A New CYP3A5 Variant, CYP3A5*11, Is Shown to Be Defective in Nifedipine Metabolism in a Recombinant cDNA Expression System

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ABSTRACT:

A new CYP3A5 variant, CYP3A5*11, was found in a white European subject by DNA sequencing. The CYP3A5*11 allele contains a single nucleotide polymorphism (SNP) (g.3775A>G) in exon 2, which results in a Tyr53Cys substitution, and a g.6986A>G splicing change, the latter SNP previously reported in the defective CYP3A5*3 allele. However, the CYP3A5*3 is not a null allele because this variant is associated with leaky splicing, resulting in small amounts of functional protein still being produced. Therefore, we constructed a cDNA coding for the newly identified CYP3A5.11 protein by site-directed mutagenesis, expressed it in Escherichia coli, and partially purified it. Whereas bacteria transformed with wild-type CYP3A5*1 cDNA expressed predominantly cytochrome P450 (P450), those transfectd with CYP3A5*11 expressed a significant amount of denatured cytochrome P420 in addition to P450, suggesting the protein to be unstable. CYP3A5.11 exhibited a 38% decrease in the Vmax for nifedipine metabolism, a 2.7-fold increase in the Km for nifedipine metabolism, and a 4.4-fold decrease in the Clint of nifedipine compared with CYP3A5.1. A polymerase chain reaction-restriction fragment length polymorphism genotyping procedure was developed and used to genotype DNA of 500 white individuals for CYP3A5*11. No additional examples of this allele were identified. In summary, individuals carrying the rare CYP3A5*11 allele are predicted to have lower metabolism of CYP3A5 substrates than individuals expressing CYP3A5*3.

Cytochromes P450 (P450s) metabolize endogenous and xenobiotic compounds to more water-soluble products that are easily eliminated from the body. CYP3A is the most abundant P450 subfamily in human liver and intestine (Watkins et al., 1987; Kolars et al., 1992, 1994; Shimada et al., 1994), accounting for 30% of total P450 in the human liver, and metabolizes approximately 50% of currently used clinical drugs (Shimada et al., 1994; Guengerich, 1999; Rendic, 2002). There are four human CYP3A genes: CYP3A4, CYP3A5, CYP3A7, and CYP3A43. In postnatal human liver, CYP3A4 and CYP3A5 are the two major CYP3A enzymes, which have overlapping substrate specificities (Guengerich, 1999; Lamba et al., 2002; Lee and Goldstein, 2005). The in vivo observed variability in clearance of CYP3A drugs can be caused by inhibition, induction, comedication, and/or the inheritance of CYP3A variant alleles encoding enzymes with altered activity (Thummel and Wilkinson, 1998). The CYP3A5 protein expression is highly variable in human liver largely because of the high frequency of an intronic single nucleotide polymorphism (SNP), CYP3A5*3 (g.6986A>G), which creates a cryptic splice site that results in aberrant splicing (Kuehl et al., 2001). Although CYP3A5 may represent 30% of the total CYP3A content in livers of individuals homozygous for CYP3A5*1, CYP3A5 protein is detected at appreciable levels in only 10 to 30% of livers from white and Asian individuals because of the high frequency of CYP3A5*3 (Kuehl et al., 2001). In contrast, CYP3A5 is highly expressed in livers of 50% of African-Americans, who have a high incidence of the CYP3A5*1 allele (Kuehl et al., 2001). CYP3A5 is also expressed in extrahepatic tissues, such as lung (Kivisto et al., 1996), kidney (Schuetz et al., 1992; Haehner et al., 1996), breast, and leukocytes (Huang et al., 1996; Janardan et al., 1996). The extent to which CYP3A5 contributes to total CYP3A activity has been a matter of debate and ranges from accounting for up to 50% of total CYP3A protein in the liver (Kuehl et al., 2001) to being only a minor contributor (Westlind-Johnsson et al., 2003). However, the fact that plasma concentration and dose requirement of the CYP3A metabolized immunosuppressive drug tacrolimus were clearly shown to be correlated to the presence of the CYP3A5*3 allele (Hesselink et al., 2003; Thervet et al., 2003, Hau-froid et al., 2004) shows that CYP3A5 activity is important for the metabolism of specific drugs. Many additional CYP3A5 SNPs have been described in recent reports (Lee et al., 2003; Saeki et al., 2003; Solus et al., 2004). Although CYP3A5*3/*3 individuals have low expression of CYP3A5 protein (Kuehl et al., 2001; Lin et al., 2002), livers of these individuals contain also normally spliced RNA next to abnormally spliced RNA caused by leaky splicing (Kuehl et al., 2001;...
Lin et al., 2002). This results in expression of some CYP3A5 protein, although at much lower amounts than in individuals containing the CYP3A5*1 allele (Hustert et al., 2001). The CYP3A5*3 allele has been associated with reduced clearance of lipid-lowering drugs such as simvastatin and lovastatin, as well as midazolam and tacrolimus (Hesselink et al., 2003; Thervet et al., 2003; Goto et al., 2004; Haufroid et al., 2004; Kivisto et al., 2004; Wong et al., 2004). Although several other CYP3A4 and CYP3A5 alleles are predicted to be defective in vitro, a clear association between CYP3A5 genotype and variability in metabolism of CYP3A substrates remains an area of active study. The association between CYP3A4 and CYP3A5 genotype and variability in substrate specificity of CYP3A4 and CYP3A5. To date, the association between CYP3A4 and CYP3A5 genotype and variability in metabolism of CYP3A substrates remains an area of active study. The association between CYP3A4 and CYP3A5 genotype and variability in metabolism of CYP3A substrates remains an area of active study. The association between CYP3A4 and CYP3A5 genotype and variability in metabolism of CYP3A substrates remains an area of active study.

