ABSTRACT

CYP2C9 was recently resequenced in 150 Asian subjects from Singapore. Several new coding variants were reported, and these variants are now named CYP2C9*14 (R125H), CYP2C9*15 (S162X), CYP2C9*16 (T299A), CYP2C9*17 (P382S), CYP2C9*18 (D397A), and CYP2C9*19 (Q454H). The CYP2C9*18 variant also contained an I359L change previously associated with the CYP2C9*3 allele. In this study, we assessed the functional consequences of the new coding changes. cDNAs containing each of the new coding changes were constructed by site-directed mutagenesis and expressed in a bacterial cDNA expression system, the allelic proteins were partially purified, and their ability to hydroxylate a prototype CYP2C9 substrate was assayed. Expression of cDNAs in Escherichia coli containing either the D397A change or the S162X (premature stop codon) could not be detected either spectrally or at the apoprotein level. CYP2C9.14 and CYP2C9.16 exhibited 80 to 90% lower catalytic activity toward tolbutamide at two substrate concentrations compared with wild-type CYP2C9.1. Kinetic analysis confirmed that CYP2C9.14 and CYP2C9.16 have a higher \( K_m \) and a >90% lower intrinsic clearance of tolbutamide compared with wild-type CYP2C9.1. Both CYP2C9.17 and CYP2C9.19 proteins exhibited modest 30 to 40% decreases in catalytic activity toward tolbutamide. Thus, CYP2C9*15 and CYP2C9*18 may represent null alleles, whereas CYP2C9*14 and CYP2C9*16 allelic variants produce proteins that are clearly catalytically defective in vitro, indicating the existence of new defective putative alleles of CYP2C9 in Asians.

CYP2C9 is known to be one of the most important members of the drug-metabolizing cytochrome P450 (P450) isoforms in the human liver. Some of these drugs have narrow therapeutic indices, such as the anticoagulant warfarin and the anticonvulsant phenytoin (Goldstein et al., 1994; Lee et al., 2002). Other drugs metabolized principally by CYP2C9 include the antidiabetic drugs tolbutamide and glipizide, the diuretic torsemide (Miners and Birkett, 1998), the anti-hypertensive drug losartan, and numerous nonsteroidal anti-inflammatories, including flurbiprofen, ibuprofen, and diclofenac.

Many polymorphic alleles of CYP2C9 have been reported, with at least eight known or putative poor metabolizer (PM) alleles of CYP2C9 found in different racial groups. Two defective alleles, CYP2C9*2 (R144C) (Crespi and Miller, 1997) and CYP2C9*3 (I359L) (Sullivan-Klose et al., 1996), have been extensively studied both in vitro and in clinical studies (Lee et al., 2002). The CYP2C9*3 allele has lower affinity and markedly lower intrinsic clearance for numerous drugs both in vitro and in vivo. The CYP2C9*2 allele is more moderately defective. These two alleles have received the most study in white populations, where the frequencies of the defective CYP2C9*2 and CYP2C9*3 alleles are 11 and 8%, respectively. However, these alleles are found in much lower frequencies in African-American (3.0 and 0.5%, respectively) and most East Asian populations (0 and 1.6%, respectively) (Xie et al., 2001, 2002). CYP2C9*4 (Ieiri et al., 2000) (I359T), which has been reported in Asians, and CYP2C9*5 (D360E) (Dickmann et al., 2001) (found in African-Americans) resemble CYP2C9*3 kinetically, exhibiting a high \( K_m \) and low intrinsic clearance for a number of drugs. Both I359 and D360 occur in the substrate binding pocket. A null allele, CYP2C9*6, containing a base deletion and frame shift has also been identified in an individual with severe phenytoin toxicity (Kidd et al., 2001). Six new coding alleles, CYP2C9*7 to *12, were recently reported by our laboratory by resequencing genomic DNA from a racially diverse population (Blaisdell et al., 2004). Of these, CYP2C9*11 and perhaps CYP2C9*12 were predicted to be putative defective alleles.
based on in vitro metabolism of tolbutamide by the recombinant enzymes. Recently, there has been a report of an individual heterozygous for CYP2C9*3 and a novel CYP2C9 allele, CYP2C9*13 (L90P), in an Asian population. The latter was suggested to contribute to the PM phenotype (Si et al., 2004).

