Asp. However, the G276C mutation and the G395A mutation resided on separate alleles. Genotyping tests of a family study of JOB1 showed that the exon 2 change occurred on the CYP2C19*2 allele, which also contained the known splice mutation in exon 5. The exon 3 mutation resided on the CYP2C19*6 allele. This new mutation now termed CYP2C19*2B was found in 0/344 (99% confidence limits of 0 to 0.9%) Caucasian population phenotyped for mephenytoin metabolism. Seven of 46 Caucasian CYP2C19*2 alleles were CYP2C19*2B (15%) and 85% were CYP2C19*2A. The Arg132Gln mutation was produced by site-directed mutagenesis and the recombinant protein expressed in a bacterial cDNA expression system. Recombinant CYP2C19 6 had negligible catalytic activity toward S-mephenytoin compared with CYP2C19 1B, which is consistent with the conclusion that CYP2C19*6 represents a PM allele. Thus, the new CYP2C19*6 allele contributes to the PM phenotype in Caucasians.

There is a well-known genetic polymorphism in the metabolism of the anticonvulsant drug mephenytoin in humans (Wilkinson et al., 1989). Individuals can be characterized phenotypically as EMs or PMs of this drug. There is a marked ethnic difference in the distribution of this polymorphism with the PMs representing 2–5% of Caucasians but 13–23% of Oriental populations. This polymorphism affects the metabolism of a number of other clinically used drugs such as the antiulcer drug omeprazole (Andersson et al., 1992), certain barbiturates (Küpfer and Branch, 1985; Adedoyin et al., 1994) and antidepressants (Sindrup et al., 1993; Baumann et al., 1986; Nielsen et al., 1994; Skjelbo et al., 1991) the antimalarial proguanil (Ward et al., 1991) and to a lesser extent the β-blocker propranolol (Ward et al., 1989) and the anxiolytic diazepam (Bertilsson et al., 1989). The enzyme responsible for this polymorphism has been identified as CYP2C19 (Wrighton et al., 1993; Goldstein et al., 1994).

Studies in our laboratory have identified two wild-type alleles (CYP2C19*1A and CYP2C19*1B) and four defective CYP2C19 alleles. The two principal genetic defects (termed CYP2C19*2 and CYP2C19*3) account for >99% of PM alleles in Orientals but only ~87% of the defective alleles in 37 putative Caucasian PMs from previous studies (de Morais et al., 1994a, 1994b, 1995; Brøsen et al., 1995; Balian et al., 1995; Xiao et al., 1997; Goldstein et al., 1997; Sarich et al., 1997; Ferguson et al., 1998; Ibeanu et al., 1998). The principal defective allele, CYP2C19*2 consists of an aberrant splice site in exon 5 (de Morais et al., 1994a). A second defective allele, CYP2C19*3, is found primarily in Orientals (~20–

ABBREVIATIONS: PM, poor metabolizer; EM, extensive metabolizer; PCR, polymerase chain reaction; HI, hydroxylation index; CYP, cytochrome P450; RFLP, restriction fragment length polymorphism.
25% of PM alleles), but is rare in Caucasians (−1% of PM alleles) (de Morais et al., 1994b; Brøsen et al., 1995; Ferguson et al., 1998). Therefore, additional defects appear to contribute to the PM phenotype in Caucasians. Subsequent studies have revealed two additional defective CYP2C19 alleles (CYP2C19*4 and CYP2C19*5A and *5B) (Ferguson et al., 1998; Xiao et al., 1997; Ibeanu et al., 1998). These defects account for 92% of the defective alleles in 37 Caucasian PMs. The present study was undertaken to identify possible defective alleles in a Caucasian PM outlier whose genotype did not appear to correlate with his PM phenotype for metabolism of mephenytoin (de Morais et al., 1994a, 1994b).

Materials and Methods

Subjects and phenotyping for mephenytoin. Racemic mephenytoin (100 mg) was administered to subjects after emptying their bladders and a 0–8 hr urine specimen was collected. The PM phenotype was defined as an HI of >0.4 (Küpper and Preisig, 1984) and/or a urinary S/R ratio of >0.9 (Wedlund et al., 1984). The HI represents the molar ratio of the dose of S-mephenytoin to the 4′-hydroxymephenytoin in the urine.