Materials and Methods

Materials. Reduced form β-NADPH nifedipine, δ-aminovaleric acid, sodium cholate, phenylmethylsulfonyl fluoride, leupeptin, aprotinin, lyso- sozyme, ampicillin, t-n-dilauril-sn-glycos dialysed-3-phospholipids, T-thioe- dieydol- sn-glycerol-3-phospholipids, and bovine brain phosphatidylserine were pur- chased from Sigma Chemical Co. (St. Louis, MO). Restriction enzymes and T4 DNA ligase were from New England Biolabs (Beverly, MA). Oligonucleotide primers were obtained from Sigma Genosys (The Woodlands, TX). QuikChange mutagenesis kit and a proofreading Pfu DNA polymerase were from Stratagene (La Jolla, CA). Escherichia coli DH5α competent cells and isopropyl b-D-thiogalactopyranoside were from Invitrogen (Carlsbad, CA). Nickel-nitrolotriacetic acid (Ni-NTA) affinity resin was from Qiagen (Valen- cia, CA). Imidazole was purchased from Calbiochem (La Jolla, CA). Human NADPH-P450 oxidoreductase (P450 reductase) and cytochrome b5 were from Oxford Biomedical Research (Oxford, MI). All the other chemicals and organic solvents for high performance liquid chromatography (HPLC) were from the highest grade from commercial sources.

Identification of CYP3A5*11. DNA samples were from 500 healthy white Dutch individuals isolated as reported previously (van Schaik et al., 2002). The study was approved by the Medical Ethical Committee of the University Hospital Rotterdam.

Construction, Expression, and Purification of CYP3A5.11. Wild-type CYP3A5 cDNA was provided by Dr. Frank Gonzales (National Cancer Insti- tute, National Institutes of Health, Bethesda, MD). The first eight amino acids in the N terminus of CYP3A5*11 cDNA were modified to MALLLAVF as described for the 17-α hydroxylase (Barnes et al., 1991), and a 5XHis tag was added to the C-terminal region. Primers for the N- and C-terminal modifications were described previously (Lee et al., 2003). The modified CYP3A5 cDNA was cloned into the pcW vector via NdeI and HindIII sites and then used as the template for mutagenesis using a QuikChange kit (Stratagene). Mutagenesis primers for the construction of CYP3A5*11 cDNA were S-‘ggaatattgctgctcgaggtg-3’ (forward) and S-‘caactcagcatcagagacaaactc-3’ (reverse). After construction, the entire CYP3A5*11 cDNA construct was analyzed by sequencing in both directions. Expression of CYP3A5 wild type and CYP3A5.11 proteins in E. coli DH5α competent cells was induced with 0.5 mM isopropyl b-D-thiogalactopyranoside and 0.5 mM δ-aminovaleric acid, and incubation proceeded at room temperature for 96 h. To determine an optimal condition for maximal P450 expression, CO difference spectroscopy were monitored from 24 to 96 h using a DW-2000/OLIS spectrophotometer. Purification of P450 proteins was performed as described previously (Lee et al., 2003, 2005). To minimize interexperimental variations in expression and purification, CYP3A5 wild type and CYP3A5*11 cDNA constructs were simultaneously expressed, solubilized by sonication with de- tergent, and purified under the same conditions twice. Two sets of purified recombinant P450s were used to verify results. To increase the purity and the recovery of P450, solubilized membrane extract was purified on Ni-NTA affinity columns using a histidine tag. The eluted P450 was dialyzed for 48 h in dialysis buffer (100 mM potassium phosphate, pH 7.4, and 20% glycerol), and P450 content was measured on a DW-2000/OLIS spectrophotometer.