It is well known that there are racial and ethnic differences in both the frequency and types of polymorphisms of CYP2C9 (Xie et al., 2001, 2002). The identification of polymorphisms in different racial and ethnic populations is vital to understanding differences in clinical responses to drugs in these populations. An extensive review of allelic variants in the intron, exon, and 3′-untranslated regions of 11 candidate phase I drug-metabolizing genes, including CYP2C9, in white, African-American, and Asian populations has recently been published (Sulos et al., 2004). This report catalogues the different polymorphisms reported in the various ethnic groups, some of which may contribute to ethnic differences in metabolism.

In our recent studies in a Southeast Asian population (59 Chinese, 37 Malay, and 26 Indians), CYP2C9 was sequenced for new mutations (Zhao et al., 2004) including the promoter, 300 base pairs downstream in the 3′-untranslated region, the splice-site junctions, and each of the nine CYP2C9 exons. The CYP2C9*2 and CYP2C9*3 alleles were found with varying frequencies in these groups (Indians > Chinese or Malay). In addition to the known coding variants, new alleles with coding changes were discovered. These included a new null variant, CYP2C9*15, containing a premature stop codon, S162X. Although warfarin dose requirements were measured in this population, the function of the new alleles could not be adequately assessed since these occurred in the heterozygous state.

In the present study, the function of the six new coding alleles found in Chinese and Indians was further assessed in a bacterial cDNA expression system. The newly discovered coding changes were introduced by site-directed mutagenesis into CYP2C9*1 and the recombinant proteins expressed in *Escherichia coli*. The recombinant CYP2C9 allelic proteins were solubilized, partially purified, and then assayed for tolbutamide hydroxylase activity. Two allelic variants, including one with a premature stop codon, S162X, and one with a D397A, could not be expressed as recombinant proteins (D397A) was introduced alone without the I359L to determine the effect of this amino acid change on CYP2C9 expression or function. All mutants were confirmed by DNA sequencing.

**Expression and Partial Purification of CYP2C9 Allelic Variant Proteins.** Each expression clone was grown in overnight cultures at 37°C from a single colony. The cultures contained Terrific Broth (ampicillin 100 μg/ml) and were then diluted 100-fold into 500 ml. Once the cultures achieved log phase (OD600 = 0.4–0.6), they were induced with isopropyl β-D-thiogalactoside (0.5 mM) and δ-aminolevulinic acid (0.5 mM) starting at 37°C for at least 72 h. Reduced CO spectra using a DW-2000 Spectrophotometer were measured to estimate cytochrome P450 content as described earlier (Omura and Sato, 1964). Once adequate levels of cytochrome P450 expression were detected (typically ~72 h), the recombinant P450 was solubilized and partially purified by hydroxypatite chromatography as previously described (Richardson et al., 1995; Ibeanu et al., 1996).

**Tolbutamide Hydroxylation Assays.** The tolbutamide assay has previously been described in detail (Blaisdell et al., 2004). Briefly, 20 pmol of partially purified recombinant P450 proteins was reconstituted with 0.3 μg/pmol P450 of 1,2-didodecanoyl-sn-glycero-3-phosphocholine, 4 pmol/pmol P450 of recombinant human NADPH-P450 oxidoreductase, and 2 pmol/pmol P450 of human cytochrome b5 at 37°C for 5 min, then placed on ice. Samples were then diluted and mixed into a final reaction volume of 250 μl containing 20 mM HEPES, pH 7.4, 0.1 mM ethylenediaminetetraacetic acid, 1.25 mM MgCl2, and [ring-U-14C]tolbutamide as substrate. Mixtures were preincubated at 37°C for 3 min, and then the reaction was initiated by the addition of 50 mM NADPH. For single-point assays.

