Defective Activity of Recombinant Cytochromes P450 3A4.2 and 3A4.16 in Oxidation of Midazolam, Nifedipine, and Testosterone

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ABSTRACT:

Cytochrome P450 3A4 (CYP3A4) is the most abundant cytochrome P450 in adult human liver and small intestine and oxidizes numerous clinically, physiologically, and toxicologically important compounds. The metabolic activity of CYP3A4 in patients varies at least 10-fold in vivo, and CYP3A4 genetic variants are considered one of the causes of individual differences. The cDNAs for the CYP3A4*2 (S222P), *7 (G66D), *16 (T185S), and *18 (L293P) mutant alleles, found in high frequencies in Caucasians or Asians, were constructed by site-directed mutagenesis and expressed in an Escherichia coli expression system. Midazolam (MDZ), testosterone (TST), and nifedipine (NIF) were used to assess the catalytic activities of the CYP3A4 wild type (CYP3A4.1) and its variants. The catalytic activities of CYP3A4.2 and CYP3A4.16 were reduced (lower V_max and increased K_m relative to CYP3A4.1) for all substrates. The CYP3A4.7 showed lower V_max values for MDZ and NIF (60 and 84%, respectively) and a higher K_m (2-fold) for TST but not for MDZ or NIF. Although CYP3A4.18 showed low V_max values for MDZ, NIF, and TST (88, 72, and 80% of CYP3A4.1, respectively), no significant differences were identified in the ratio V_max/K_m. In summary, CYP3A4.2 and CYP3A4.16 exhibited significantly lower activity for MDZ, TST, and NIF oxidations than CYP3A4.1. Therefore, drugs metabolized by only CYP3A should be carefully administered to patients with these alleles.

The CYP3A subfamily of cytochrome P450 enzymes comprises approximately 30 to 60% of total cytochrome P450 in human liver (Shimada et al., 1994). The CYP3A enzymes are responsible for the metabolism of more than 50% of clinically used drugs and also some steroids and environmental chemicals (Li et al., 1995; Evans and Relling, 1999; Guengerich, 1999). Four human CYP3A enzymes—CYP3A4, CYP3A5, CYP3A7, and CYP3A43—have been identified, and CYP3A4 is regarded as the most dominant CYP3A enzyme in the liver and small intestine (Wrighton et al., 1990; Shimada et al., 1994; Hustert et al., 2001; Koch et al., 2002). It has been reported that CYP3A4 expression shows large interindividual variation (Guengerich, 1999; Ozdemir et al., 2000; Lin et al., 2002) and that these variations can lead to different responses to human drugs that are substrates for CYP3A4. Because the expression of CYP3A4 and CYP3A5 can be induced by pregnane X receptor, constitutive androstane receptor, and glucuronidate receptor ligands, both environmental and genetic factors can influence the CYP3A activity; however, the genetic contribution has been estimated to be larger (Ozdemir et al., 2000), suggesting that polymorphisms in CYP3A4 may predict CYP3A phenotype, at least in part. Genetic analysis is needed to understand interindividual variability. Extensive studies searching allelic variants in the coding regions of CYP3A4 have been done, and currently 20 different CYP3A4 variant proteins have been described, some of them representing proteins of either decreased or increased activity. Those polymorphisms are reported on the Human Cytochrome P450 (P450) Allele Nomenclature Committee Web site (http://www.cypalleles.ki.se/cyp3a4.htm). Decreased activities for the CYP3A4*8, *11, *12, *13, *16, and *17 alleles, increased activity of CYP3A4*18, and lack of expressed protein for CYP3A4*20 are indicated. However, there are some conflicting reports, depending on the substrates or experimental systems.

To identify the effect of genetic variation on catalytic activity of CYP3A4, we expressed CYP3A4*1 (wild type), *2 (2.7% in Caucasian) (Sata et al., 2000), *7 (1.4–3% in Caucasian) (Eiselt et al., 2001; Lamba et al., 2002), *16 (1.4–5% in Japanese) (Lamba et al., 2002; Fukushima-Uesaka et al., 2004), and *18 (1.3–2.8% in Japanese) (Dai et al., 2001; Yamamoto et al., 2003; Fukushima-Uesaka et al., 2004) in Escherichia coli using a bacterial cDNA expression system. This system highly expresses functional CYP3A4 protein cost effectively (Parikh et al., 1997). Catalytic activities of wild-type CYP3A4 and allelic variants were compared using the clinically used drugs midazolam (MDZ) and nifedipine (NIF) and an endogenous CYP3A4 substrate, testosterone (TST), as typical CYP3A4 substrates.

