DIFFERENTIAL ENANTIOSELECTIVITY AND PRODUCT-DEPENDENT ACTIVATION AND INHIBITION IN METABOLISM OF VERAPAMIL BY HUMAN CYP3A \textsc{S}

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ABSTRACT:

In vitro studies of enantioselective metabolism of \( R-\) and \( S-\) verapamil (VER) were conducted using human cDNA-expressed CYP3A isoforms, CYP3A4, CYP3A5, and CYP3A7. \( N\)-dealkylated products nor-VER \([2,8\text{-bis-(3,4-dimethoxyphenyl)-2-isopropyl-6-azaoctanitrile}] \) and D617 \([2-(3,4\text{-dimethoxyphenyl)-5-methylamino-2-isopropylvaleronitrile}] \) were the major metabolites for all CYP3A isoforms regardless of enantiomer. Enantioselectivity of CYP3A4 and CYP3A7 was most similar among the three isoforms. This coincides with the degree of homology of amino acids at the active sites and in the total amino acid sequences of the enzymes. Bisphenolic substrate inhibition was observed for the formation of nor-VER and D617, whereas simple bisphenolic kinetics were observed for the formation of \( O\)-demethylated products for both enantiomers with CYP3A4. The bisphenolic substrate inhibition was observed only for nor-VER, and simple bisphenolic kinetics were observed for D617 and \( O\)-demethylated products for both enantiomers with CYP3A5. However, with CYP3A7, D617 and \( O\)-demethylated products showed typical Michaelis-Menten kinetics, and only nor-VER displayed substrate (monophasic) inhibition. When metabolic rates of VER were determined in the presence of three different effectors, midazolam, testosterone, and nifedipine, activation, inhibition, or activation and inhibition of VER metabolism was observed depending on the enantiomers, metabolites, effectors, and cytochrome P450 isoforms. Addition of anti-CYP3A4 antibody inhibited formation of all metabolites for both CYP3A4 and CYP3A5. The atypical phenomena (bisphenolic substrate inhibition, activation, and inhibition depending on product formation) of VER kinetics could be adequately explained by introducing the concept of steric interaction into a two binding-site model.

Cytochrome P450 (P450\(^1\) \( 3A \)) is the most important human P450 subfamily due to its high relative abundance in the liver and its broad substrate specificity (Thummel and Wilkinson, 1998). The enzymes within this subfamily are estimated to participate in the metabolism of approximately 50% of marketed drugs known to undergo oxidative metabolism (Benet, 1996) and are responsible for many metabolism-based drug-drug interactions. Four members of the CYP3A subfamily have been reported in humans: CYP3A4, CYP3A5, CYP3A7 (Nelson et al., 1996), and CYP3A43 (Domanski et al., 2001a). CYP3A4 is the most abundant hepatic and intestinal form. CYP3A5 is the second most abundant CYP3A in adult human liver and shows reduced metabolic capability compared with CYP3A4 in most cases. Recently, however, CYP3A5 is reported to be more abundant than CYP3A4 in the lung and kidney (Ding and Kaminsky, 2003). CYP3A4-specific probe substrates and/or inactivators have been identified (Cook et al., 2002; Khan et al., 2002), but specific probe substrates for CYP3A5 have not yet been reported. CYP3A7 is the major fetal form and is rarely expressed in adults. Comparative in vitro metabolic activities of CYP3A4, CYP3A5, and CYP3A7 have been extensively investigated using model substrates (Williams et al., 2002). CYP3A43 was discovered in the prostate and determined to be present in very low amounts in the liver.

In vitro studies have revealed that some CYP3A4 substrates do not obey classical Michaelis-Menten hyperbolic kinetics and show some atypical kinetic properties. These types of nonhyperbolic kinetics include autoactivation (Etkins et al., 1998), substrate inhibition, and bisphenolic kinetics. Furthermore, these enzymes demonstrate activation with the addition of a second compound, such as flavonoids or steroid hormones (Ueng et al., 1997; Korzekwa et al., 1998; Ludwig et al., 1999; Houston and Kenworthy, 2000). Interestingly, product-dependent activation and inhibition were observed with some substrates in the presence of a second compound (Wang et al., 2000; Schrag and Wienkers, 2001). These nonhyperbolic kinetics with CYP3A4 were hypothesized to reflect the binding of two substrates simultaneously in the active site (Korzekwa et al., 1998). Recently, Domanski et al. (2001b) demonstrated that multiple substrate molecules can bind within the CYP3A4 active site using a method of site-directed mutagenesis of the substrate recognition site (SRS). They proposed that...
at least three subpockets might exist for substrate binding to the active site, including one allosteric “effector” site too distal from the heme for metabolism to occur. An alternative hypothesis has also been made that multiple conformations of CYP3A4 may exist (Koley et al., 1997). More recently, the concept of “nested allosterism” has been proposed, in which the relative proportions of multiple conformers are determined by allosteric effectors (Atkins et al., 2001). However, more examples of these types of atypical substrate behavior are required to better understand the role of CYP3A in drug metabolism and thus improve the current capabilities for predicting which drugs are metabolized by these enzymes.