**Materials and Methods**

[ring-U-14C]Tolbutamide was purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Human cytochrome b5, as well as NADPH-P450 oxidoreductase were purchased from Oxford Biochemical Research (Oxford, MI). All restriction enzymes used were purchased from New England Biolabs (Beverly, MA). All chemicals used were purchased from Sigma-Aldrich (St. Louis, MO) or were the highest grade commercially available.

**Site-Directed Mutagenesis of Expression Plasmids.** Cloning and modification of the wild-type CYP2C9*1 cDNA for expression in pCW ori+ in a bacterial expression system was performed as previously described for other alleles (Ibeanu et al., 1996). The newly discovered coding SNPs were introduced into the wild-type construct using a Quick Change Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) with primers listed in Table 1. The SNP 1190A→C (D397A) was introduced alone without the I359L to determine the effect of this amino acid change on CYP2C9 expression or function. All mutants were confirmed by DNA sequencing.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Mutation</th>
<th>Primer Sequence</th>
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<tbody>
<tr>
<td>2C9Exon3/371Forward</td>
<td>Arg124Gln</td>
<td>ATGGAAAGGAGATCCAGGTCGTTTCCT</td>
</tr>
<tr>
<td>2C9Exon3/371Reverse</td>
<td>Arg125His</td>
<td>GAGAAACGCTGGATCCTCCATCCAT</td>
</tr>
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<td>Arg125His</td>
<td>GAGATCCGGGATTTTCTCCCTCATAGG</td>
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<td>2C9Exon3/374Reverse</td>
<td>Ser162X</td>
<td>CGTCGACTAGGAGGAAATTTCGCGATTC</td>
</tr>
<tr>
<td>2C9Exon4/485Forward</td>
<td>Thr299Ala</td>
<td>AAAACGCGCTACCCGCTGTGATCCAC</td>
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<td>Thr299Ala</td>
<td>GTGGGATCACAGGGTTAGGCCTTGG</td>
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<td>2C9Exon6/895Forward</td>
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<tr>
<td>2C9Exon6/895Reverse</td>
<td>His397Asp</td>
<td>CGTTTGAGCTGCGGACAGCAGAAG</td>
</tr>
</tbody>
</table>

**TABLE 1**

Primers used for site-directed mutagenesis of CYP2C9*1

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DeLozier et al.
three to four separate purified preparations of each allele were reconstituted and then assayed with either 0.1 or 1 mM [ring-U-14C]tolbutamide; for kinetics, preparations from a single date were assayed with varying concentrations (0.075–3 mM) of substrate. All reactions were linear with time and protein under these conditions. The reactions were stopped after 45 min by the addition of methanol in an equal volume to reaction mixture. After centrifugation at 10,000 g for 10 min, an aliquot of the supernatant was checked for total radioactivity by liquid scintillation (Beckman LS6500 Multi-Purpose Scintillation Counter; Beckman Coulter, Fullerton, CA). High-performance liquid chromatography was used to separate and quantitate metabolites as described previously (Blaisdell et al., 2004), with the mobile phase consisting of acetonitrile/0.1% trifluoroacetic acid (40:60). Retention times of the radiolabeled substrate and metabolite were compared with those of the unlabeled tolbutamide and hydroxytolbutamide standards (Sigma-Aldrich; BD Gentest, Woburn, MA).

Statistical Analysis. For each allelic protein, three separate rounds of expression and purification were performed concurrently with expression and purification of CYP2C9.1. The resulting three preparations of each mutant allele and four preparations of CYP2C9.1 were assayed for tolbutamide activity in triplicate. The data for each mutant were calculated as a percentage of the activity of the CYP2C9.1 wild type, which was copurified on the same date to prevent P450 expression in bacteria. Despite three separate attempts to express and purify any solubilized P450 proteins, expression of CYP2C9.15 could not be detected spectrally in bacteria through 72 h, and Western blot analysis of the partially purified bacterial extracts with a polyclonal antibody to CYP2C9 showed no evidence of CYP2C9 apoprotein (data not shown). This is not surprising since the CYP2C9.15 allele has a premature stop codon. However, it was somewhat surprising that the mutant allele containing D397A (found in the CYP2C9*18 allele) could also not be expressed in bacteria at the spectral or apoprotein level (Western blotting of the solubilized bacterial extracts showed no apoprotein in contrast to that seen with wild-type CYP2C9.1 in three separate experiments). We made no subsequent attempts to construct the D397A amino acid change on the defective CYP2C9*3 background since the single mutation D397A appeared to prevent P450 expression in bacteria.