A Swiss subject JOB1 was identified as an outlier in our earlier studies of CYP2C19*2 and CYP2C19*3 (de Morais et al., 1994a, 1994b), because his genotype (CYP2C19*1/CYP2C19*2) was inconsistent with his PM phenotype (based on an HI of 2.79). After analysis of additional PM alleles (CYP2C19*4 and CYP2C19*5), JOB1 remained an outlier (Ferguson et al., 1998; Ibeanu et al., 1998). Family members of JOB1 were also phenotyped by HIs. Urine is not presently available for determination of S/R ratios on these individuals.

CYP2C19 allele frequencies were determined in a French European Caucasian population (172 individuals) consisting of the control group from a case-control study of tobacco related cancers in smokers (Benhamou et al., 1997; Ferguson et al., 1998) which had been phenotyped in vivo for mephenytoin metabolism using HI values. S/R ratios before and after acidification of the urine were also determined on samples with HI values of >18 before assigning pheno types. The S/R ratio is done before and after acidification to circumvent misclassification from an acid labile metabolite in urine of some EMs (Zhang et al., 1992) (Wedlund et al., 1987) An S/R ratio of >0.9 and an HI of >40 were consistent with the PM phenotype.

Amplification and sequencing of CYP2C19. Genomic DNA from the Swiss PM outlier JOB1 (de Morais et al., 1994a, 1994b) was amplified across all exons using CYP2C19 intron-specific primers. Sequencing was performed on PCR products using an ABI automated sequencer with a PRISM Dye Terminator Cycle Sequencing Kit and sequence comparisons made using the University of Wisconsin GCG software package.

Genotyping tests. Genomic DNA was isolated from blood using QIAamp blood kits (Qiagen, Chatsworth, CA) according to the manufacturer’s protocol. PCR-RFLP tests for the defective CYP2C19*2 and CYP2C19*3 were previously described (Goldstein and Blaisdell, 1996). Mismatch PCR-RFLP genotyping tests have been described for the CYP2C19*4 (Ferguson et al., 1998) and CYP2C19*5 alleles (Xiao et al., 1997).

New PCR-RFLP genotyping tests were developed to detect the presence of base changes in exons 2 and 3 of JOB1. 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′-ATACAATTGAAATTGATCTAAG-3′, 5′-CAGGACTCCTAAATTGACTG-3′) flanking exons 2 and 3 were used. Two reactions were performed for each sample and the resulting 675 bp products were digested overnight with either 4 units of BsmI I (55′) or 10 units of Pst I (37′) and analyzed on 3% agarose gels. BsmI I cuts the PCR product containing the exon 2 mutation into 490 bp and 185 bp fragments while Pst I digests the PCR product with the exon 3 mutation into fragments of 483 bp and 192 bp. The 675 bp products generated from other CYP2C19 alleles remained uncut. Frequencies and confidence limits for the CYP2C19 alleles were calculated as described by Hahn and Meeker (1991).

Expression of 2C19*6 in a Bacterial cDNA Expression System

Construction of expression plasmids. CYP2C19*1 cDNA was excised from the yeast vector pAAH5 (Goldstein et al., 1994) and ligated in the Hind III site of pUC19 to generate plasmid pUC2C19 (Ibeanu et al., 1996). The clone was then modified at the N-terminus using a 5′-mutagenic oligonucleotide (5′-CTCTTAGACAGTG-GCTCTGTATTAGCAGITTTCCTCTGCTCTTGCTTCTC-3′) designed to replace the first eight N-terminal amino acid codons of the cDNA with those of bovine cytochrome P450 17α-hydroxylase (CYP17) (bold) as described by Barnes et al. (1991) and to introduce Xba I and Nde I sites (underlined) to expedite plasmid construction. PCR was performed using the synthetic mutagenic primer and a template specific 3′ anti-sense primer (5′-TCTTCCAGAAACACTCCTCCCA3′) to generate a ~200 bp fragment. The following PCR conditions were employed: a 20 sec denaturation at 95°C, annealing at 55°C for 20 sec and a 45 sec extension at 70°C for a total of 35 cycles. The PCR products were cut at the engineered Xba I site and at a unique Sac I site present downstream of the modified N-terminal coding sequence. This modified N-terminal fragment was ligated to Xba I-Sac I digested pUC2C19 (*1A) plasmid. The PCR generated segment of the plasmid was sequenced, and the cDNA insert excised and cloned in the Nde I-Hind III sites of the vector pCW Ori+ (kindly provided by Dr. A. Roth with permission from Dr. F. W. Dahlquist, University of Oregon) to generate the bacterial expression constructs pCW2C19. Transformants were selected on LB-Ampicillin plates, minipreps prepared, and inserts confirmed by Nde I-Hind III digestion.