We have selected verapamil (VER) to study enantio- and regioslective metabolism by CYP3A isoforms. VER is a calcium channel blocker and has been widely used in the treatment of hypertension, stable angina, and narrow QRS supraventricular arrhythmias. This blocker and has been widely used in the treatment of hypertension, and thus the current capabilities for predicting which drugs are metabolized by these enzymes.

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Materials and Methods

Materials. R(+)-VER, S(−)-VER, nor-VER [2,8-bis-(3,4-dimethoxyphenyl)-2-isopropyl-6-azaocanitride], D617 [2-(3,4-dimethoxyphenyl)-5-methylamino-2-isopropylvaleronitrile], M1 [2-(3,4-dimethoxyphenyl)-8-(4-hydroxy-3-methoxyphenyl)-6-methyl-2-isopropyl-6-azaocanitride], M2 [2-(3,4-dimethoxyphenyl)-8-(3-hydroxy-4-methoxyphenyl)-6-methyl-2-isopropyl-6-azaocanitride], M3 [2-(4-hydroxy-3-methoxyphenyl)-8-(3,4-dimethoxyphenyl)-6-methyl-2-isopropyl-6-azaocanitride], M4 [2-(3-hydroxy-4-methoxyphenyl)-8-(3,4-dimethoxyphenyl)-6-methyl-2-isopropyl-6-azaocanitride], and benzphetamine were obtained from Pfizer Corporation (Kalamazoo, MI). Acetonitrile, ammonium acetate, K2HPO4, phosphoric acid, D-glucose 6-phosphate, glucose-6-phosphate dehydrogenase, β-nicotinamide adenine dinucleotide phosphate, EDTA disodium salt, phosphoric acid, MgCl2·6H2O, midazolam, testosterone, and nifedipine were purchased from Sigma-Aldrich (St. Louis, MO). Microsomes from baculovirus-insect cell that is genetically engineered to express human cytochrome isozymes CYP3A4, CYP3A5, CYP3A7, and monoclonal antibody inhibitory to CYP3A4 were purchased from BD Gentest (Woburn, MA).

Metabolism Kinetics Experiments. Metabolic rates of VER by human CYP3A isoforms were determined in triplicate as follows under conditions in which less than 0.5% acetonitrile was present. Human cDNA-expressed CYP3A4 (31 μg), CYP3A5 (120 μg), or CYP3A7 (150 μg) was incubated with various concentrations of R(+)-VER or S(−)-VER in 100 mM potassium phosphate buffer (pH 7.4) with 1 mM EDTA, 6 mM MgCl2, and an NADPH-generating system consisting of 10 mM β-glucose 6-phosphate, 1 mM NADP, and 0.14 U of glucose-6-phosphate dehydrogenase in a total volume of 0.2 ml. Incubations were carried out in a 37°C water bath for 10 min. A preliminary experiment indicated that the formation of R(+)-VER and S(−)-VER metabolites was linear for at least 12 min. Reactions were stopped by the addition of 200 μl of an ice-cold, 100 ng/ml solution of benzphetamine (used as an internal standard for analysis) in acetonitrile. Samples were filtered through a 96-well protein precipitation plate after vortexing and then were directly used for high-performance liquid chromatography/tandem mass spectrometric (LC-MS/MS) analysis. Each set of incubations was carried out with 16 concentrations of substrate from 0.19 to 609 μM. In the kinetic studies as well as in the inhibition studies below, each P450 enzyme obtained from BD Gentest was used directly without adding any additional cytochrome c reductase. The ratios of the reductase to P450 in the incubation mixture were 0.5, 0.6, and 4.0 for CYP3A4, CYP3A5, and CYP3A7, respectively.

Metabolism Inhibition Experiments. Metabolic rates of VER by human CYP3A4, 3A5, and 3A7 were determined in the presence of midazolam, testosterone, and nifedipine as follows: cDNA-expressed CYP3A4 (0.05 and 0.2 mg/ml), CYP3A5 (0.11 and 0.44 mg/ml), and CYP3A7 (0.17 and 0.7 mg/ml), which were preincubated with different concentrations of midazolam
(0.2–20.6 μM), testosterone (1.7–35 μM), and nifedipine (0.7–36 μM) for 10 min at 37°C, were incubated with 5 μM R(+)-VER or S(−)-VER for an additional 10 min at 37°C in a total volume of 0.2 ml. Metabolic rates of VER by human CYP3A4 and CYP3A5 were also determined in the presence of monoclonal antibody, anti-CYP3A4. After cDNA-expressed CYP3A4 (10 or 40 μg) or CYP3A5 (22 or 88 μg) was preincubated with different amounts of anti-CYP3A4 (5–160 μg) for 30 min at 4°C, 5 μM R(+)-VER or S(−)-VER was added, and the reaction mixture was incubated for an additional 10 min at 37°C in a total volume of 0.2 ml. All reactions were stopped by the addition of 200 μl of ice-cold 100 ng/ml benzphetamine acetonitrile solution and prepared for LC-MS/MS analysis as described below.