Materials and Methods

Chemicals and Materials. NdeI and XbaI restriction endonucleases were purchased from New England Biolabs (Beverly, MA). Baculovirus-insect cell
expressed CYP3A4 were purchased from BD Gentest (Woburn, MA). Primers were purchased from KURABO Industries Ltd. (Osaka, Japan). Nifedipine and hydrocortisone were purchased from Sigma-Aldrich (St. Louis, MO). Luteopin, TST, and iso-propyl-β-D-thigalactopyranoside were purchased from Wako Pure Chemicals (Osaka, Japan). Mouse anti-human CYP3A4, rabbit anti-mouse IgG antibodies, 1'-hydroxymidazolam, 6β-hydroxy-TST, and 2,6-dimethyl-4-(2'-nitrphenyl)-3,5-pyridinedicarboxylic acid dimethyl ester (oxidized nifedipine) were purchased from Daiichi Pure Chemical (Tokyo, Japan). Midazolam injections (Dormicum injections) were purchased from Astellas Pharma Inc. (Tokyo, Japan) and used (in in vitro studies) without purification. Diazepam was purchased from Dainippon Sumitomo Pharma Co., Ltd. (Osaka, Japan). All other chemicals and reagents were of analytical grade and were obtained from commercial sources and used without further purification.

**Modification of CYP3A4 cDNA.** The expression vectors pCW-OR and pBL3A4 were described previously (Parikh et al., 1997) and were digested with NdeI and Xbal. To achieve expression, the N terminus of protein was modified as described by Parikh et al. (1997) and Guengerich et al. (1996). After electrophoresis on a 2% agarose gel, bands at 7025 base pairs (pCW-OR vector) and 1500 base pairs (CYP3A4 cDNA) were cut out. DNA fragments were extracted with a QIAEX II gel extraction kit (QIAIEN, Valencia, CA), and those fragments were ligated with a DNA ligation kit (TAKARA, Otsu, Japan) to construct wild-type pCW3A4b, the plasmid for expressing CYP3A4 and NADPH-P450 reductase (OR) bicistronically.

**Site-Directed Mutagenesis.** Expression vectors for CYP3A4.2 (S222P), 0.7 (G56D), 0.16 (T185S), and 0.18 (L293P) were made using site-directed mutagenesis. A QuikChange site-directed mutagenesis kit from Stratagene (La Jolla, CA) was used to introduce single nucleotide changes (primers used to introduce single nucleotide polymorphisms are available upon request). The entire coding region, including the mutated sites, was confirmed by direct sequencing. The entire cDNA was then excised and subcloned into a new pCW/OR plasmid to avoid any accidental mutations in the plasmid caused by the mutagenesis procedure.

**Expression of CYP3A4s and Preparation of Membrane Fraction of E. coli.** Expression of CYP3A4 wild-type and variant proteins in E. coli DH5α F’IQ cells and preparation of the membrane fraction were carried out according to Gillam et al. (1993). After determination of the protein content according to Lowry et al. (1951) with a DC protein assay kit (Bio-Rad, Hercules, CA), the P450 content was determined by the CO difference spectroscopy method of Omura and Sato (1964) as modified by Iwata et al. (1998), after dilution of the suspension with 100 mM of potassium phosphate buffer (pH 7.4) containing 20% (v/v) glycerol and 0.2% (w/v) Emulgen 911. The difference spectra were recorded by using a Shimadzu UV-visible spectrophotometer model MPS-1600. The membrane fractions were frozen and stored at −80°C until use. Three lots of membrane fractions expressing wild-type or the variants of CYP3A4 were used in further experiments.

**Immunoblot Analysis.** SDS-polyacrylamide gel electrophoresis and Western blotting were performed according to the methods of Guengerich et al. (1982) with 12% (w/v) polyacrylamide gels. Immuno detection was performed with a WB-MAB3A/human CYP3A4/3A5 WB kit according to the manufacturer’s instructions, using diaminobenzidine as the peroxidase substrate.

**Enzyme Activity.** MDZ 1'-hydroxylation activity was determined as described by Yamaori et al. (2004). The reaction mixture (total 450 μl) for the assay consisted of 0.1 M potassium phosphate buffer (pH 7.4), 0.1 mM EDTA, an NADPH-generating system (5.0 mM glucose 6-phosphate, 0.5 mM NADP+, and 1 unit/ml glucose 6-phosphate dehydrogenase) and membrane fraction (0.025 nmol of P450). After preincubation at 37°C for 5 min, the reaction was initiated by addition of 50 μl of midazolam in 50% methanol (0–150 μM final concentration). The reaction mixture was incubated at 37°C for 5 min, and the reaction was terminated by adding 500 μl of ice-cold acetonitrile, followed by the addition of 50 μl of 10 mg/ml diazepam and 2 mM hydrocortisone, respectively, as internal standards. After centrifugation, the concentration of oxidized NIF or 6β-hydroxy-TST in the supernatants was determined by HPLC.