Site-directed mutagenesis. The mutagenesis of CYP2C19*1A to yield CYP2C19*1B and CYP2C19*6 was performed as described by Deng and Nickoloff (1992) with minor modifications (Ibeanu et al., 1996). The Ile331Val (*1B) change was introduced directly in pCW2C19 using the mutagenesis primer (5′-GACGCTGTCGTTG-GCAGAAACC-3′) and a second vector specific primer (5′-CCCTCCTCCCTCCA-3′) which abolished a unique restriction site in the pCW Ori+ DNA. The Arg132Gln (*6) change inactivating mutation was thereafter incorporated in the *1B clone with the mutagenesis primer (5′-GACGCTGTCGATTATTTTGCCGAA-3′) and a new vector primer (5′-CCCCCTAGCAGCTTCCGGCAGAAG-3′) which restored the original restriction enzyme site. Mutants were confirmed by sequencing.

Bacterial expression of CYP2C enzymes and membrane isolation. Expression of CYP2C enzymes was accomplished in E. coli DH5α. Overnight cultures of cells containing 2C19*1B and mutant 2C19*6 plasmids were diluted 50-fold in 250 ml of Terrific broth (Tartof and Hobbs, 1987) supplemented with 200 μg/ml ampicillin and 0.5 mM of the heme precursor δ-aminolevulinic acid. Cells were cultured for ~3 hr at 37°C with vigorous shaking and cooled to 25°C. Isopropyl β-d-thiogalactoside (IPTG) was added to a final concentration of 0.1 mM and incubation resumed by gentle shaking at 150 rpm in an orbital shaker incubator at 25°C for 48 hr. The cultures were centrifuged at 5000 × g and the cell pellet resuspended in 75 ml of ice cold sonication buffer containing 20 mM potassium phosphate (pH 7.2), 100 mM potassium chloride, 1 mM EDTA, 1 mM DTT and 1 mM phenylmethylsulfonyl fluoride. Subsequent steps were performed at 4°C. The cells were disrupted with at least fifteen 30 sec pulses at 40% power using a Branson 200 series sonicator. Cell disruption was monitored by protein quantitation using the method of Bradford (1976) and sonication discontinued when no further increases in protein concentration was observed. The suspension was centrifuged at 150,000 × g for 1 hr and the isolated membranes were resuspended to 1 mg/ml by homogenization in 10 mM phosphate buffer, pH 7.4, containing 20% glycerol, 0.1 mM EDTA and 1 mM DTT.
Cytochrome P450 content was determined as described by Omura and Sato (1964).

Partial purification of P450 proteins was performed according to the procedure of Richardson et al., (1995). Nonidet-P40 was added to the membrane suspension to a final concentration of 0.3% with continuous stirring at 4°C for 30 minutes. The solubilized membranes were centrifuged at 150,000 \( \times \) g and the supernatant loaded onto hydroxyapatite resin (Clarkson, Williamsport, PA.) equilibrated with 2 volumes of homogenization buffer. After extensive washing with at least 15 volumes of buffer, the proteins were eluted in 0.5 M phosphate buffer, pH 7.4, containing 20% glycerol, 1 mM EDTA and 1% cholate, and dialyzed for 48 hr against detergent-free 0.1 M phosphate-glycerol buffer.

**Results**

Sequence analysis of CYP2C19 in the outlier JOB1 revealed that this individual was heterozygous for CYP2C19*2 and for two new mutations in exon 2 (G\(_{276}\)C, Glu\(_{92}\)Asp) and exon 3 (G\(_{990}\)A, Arg\(_{132}\)Gln). A new BsmB I site was introduced by the base transition in exon 2 while the change in exon 3 generated a new Pst I restriction site in the DNA. To determine if both changes occurred on the same or separate alleles, genomic DNA was amplified across exons 2 and 3 using CYP2C19 intron specific primers and double digested with BsmB I and Pst I. Analysis of the restricted fragments showed complete disappearance of the 675 bp PCR products (data not shown) indicating that the defects occurred on separate alleles.

To determine which mutation was on the CYP2C19*2 allele and which segregated with the new defective allele, genotyping was performed on DNA from a family study of JOB1 (fig. 1). This figure clearly demonstrates the inheritance pattern of the two alleles. The mutation in exon 2 is present on the CYP2C19*2 allele (a new variant named CYP2C19*2B) (C\(_{99}\)T,G\(_{276}\)C, Glu\(_{92}\)Asp,G\(_{685}\)A,C\(_{990}\)T,A\(_{991}\)G, Ile\(_{331}\)Val) which
differs from the previously described variant now named CYP2C19*2A (C99T;G681A,A991G, Ile331Val). As shown in figure 2, both CYP2C19*2 alleles contain the inactivating splice mutation in exon 5. The exon 3 mutation resides on a separate allele (now termed CYP2C19*1A) by a silent base change and one coding change (C99T;A991G, Ile331Val). The sequence of this allele was otherwise identical to the CYP2C19*1B wild-type allele which differs from CYP2C19*1A by a silent base change and one coding change (C99T;A991G, Ile331Val). The known CYP2C19 alleles are shown in figure 2.