P450 Expression. In the present study, each of the new coding changes was introduced individually by site-directed mutagenesis, the cDNAs were expressed in E. coli, and the recombinant proteins were solubilized after ~72 h and partially purified. Of the six mutant alleles, only four could be expressed in bacteria. Since there were four preparations of wild type and three of each mutant allele, analysis of variance was followed by Dunn’s test, which allows for comparison of unequal groups of values. A single rectangular hyperbola nonlinear regression model (Michaelis-Menten) was used to analyze kinetic data using SigmaPlot version 9.0 (Systat Software Inc.).

Results

In the present study, we assess the functional consequences of six new alleles recently reported by one of our laboratories (Zhao et al., 2004). These new alleles are as follows: CYP2C9*14 (R125H), CYP2C9*15 (S162X), CYP2C9*16 (T299A), CYP2C9*17 (P382S), CYP2C9*18 (D397A/I359L), and CYP2C9*19 (Q454H). Their linkages to the CYP2C9.1 wild type, which was copurified on the same date to prevent P450 expression in bacteria, were determined through four rounds of expression and purification of CYP2C9.1. The resulting three alleles in Southeast Asians were typed Chinese (for the three Asian alleles typed) and Indian (for the allele containing D397A). However, it was somewhat surprising that the mutant allele containing D397A (found in the CYP2C9*18 allele) could also not be expressed in bacteria at the spectral or apoprotein level (Western blotting of the solubilized bacterial extracts showed no apoprotein in contrast to that seen with wild-type CYP2C9.1 in three separate experiments). We made no subsequent attempts to construct the D397A amino acid change on the defective CYP2C9*3 background since the single mutation D397A appeared to prevent P450 expression in bacteria.

Table 2

<table>
<thead>
<tr>
<th>Allele</th>
<th>cDNA Positiona</th>
<th>Amino Acid</th>
<th>Gene Positionb,c</th>
<th>Race</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2C9*14</td>
<td>374G&gt;Aa</td>
<td>R125H</td>
<td>−1188C&gt;T; 3552G&gt;A</td>
<td>Indian</td>
</tr>
<tr>
<td>CYP2C9*15</td>
<td>485C&gt;A</td>
<td>S162X</td>
<td>9109C&gt;A (possibly linked to −1188C&gt;T)</td>
<td>Indian</td>
</tr>
<tr>
<td>CYP2C9*16</td>
<td>895A&gt;G</td>
<td>T299A</td>
<td>33491T&gt;A</td>
<td>Chinese</td>
</tr>
<tr>
<td>CYP2C9*17</td>
<td>1144C&gt;T</td>
<td>P382S</td>
<td>1188C&gt;T; 46864C&gt;T</td>
<td>Chinese</td>
</tr>
<tr>
<td>CYP2C9*18</td>
<td>1190A&gt;C</td>
<td>D397A</td>
<td>−1911T&gt;C; −1885C&gt;G; −1537G&gt;A; −1188T&gt;C, and −982G&gt;A</td>
<td>Indian</td>
</tr>
<tr>
<td>CYP2C9*19</td>
<td>1425A&gt;T</td>
<td>Q454H</td>
<td>47391A&gt;C; 50298A&gt;T</td>
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<tr>
<td></td>
<td>1362G&gt;C</td>
<td>50235G&gt;C</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Bold lettering denotes those SNPs that produce coding changes. Linkages are considered unequivocal for these SNPs that occur in individuals who are homozygous for the associated linked SNPs. For CYP2C9*15, the individual was heterozygous for both SNPs; therefore, linkage remains to be determined in larger studies.

b Based on GenBank AL359672.