Reconstitution and Enzyme Assays. The ratio of P450/P450 reductase/ cytochrome b5 was 1:4:2 as optimized previously (Lee et al., 2003). Enzyme activity was reconstituted with incubation mixtures containing 4 pmol of P450 (determined spectrally), human P450 reductase (16 pmol), cytochrome b5 (8 pmol), 1:1:1 ratio of lipid mix (2 μg/reaction), sodium cholate (0.05 μmol/ reaction), and MgCl2 (30 mM in final) in a total reaction volume of 0.1 ml in 50 mM potassium buffer, pH 7.7. Nifedipine was protected from light during the experiment. Nifedipine concentrations for the kinetic analyses were 10, 20, 40, 60, 120, 240, and 480 μM. These substrate concentrations were above the lower limit of HPLC detection (6.25 μM) and were within the linear range for enzyme activity. Reactions were preincubated for 3 min at 37°C and initiated with NADPH (1 mM in final) at 37°C for 10 min. All the assays were performed in triplicate. The reactions were stopped with the addition of 50 μl of methanol and vigorously vortexed for 2 min followed by centrifugation at 10,000g for 15 min. The oxidized metabolite in the supernatant was analyzed by HPLC as described earlier (Lee et al., 2003). There was no catalytic activity in the absence of NADPH.

Development of a Genotyping Test. A specific polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) test was developed to detect this novel mutation. For a 50-μl PCR, 10 ng of genomic DNA was incubated in a PCR mixture containing 1× buffer (10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl2, 50 mM KCl, and 10 μg/ml gelatin (Perkin-Elmer)), 0.2 mM each of the deoxynucleotide triphosphates (Roche), 1.25 μl of AmpliTaq Gold (Perkin-Elmer), and 40 pmol of primers P1 (5′-CTTATACCCCTCCTGTC- GAC-3′) and P2 (5′-CTCAAACACTTCACTGCG-3′). The italicized, lower case nucleotides are mismatches with the CYP3A5 sequence, creating restric- tion sites in the PCR product for the detection of the CYP3A5*11 SNP, but also creating an internal control for the digestion. PCR conditions were 7 min at 94°C; 30 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C; and finally 7 min at 72°C. The size of the amplified product was 213 bp. The PCR product (10 μl) was digested with SaII (New England Biolabs) in a total volume of 15 μl for 2 h at 37°C and subsequently analyzed on a 3% agarose/Tris-borate- EDTA gel with ethidium bromide staining. The fragments obtained for the wild-type allele were 196 and 17 bp; for the CYP3A5*11 variant allele, the expected fragment were 175, 21, and 17 bp. The sample identified as a heterozygote CYP3A5*11 by sequencing was used as a positive control for the PCR-RFLP.

Analysis of Enzyme Kinetics and Statistics. Nifedipine oxidation was analyzed by nonlinear regression using GraphPad Prism 4.0 (GraphPad Soft- ware Inc., San Diego, CA). The goodness of fit was determined by the degrees of freedom, absolute sum of squares, and the standard error of the parameter estimates. Kinetics for nifedipine metabolism followed the Michaelis-Menten equation for hyperbolic kinetics as the best fit of the data as described previously (Williams et al., 2002; Lee et al., 2003). Values represent the mean ± S.E. of at least two independent experiments, each in triplicate. Statistical significance (p < 0.001) was determined using a two-tailed Student’s t test.

Results

We found a novel SNP while validating a wild-type sample for our CYP3A5*8 PCR-RFLP assay by sequencing DNA samples from healthy white subjects. One sample showed a heterozygous peak at a different site than expected for the CYP3A5*8 polymorphism. The sample was resequenced (in both directions) to confirm the presence of the newly discovered g.3775A→G SNP (Fig. 1). Analysis of this sample for the intronic CYP3A5*3 SNP (van Schaik et al., 2002) revealed that this individual was homozygous for a g.6986A→G SNP associated with the CYP3A5*3/*3 allele. Subsequent sequencing of all the CYP3A5 exons in both directions indicated that no other CYP3A5 SNP was present (results not shown).