**Measurement of Metabolites by HPLC.** Concentrations of 1'-hydroxy-MDZ were determined according to the method of Qi et al. (2005). Oxidized NIF and 6β-hydroxy-TST were determined using similar conditions. For the determination of oxidized NIF and 6β-hydroxy-TST, an Intersil ODS-2 (4.6 × 150 mm) column was used with a gradient system beginning with methanol-water (45:55, v/v) from 0 to 10 min, the acetonitrile concentration was programmed to linearly increase from 0 to 25% (v/v) during 10 min and then returned to the starting mobile phase from 10 to 12 min. The absorbance of oxidized NIF and 6β-hydroxy-TST were detected at 220 and 239 nm, respectively.

**Data Analysis.** The kinetic parameters Kmax and Vmax were estimated by analyzing Michaelis-Menten plots using GraphPad Prism software (version 4.0; GraphPad Software Inc., San Diego, CA). The equation v = Vmax[S/(Kmax + S)] was fitted to the measured data for initial velocity (v) at varying concentrations of substrate (S) using nonlinear regression. Values of steady-state catalytic parameters are considered as the ratio Vmax/Kmax. Statistical comparisons were made with Student’s t test with the Bonferroni revision, and differences were considered statistically significant when the p value was <0.05.
E. coli

Results

Expression of Wild-Type and Variant CYP3A4s in E. coli. The introduction of four SNPs into CYP3A4 cDNAs was confirmed by direct sequencing (data not shown). The expression levels of CYP3A4 proteins in membrane fractions obtained from E. coli DH5α F’1Q transformed with CYP3A4*1 (wild-type), CYP3A4*2, CYP3A4*7, CYP3A4*16, and CYP3A4*18 were examined by reduced CO difference spectral and immunoblot analyses. As shown in Fig. 1C, the reduced CO difference spectra of CYP3A4s showed Soret peaks at 450 and approximately 420 nm. The expression levels of wild-type and variant CYP3A4 proteins in E. coli membrane fractions were also assessed by immunoblotting. The stained CYP3A4 bands were at 52 kDa, the same as human liver microsomes or CYP3A4 expressed by a baculovirus-insect cell system containing CYP3A4 cDNA (Fig. 1A). The expression levels of CYP3A4*1 and variants did not differ in three independent experiments.

Comparison of Activities of Wild-Type and Variant CYP3A4s Expressed in E. coli. The MDZ 1'-hydroxylation, NIF oxidation, and TST 6β-hydroxylation activities of CYP3A4.1, CYP3A4.2, CYP3A4.7, CYP3A4.16, and CYP3A4.18 were examined. The relationships between substrate concentrations and reaction velocities are shown in Fig. 2, and the Vmax and Km values are compiled in Table 1. The Vmax/Km values, which predict intrinsic clearance in vivo, were calculated and compared with those for CYP3A4.1. The activities of CYP3A4.2 and CYP3A4.16 were reduced (lower Vmax and increased Km relative to CYP3A4.1) for all substrates. CYP3A4.7 had a decreased Vmax for MDZ and NIF (to 60 and 84%, respectively) and increased (2-fold) Km for TST but not for MDZ or NIF. Although CYP3A4.18 showed a reduced Vmax for MDZ, NIF, and TST (to 88, 72, and 80%, respectively), no significant differences were identified in the ratio Vmax/Km.