c See Table 1.
defective in the metabolism of tolbutamide, exhibiting a >80% decrease in catalytic activity at low and high concentrations of substrate (0.1 and 1.0 mM). These results were significantly different from wild type ($P < 0.05$). Moreover, these data represent three separate preparations of each enzyme, ruling out variation in purification. Detailed kinetic analysis of the CYP2C9.14 allelic protein versus wild-type demonstrated a 5-fold increase in the $K_m$, a 65% decrease in the $V_{max}$ and a >93% decrease in the intrinsic clearance compared with wild-type CYP2C9.1 (Table 3 and Fig. 2). Examination of the crystal structure of CYP2C9 indicates R125 is on the surface of the protein near the heme. This amino acid is equivalent to R126 in CYP2B6, which is one of several amino acids that appears to be part of binding sites for cytochrome $b_5$ and cytochrome P450 reductase (Bridge et al., 1998). Recombinant CYP2C9.16 had a 3.5-fold increase in the $K_m$, a 69% decrease in the $V_{max}$ and a >92% decrease in the intrinsic clearance of tolbutamide compared with CYP2C9.1. The T299A substitution found in CYP2C9.16 is on the back side of the I helix, close to the predicted oxygen binding site. Threonine 299 is highly conserved in family 2. CYP2C9.17 (P382S) and CYP2C9.19 (Q454H) exhibited somewhat lower tolbutamide hydroxylase activity than wild-type CYP2C9 (40 and 20% decreases, respectively), but this was not statistically significant with the number of samples analyzed. Moreover, more complete kinetic analysis indicated that the $K_m$, $V_{max}$ and intrinsic clearance of CYP2C9.17 and CYP2C9.19 were similar to CYP2C9.1.

### Discussion

Polymorphisms of CYP2C9 have been studied extensively in whites. CYP2C9*2 and CYP2C9*3 are the most common defective alleles in the white populations (11 and 8.4%, respectively) but have significantly lower frequencies in African populations (2.9 and 1.3%, respectively) and Eastern Asian populations such as Chinese (0 and 3.3%), Japanese (0 and 2.2%), and Koreans (0 and 1.1%, respectively) (Xie et al., 2001, 2002). However, higher frequencies of these two alleles have been reported in Indian populations compared with Eastern Asians (4–6% and 8–13.8%) (Zainuddin et al., 2003; Jose et al., 2005). Similarly, recent studies from one of our laboratories (Zhao et al., 2004) reported frequencies of 4 and 11.5%, respectively, for the CYP2C9*2 and CYP2C9*3 alleles in Indians, 0 and 3.3% in Chinese, and 0 and 6.3% in Malays from Singapore who were receiving maintenance warfarin therapy. Importantly, several new alleles with coding changes in CYP2C9 were discovered in Asians. Three of the new coding mutations were detected in Indians and three in Chinese. Since these new mutations were found in single individuals in the heterozygous state, it was difficult to draw meaningful conclusions concerning the functional consequences of these new variant alleles based on warfarin dosage requirements. Therefore, in the present study, we assessed the function of these new alleles using a cDNA expression system and tolbutamide as the probe drug to study metabolism of the recombinant proteins in vitro.