Maximal expression obtained for wild-type CYP3A5.1 was 100 to 150 nM in E. coli at 72 h. CYP3A5.11 exhibited approximately 50% lower P450 expression of 40 to 70 nM at 72 h with an additional peak at 420 nm, suggesting that the protein might be unstable. CYP3A5.1
protein did not contain a 420-nm peak at 72 h of expression in E. coli. Purification of P450s by Ni-NTA affinity column through the histidine tag resulted in approximately 40% recovery for wild-type and ~10% recovery for CYP3A5.11. Purified CYP3A5.1 protein contained only a small peak at 420 nm, but CYP3A5.11 protein exhibited comparable amounts of denatured cytochrome P420 and P450 despite two different rounds of expression and purification (Fig. 2). For enzyme assays, the reaction was normalized to equal amounts of P450, not cytochrome P420. Because kinetic analysis requires large amounts of enzyme, we initially compared the statistical differences in turnover number by two aliquots of CYP3A5.11 and CYP3A5.1 proteins purified on separate days at two different concentrations of substrate. The first concentration of nifedipine, 60 μM, approximates the $K_m$ value of wild-type CYP3A5 as determined in our laboratory, and the second higher concentration of 240 μM approaches the $V_{max}$ of the enzyme. Figure 3 shows the results of two independent sets of assays. CYP3A5.11 had significantly lower (67 and 70%; $p < 0.001$) turnover numbers for oxidation of nifedipine at the low substrate concentration and 44 and 50% ($p < 0.001$) lower activity at the high concentration of nifedipine for the two preparations. There was no statistically significant difference in nifedipine oxidation by two preparations of CYP3A5.11 or CYP3A5.1, suggesting low interexperimental variation in expression and purification. Figure 4 and Table 1 compare kinetic parameters for nifedipine metabolism by CYP3A5.11 with that of wild-type. Kinetic parameters for nifedipine metabolism by CYP3A5.11 protein exhibited a 39% decrease in the $V_{max}$, a 2.7-fold increase in the $K_m$, and a 4.3-fold decrease in the intrinsic clearance compared with wild-type.

We screened 500 healthy white Dutch individuals for this novel CYP3A5*11 polymorphism but could not identify additional individuals with this SNP, thus resulting in an allelic frequency of 0.1% in this population.

**Discussion**

Genetic variation in the CYP3A genes may influence the metabolism and elimination of CYP3A substrates in humans. Because CYP3A4 and CYP3A5 have overlapping substrates, it has been difficult to determine the effect of each CYP3A genotype on in vivo
FIG. 3. Nifedipine metabolism by CYP3A5.1 and CYP3A5.11 proteins. Reactions were performed with spectrally determined P450 (4 pmol), 16 pmol of reductase, and 8 pmol of cytochrome b₅ (the ratio of P450/reductase/cytochrome b₅ = 1:4:2) in 0.1 ml of reaction buffer for 10 min. All the values plotted are means of triplicate measurements ± S.E. The preparation of proteins and details of reaction mixtures are described under Materials and Methods. Two different concentrations were used: 60 μM, which is near the Kₘ value, and 240 μM, which is near the Vₘₐₓ, * significantly lower (p < 0.001) than CYP3A5.1 as analyzed by two-tailed Student’s t test.

FIG. 4. Catalytic profiles of nifedipine metabolism by CYP3A5.1 (closed circles) and CYP3A5.11 (open circles). The preparation of proteins and details of reaction mixtures and kinetics are described under Materials and Methods. All the reactions were performed with spectrally determined P450 (4 pmol), 16 pmol of reductase, and 8 pmol of cytochrome b₅ (the ratio of P450/reductase/cytochrome b₅ = 1:4:2) in 0.1 ml of reaction buffer for 10 min. All the values plotted are means of triplicate experiments ± S.E.

phenotype. The variant g.6986A>G (CYP3A5*3) is responsible for dramatic decreases in hepatic expression of CYP3A5 protein (Kuehl et al., 2001) and is a major determinant in low CYP3A5 protein expression in the liver because of its high frequency in many populations. However, individuals having the CYP3A5*3/*3 genotype still express low amounts of CYP3A5 protein (Hustert et al., 2001; Westlind-Johnsson et al., 2003). Several studies have identified protein coding variants of CYP3A5, as well as splice variants (Jounaidi et al., 1996; Chou et al., 2001; Hustert et al., 2001; Kuehl et al., 2001; Lee et al., 2003; Solus et al., 2004). One such allele, CYP3A5*10, contains an F446S change in the heme-binding region, in addition to the g.6986A>G change associated with the major splice variant (Lee et al., 2003), for which the recombinant CYP3A5.10 (F446S) protein exhibited a greater than 95% decrease in the intrinsic clearance for testosterone and nifedipine compared with wild-type CYP3A5.1 (Lee et al., 2003).