The wild-type allele reported by Romkes et al (1991) is denoted CYP2C19*1A and one coding change (C99T;A991G, Ile331Val). The wild-type allele reported by Romkes et al (1991) is denoted CYP2C19*1A and one coding change (C99T;A991G, Ile331Val). The sequence of this allele was otherwise identical to the CYP2C19*1B wild-type allele which differs from CYP2C19*1A by a silent base change and one coding change (C99T;A991G, Ile331Val). The known CYP2C19 alleles are shown in figure 2.

The incident of all known PM alleles was determined in a European Caucasian control population using the new genotyping tests (table 1). The exon 2 mutation was found only in individuals containing at least one CYP2C19*2 allele, indicating that it cosegregated with the CYP2C19*2 allele in this population. About 85% of the forty six CYP2C19*2 alleles were the original CYP2C19*2A variant and 15% were the new CYP2C19*2B variant. No additional CYP2C19*6 alleles were detected in this population and CYP2C19*6 is concluded to occur at a low frequency (0–0.9%, 95% confidence limits). However, it accounted for one of 74 PM alleles in 37 PMs (CYP2C19*6) and was the most defective allele (28% activity compared to wild-type CYP2C19 1B toward both mephenytoin and tolbutamide (table 2).

Discussion

Two new CYP2C19 alleles have been identified in a Caucasian PM of mephenytoin (JOB-1) in the present study. A new PM allele (CYP2C19*6) (C99T;G681A, Arg132Gln; A991G, Ile331Val) was identical to the wild-type CYP2C19*1B allele except for a mutation in exon 3 (G395A) which results in the coding change Arg132Gln. Family studies showed that a second base pair substitution (G276C) in exon 2 resulting in Glu92Asp coding change occurred on a separate allele (CYP2C19*2B)(C99T;G276C, Glu92Asp;G681A; C990T;A991G; Ile331Val) which was otherwise identical to the previously known splice variant CYP2C19*2A (de Morais et al., 1994a).

<table>
<thead>
<tr>
<th>Allele</th>
<th>Trivial Name</th>
<th>Effect of Nucleotide Changes</th>
<th>Enzyme Activity</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2C19*1A</td>
<td>CYP2C19wt</td>
<td></td>
<td>Active</td>
<td>1 2 3 4</td>
</tr>
<tr>
<td>CYP2C19*1B</td>
<td>CYP2C19wt</td>
<td>Ile331Val</td>
<td>Inactive</td>
<td>0</td>
</tr>
<tr>
<td>CYP2C19*2A</td>
<td>CYP2C19m1A</td>
<td>Splicing Defect</td>
<td>Inactive</td>
<td>0</td>
</tr>
<tr>
<td>CYP2C19*2B</td>
<td>CYP2C19m1B</td>
<td>Glu26 Asp Splicing Defect</td>
<td>Inactive</td>
<td>0</td>
</tr>
<tr>
<td>CYP2C19*3</td>
<td>CYP2C19m2</td>
<td>Stop Codon</td>
<td>Inactive</td>
<td>0</td>
</tr>
<tr>
<td>CYP2C19*4</td>
<td>CYP2C19m3</td>
<td>GTG Initiation Codon</td>
<td>Inactive</td>
<td>0</td>
</tr>
<tr>
<td>CYP2C19*5A</td>
<td>CYP2C19Arg353Trp</td>
<td>Arg435Gln; Ile331Val; Arg435Trp</td>
<td>Inactive</td>
<td>0</td>
</tr>
<tr>
<td>CYP2C19*6</td>
<td>CYP2C19Arg353Trp</td>
<td>Arg435Gln; Ile331Val; Arg435Trp</td>
<td>Inactive</td>
<td>0</td>
</tr>
</tbody>
</table>