![Fig. 1. Tolbutamide hydroxylase activity of recombinant mutant CYP2C9 allelic proteins compared with that of wild-type CYP2C9.1 protein. The new mutant CYP2C9 cDNAs were constructed by site-directed mutagenesis from the wild-type CYP2C9*1 cDNA, expressed in E. coli and partially purified as described under Materials and Methods. Separate rounds of expression and purification resulted in three purified preparations of each variant allele, which were expressed and purified simultaneously with wild-type CYP2C9.1. All 16 partially purified preparations (three of each mutant allele and four of wild-type CYP2C9.1) were assayed the same day in triplicate at high (1 mM) and low (0.1 mM) substrate concentrations. The values of each preparation of the mutant alleles were calculated as a percentage of enzymatic activity of the wild-type allele that had been purified on the same day. * significantly different from wild-type CYP2C9, $P < 0.05$.](Image)
The CYP2C9*18 allele, occurring in a single Indian (1 of 52 alleles), contained two coding mutations, D397A and I359L (also found on the defective CYP2C9*3 allele). Since the CYP2C9*3 allele itself is markedly defective, we first tested the effect of the single mutation D397A. Similar to the null CYP2C9*3 allele, the recombinant allelic protein had a markedly decreased catalytic activity. The results with recombinant CYP2C9.17 protein indicated a possible moderate reduction in tolbutamide hydroxylase activity, but this difference was not statistically significant. Moreover, kinetic parameters of this enzyme were similar to those of CYP2C9.1. The P382 is located on the surface of the enzyme in a turn following β-sheet 2, and it has been found to be highly conserved in all structurally characterized microsomal P450s. The CYP2C9*19 allele was discovered in one individual of Chinese origin (1 of 118 alleles). The tolbutamide hydroxylase activity of the recombinant CYP2C9.19 was also 20 to 30% lower than wild type, but again not statistically significant, nor did kinetic parameters appear to differ from wild-type CYP2C9.1. Q454 is also on the surface at the end of a helix. These two latter alleles will require further clinical study to determine whether they have modest effects on CYP2C9 activity in vivo.

Warfarin maintenance doses for Asians are known to be lower than those of whites (Loebstein et al., 2001; Zhao et al., 2004). However, the frequency of PM alleles is lower in most Asian groups than in whites. This discrepancy could be explained by the possible existence of new undiscovered alleles in Asians, including SNPs in the introns or upstream regions. However, warfarin dosage is also known to be affected by dietary changes and drug interactions, including induction of CYP2C9 by prior exposure to drugs and herbal remedies (Loebstein et al., 2001; Chen et al., 2004). Various upstream SNPs are more frequent in Asians, namely an SNP at −1188 (GenBank National Center for Biotechnology Information no. AL359672) (Shintani et al., 2001; King et al., 2004). This SNP is identical to the −1189 denoted in our earlier study (Zhao et al., 2004) due to the use of an alternate older reference sequence for numbering in the Asian literature (GenBank National Center for Biotechnology Information no. L16877). In Asians, the most frequent allele is −1188C, whereas in whites, the more frequent allele is −1188T. Several studies have addressed the possible effects of upstream polymorphisms on warfarin dosage (Shintani et al., 2001; King et al., 2004).
2004; Takahashi et al., 2004). To date, there is no clear evidence that any of these SNPs have a role in promoter activity. Recently, new studies have shown that polymorphisms in the vitamin K epoxide reductase complex 1 (the molecular target of warfarin) and other vitamin K-dependent proteins (factor II and factor VII genes) also modulate the anticoagulant effects of warfarin, thereby altering dose requirements (D’Ambrosio et al., 2004; Rost et al., 2004; D’Andrea et al., 2005; Yuan et al., 2005). Yuan et al. (2005) suggested the possibility that ethnic differences in warfarin dose requirements might be attributed to ethnic differences in the frequency of a mutation found in the promoter region of the VKORC1 gene in Chinese versus whites. Therefore, multiple genetic factors (including polymorphisms in CYP2C9 as well as vitamin K-dependent genes) modulate warfarin dosage.

In summary, the present study functionally assessed novel coding SNPs discovered in Chinese and Indians in a recombinant cDNA expression system. We confirm the existence of at least one null allele (CYP2C9*15) and suggest that CYP2C9*18 may also potentially represent a null allele based on the lack of expression in a cDNA expression system. In addition, CYP2C9*14 and CYP2C9*16 represent new potentially defective alleles in Asian populations based on markedly decreased intrinsic clearance of tolbutamide by both recombinant allelic proteins. This study highlights the existence of ethnic variability in the pharmacokinetics and pharmacodynamics of common drugs metabolized by CYP2C9. Clinical studies of these variants in larger Asian populations are needed to assess the role of these mutations in man. Our results show that defective alleles of CYP2C9 are more common than previously realized in various Asian populations, and additional genetic testing for the new defective alleles may be helpful in these populations.

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