Discussion

A wide interindividual variability exists in CYP3A substrate clearance in vivo (Wrighton et al., 1990; Shimada et al., 1994; Hustert et al., 2001; Kuehl et al., 2001; Koch et al., 2002), but there is very limited evidence linking individual genetic polymorphisms to variations in metabolism. The present study shows that recombinant CYP3A4.2 (S222P) is decreased (to 37%, Vmax) for NIF oxidation, with a 3.4-fold increased Km, compared with wild type, and this result indicated lower activity for NIF than for the other two substrates. In a previous study, recombinant CYP3A4.2 exhibited a lower Vmax for NIF metabolism (~36% decrease) but not for TST compared with CYP3A4 wild type (Sata et al., 2000). In the present study, expressed CYP3A4.2 is markedly defective in the oxidation of TST (Table 1). The difference with the previous work (Sata et al., 2000) may be the result of the difference in the range of substrate concentrations, expression level of reductase, and other factors in the reaction mixture. The mechanism of the change in kinetics on metabolism of the CYP3A4 substrates may be caused by the disruption of protein conformation by the serine to proline substitution. Codon 222 in CYP3A4 is thought to form a membrane interaction domain (Williams et al., 2000). In a previous study, a CYP3A4*2 homozygous individual was found in the group of slow metabolizers for cortisol 6β-hydroxylation (Shcheopotina et al., 2006). It is predicted that the CYP3A4*2 allele may result in lower CYP3A4 activity in vivo, although in vivo activity of this allele for NIF oxidation has not been reported.

The present study shows that recombinant CYP3A4.16 is markedly defective in MDZ, NIF, and TST oxidation in vitro, exhibiting approximately 65% decreases in Vmax and increased Km values (3.6- to 5.9-fold) compared with the wild type. This result is consistent with a report that the CYP3A4*16 allele caused a 60% decrease activity in TST 6β-hydroxylation (Murayama et al., 2002). In the comparison between CYP3A4.1 and CYP3A4.16, CYP3A4.16 showed lower activity than in the report from Murayama et al. (2002). Possible reasons for this disagreement are differences in the experimental systems, i.e.,
Miyazaki et al. used mammalian cells expressing P450 and substrates were added to the culture media. Recombinant CYP3A4.17 was markedly defective in NIF oxidation in vitro, i.e., a $>99\%$ decrease in $V_{\text{max}}$ and $V_{\text{max}}/K_m$ (Lee et al., 2005). The amino acid changes in CYP3A4.16 (T185S) and CYP3A4.17 (F189S) are located in helix E and appear to be involved in packing with residues on helices G and H and the turn between helices G and H (Yano et al., 2004). The region including these amino acids can be significant for CYP3A4 catalytic activities, even though these amino acids are not located in the putative substrate recognition site of CYP3A4 reported previously (Gotoh, 1992; Wang et al., 1998; Khan et al., 2002). The residues of CYP3A4.16 and CYP3A4.17 are non-conservative mutations in tightly packed regions, which could conceivably affect the conformation of the protein, substrate access, and/or catalytic activity. In both the present and a previous study, recombinant CYP3A4.16 and CYP3A4.17 showed large peaks around 420 nm in the CO difference spectra compared with wild-type or other variant CYP3A4s. These mutations could result in influences on other parts of the protein and conformational changes that affect stability and indirectly affect activity.

The CYP3A4*16 allele has been reported at a frequency of 1.4 to 5% in Asians and other ethnic groups (Lamba et al., 2002; Fukushima-Uesaka et al., 2004), so marked in vitro changes in metabolic activity provide important information for pharmacotherapeutics. However, there have been no reports suggesting that the CYP3A4*16 allele causes changes in activity in vivo because the CYP3A4*16 allele has been detected only in heterozygotes. Thus, the clinical relevance of the CYP3A4*16 homozygote remains to be further evaluated. CYP3A4*16 in the homozygote state (in vivo) would be expected to reduce the enzyme activity and produce a “slow metabolizer” or “poor metabolizer” phenotype, such as CYP3A4*2. Furthermore, the contribution of other drug-metabolizing enzymes can lead to underestimation of genetic influence. The CYP3A4*16 allele manifests small renal excretion or high specificity for CYP3A4 should be administrated with some caution.

The present study shows that recombinant CYP3A4*7 is defective in testosterone metabolism in vitro, exhibiting an increase in $K_m$ compared with the wild type. However, no differences were identified in the $V_{\text{max}}/K_m$ ratio between the substrates. Although the CYP3A4*7 allele was found at a 1.4 to 3% allele frequency in Caucasians, no other report has been published about activities in vivo or in vitro. Our present study suggests that the CYP3A4*7 allele does not cause differences in catalytic activity in the three typical CYP3A4 substrates, and additional work is needed to further define the property of this allele.

Recombinant CYP3A4.18 exhibited a 2-fold higher activity toward the insecticide chlorpyrifos in an earlier study (Dai et al., 1995) compared with wild type, with no significant change in $V_{\text{max}}$ and $V_{\text{max}}/K_m$ attributable to CYP3A4.18. According to the study, the contribution of other drug-metabolizing enzymes can lead to underestimation of genetic influence. The CYP3A4*16 allele manifests small renal excretion or high specificity for CYP3A4 should be administrated with some caution.

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**References**


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