Fig. 2. CYP2C19 alleles. The positions of the various polymorphisms compared to the wild-type CYP2C19 (CYP2C19*1A allele) are indicated. The original CYP2C19 allele nomenclature (De Morais et al., 1994a,1994b) and the new recommended nomenclature (Daly et al, 1996) are both indicated. The wild-type allele reported by Romkes et al, 1991 is denoted CYP2C19*1A. A second catalytically active wild-type allele (C99T;A991G, Ile331Val) (Richardson et al, 1995; Goldstein et al, unpublished data) is designated CYP2C19*1B. The defective PM alleles previously reported by our laboratory (deMorais et al., 1994a,1994b; Xiao et al, 1997; Ferguson et al., 1998) are designated as follows: CYP2C19*4 is designated CYP2C19*2A (C99T;G681A; C990T;A991G; Ile331Val). A new CYP2C19*2 allele (CYP2C19*2B)(C99T;G276C, Glu92Asp;G681A; C990T;A991G; Ile331Val) is described in this paper. CYP2C19*6 is designated CYP2C19*3, CYP2C19*14 is designated CYP2C19*4, CYP2C19*15 is designated CYP2C19*5. Two variant CYP2C19*5 alleles are termed CYP2C19*5A (C990T;A991G, Ile331Val) and CYP2C19*5B (C990T;A991G; Ile331Val; C1297T;TAr435Trp). CYP2C19*6 (C99T;G681A; Arg132Gln; A991G; Ile331Val).
Genotyping tests indicate that CYP2C19*6 is rare in a French Caucasian population (0/344 alleles with 95% confidence limits of 0–0.9%). Of the forty-six CYP2C19*2 alleles present in this Caucasian population, 15% were the new CYP2C19*2B allele in the present population. The new allele had negligible activity toward two CYP2C19 substrates, mephenytoin and tolbutamide in a cDNA expression system. Thus CYP2C19*6 is a rare defective allele that contributes to the PM phenotype in Caucasians.

Acknowledgments
The authors thank Richard W. Morris, of Analytical Sciences, Inc., Research Triangle Park, NC, for his expert statistical analyses.

References


Ibeanu GC, Ghanayem BI, Linke P, Leiping L, Pedersen LG and Goldstein JA (1996) cDNA expression studies demonstrated that the single amino acid change Arg132Gln in exon 3 abolishes the catalytic activity of CYP2C19 6 protein toward both S-mephenytoin and tolbutamide compared with wild-type CYP2C19 1B protein. The absence of catalytic activity is consistent with the high HI index of the PM outlier JOB 1 when phenotyped with mephenytoin in vivo. This amino acid is not in any known substrate binding site (SRS). However, the positively charged Arg132 is conserved in the human and rodent CYP2 family (Gotoh, 1992). This residue is within the C helix (Hasemann et al., 1995) and could be involved in a salt bridge that is important for structure or catalytic activity within the CYP2 family. CYP2C19 6 protein still exhibited a CO-binding spectrum indicating that it bound heme. The conservation of this amino acid in the CYP2 family is consistent with the fact that an allele lacking this amino acid is catalytically inactive.

In summary, this study identifies a new CYP2C19 allele (CYP2C19*6) in a PM of mephenytoin which accounts for ~1.4% of the defective alleles in 37 Caucasian PMs. This allele had negligible activity toward two CYP2C19 substrates, mephenytoin and tolbutamide in a cDNA expression system. Thus CYP2C19*6 is a rare defective allele that contributes to the PM phenotype in Caucasians.

TABLE 2

<table>
<thead>
<tr>
<th>S-Mephenytoin hydroxylase</th>
<th>Tolbutamide hydroxylase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol/min/nmol P450</td>
</tr>
<tr>
<td>2C19*1B</td>
<td>23.0 ± 0.2</td>
</tr>
<tr>
<td>2C19*6</td>
<td>ND*</td>
</tr>
</tbody>
</table>

* ND = <0.06 nmol/min/nmol.

** TABLE 2**

Catalytic activity of recombinant CYP2C19 proteins purified from bacteria.

CYP2C19*6 differs from wild-type CYP2C19*1B by an Arg132Gln change in exon 3.

**Fig. 3.** Reduced carbon-monoxide difference binding spectra of membrane proteins of bacteria transformed with CYP2C19*1B or CYP2C19*6.


Send reprint requests for: Joyce Blaisdell (C3–01), NIEHS, P.O. Box 12233, Room C324, 111 Alexander Drive, Research Triangle Park, NC 27709. E-mail: Blaisde1@NIEHS.NIH.GOV

**ERRATUM**


The publisher regrets that Table 4 (Antipyrine clearances and volumes of distribution) on page 59 was incorrectly labeled as Table 2 (Zidovudine clearances and volumes of distribution). The data for this table were presented accurately.