In the present study, we identified a new CYP3A5 variant, CYP3A5*11, in white Europeans containing a single base pair change coding for Tyr53Cys. We investigated metabolism of the antihypertensive drug nifedipine by recombinant CYP3A5.11 protein, showing a defective variant with a 38% decrease in the Vₘₐₓ, a 2.7-fold increase in the Kₘ, and a 4.3-fold decrease in the intrinsic clearance compared with wild-type CYP3A5.1. Because this allele also contained the g.6986A>G SNP, already lower amounts of functional CYP3A5 protein are being produced (Hustert et al., 2001; Westlind-Johnsson et al., 2003). Therefore, the presence of the Tyr53Cys variant affects this residual CYP3A5 activity. The exact importance of this new SNP, in the context of the *3 splice variant, needs further study. Although we have assumed that the *3 and *11 SNPs function independently—one affecting mRNA splicing, the other decreasing the activity of residual protein formed—expression of the CYP3A5*11 allele in a mammalian expression system may be a better way to study this combination of SNPs. However, the Tyr53Cys variant may also occur without the g.6986A>G SNP and would then be expected to have a more profound effect on total CYP3A5 activity. It remains to be studied whether the observed decrease in activity on nifedipine also holds true for other CYP3A5 substrates.

CYP3A4 and CYP3A5 have 84% amino acid sequence identity: alignment of CYP3A5 Cys-53 with CYP3A4 revealed that the CYP3A5 Cys-53 was not located in the known substrate recognition sites or in the active site cavity of CYP3A4 (Harlow and Halpert, 1997; Domanski et al., 1998, 2000; Yano et al., 2004). The region containing Cys-53, however, is well conserved in the CYP3A4 and CYP3A5 primary amino acid sequences. In the crystal structure of CYP3A4 protein, Cys-53 appears to be located around the N-terminal “A” helix (Williams et al., 2004). A notable hydrophobic region was found around the loop following helix A in CYP3A4, and this hydrophobic region was suggested to be mediated with the microsomal membrane interaction (Williams et al., 2004; Scott and Halpert, 2005). The change from the tyrosine, which is a hydrophobic and aromatic amino acid, to the sulfur-containing hydrophilic amino acid cysteine could cause structural changes in the membrane binding regions. The Tyr-53 in CYP3A4 was reported to be one of the amino acids involved in a hydrogen-bonding network with Arg-106, Glu-374, Asp-376, Arg-372, and Asp-61 (Yano et al., 2004). CYP3A4 and CYP3A5 have identical amino acids at all these locations except that CYP3A5 contains a Glu-76. This hydrogen-bonding network has been suggested to have an important role in the solvent access channel in an α-helix (Williams et al., 2004). A notable hydrophobic region was considered in the ratio of mixture (P450/reductase/cytochrome b₅ = 1:4:2). Data were obtained from 4 pmol of P450 per reaction with nifedipine concentrations of 10, 20, 40, 60, 120, 240, and 480 μM.

### Table 1

<table>
<thead>
<tr>
<th>P450</th>
<th>Vₘₐₓ (nmol/min/mmol P450)</th>
<th>Kₘ (μM)</th>
<th>CLₘₐₓ (ml/min/mmol P450)</th>
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<tbody>
<tr>
<td>CYP3A5.1</td>
<td>144.1 ± 2.5</td>
<td>44.1 ± 2.6</td>
<td>3.23</td>
</tr>
<tr>
<td>CYP3A5.11</td>
<td>88.2 ± 2.6 (↓ 39%)</td>
<td>118.7 ± 8.8 (↓ 2.7 × 10⁻²)</td>
<td>0.74 (↓ 4.3 × 10⁻²)</td>
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*Fold and % difference compared with values of wild type.
A PCR-RFLP genotyping test, developed for the detection of the g.3775A>G SNP found in CYP3A5*11, detected no additional examples of this SNP in 500 genomic DNA samples from healthy white individuals, suggesting that CYP3A5*11 is a very rare allele in whites. One of our previous studies detected CYP3A5*10 only in certain white ethnic groups (Adeyi et al., 2005), and it is possible that also the CYP3A5*11 allele is more frequent in other ethnic groups. The present genetic test with functional information will be useful for future clinical studies to link its association with other CYP3A SNPs, particularly in individuals who show defective metabolism of CYP3A substrates.

References


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