**LC-MS/MS Analysis.** Quantitative analysis of metabolites of R(+)-VER or S(−)-VER was performed using a Shimadzu LC-10Advp (Shimadzu Corporation, Kyoto, Japan) high-performance liquid chromatography pump and a PerkinElmer Series 200 autosampler (PerkinElmer Life and Analytical Sciences, Boston, MA) coupled to a Sciex API 3000 mass spectrometer (PerkinElmerSciex Instruments, Boston, MA) operated in the positive ion mode. Separation of the metabolites was achieved on a BDS Hypersil C18 3.0 × 50 mm column (Thermo Hypersil, Keystone Scientific Operations, Bellefonte, PA) after gradient elution using acetonitrile containing 4 mM ammonium acetate (pH 7.4) and water containing 4 mM ammonium acetate (pH 7.4). The acetonitrile concentration was increased from 4 to 95% over 42 min and held at 95% for 3 min. The internal standard was benzphetamine. Metabolites and internal standard were detected by monitoring the appropriate transitions in a multiple reaction-monitoring mode under electrospray ionization conditions.

**Kinetic Analysis.** Triplicate values for the rate of metabolite formation for each substrate concentration were fit using GraFit 3 software (Erithacus Software, Horley, Surrey, UK) to equations describing a typical Michaelis-Menten hyperbolic curve for one-site binding (eq. 1) (Segel, 1975), substrate inhibition for one-site binding (eq. 2) (Houston and Kenworthy, 2000), biphasic substrate inhibition model for two-site binding (eq. 3), or simple two-site binding without binding ligands interaction (eq. 4, when c and d become infinite in eq. 3). The biphasic substrate inhibition model for two-site binding is shown in Fig. 2. In the model, the two binding sites are assumed to be independent until both binding sites are occupied by the substrate.

\[ v = \frac{\nu_{\text{max}}[S]}{K_s + [S]} \]  
\[ v = \frac{\nu_{\text{max}}[S]}{K_s + [S]/K_i} \]  
\[ v = \frac{\nu_{\text{max}}[S]}{K_{s1} + [S]/[S]/K_{i1}} \]  
\[ v = \frac{\nu_{\text{max}}[S]}{K_{s1} + [S]/K_{s2} + [S]/[S]/K_{i2} + [S]/[S]/K_{i3} + [S]/[S]/K_{i4}} \ ]

In addition, apparent \( K_m \) and \( V_{\text{max}} \) values were also calculated from the metabolic data with CYP3A4 and CYP3A5 according to the Michaelis-Menten equation (eq. 1).

**Results**

**Metabolism Kinetics.** The formation rates and Eadie-Hofstee plots of nor-VER, N-dealkylated metabolite D617, and four O-demethylated metabolites by CYP3A4 are shown in Fig. 3. As characterized by Eadie-Hofstee plots, the formation rates of all six metabolites from R(+)- and S(−)-VER exhibited biphasic kinetic profiles. This was evidenced by the fact that declining phases of Eadie-Hofstee plots for these metabolites were not linear but curved as the V/S values increased. In addition, formation rates of nor-VER and D617 reached maximum levels and then declined thereafter, indicating substrate inhibition kinetics for these metabolites. In some of the Eadie-Hofstee plots, it looks like a “hook” is starting to form at high V/S values, suggesting that autoinduction may have occurred at these concentrations. However, there were only two data points involved in the hook, and these data points are at very low S values. We considered that these phenomena may have been more due to an experimental error than an autoinduction hook in the kinetic analysis. A simple substrate inhibition model for one binding site (eq. 2) could not fit the data of nor-VER and D617. Therefore, the data were analyzed according to a biphasic substrate inhibition model shown in Fig. 2 using eq. 3 (Table 1). The a and b values of two enantiomers are all smaller than 1, which suggests the substrate binding in one site leads to a lower product formation rate from the other binding site. The c and d values are all greater than 40 for both enantiomers, which suggests that the substrate binding in one site leads to lower affinity binding to the other site. In the initial fitting, the b value of nor-VER formed from S-VER was less than 0.01, and thus, the b value was set to zero in the final fitting to minimize the number of parameters to be estimated. The c value of D617 formed from S-VER was almost infinite (greater than 4,000,000), and the terms containing c became approximately zero. The formation rates for M1, M2, M3, and M4 were analyzed by both a biphasic substrate inhibition model (eq. 3) and a simple two-site binding model using eq. 4 (Table 1). Both models fit the data reasonably well, and the more complex biphasic substrate inhibition model did not appear to be necessary for the data. Thus, the simple two-site binding model was chosen for final analysis (Table 1).

With CYP3A5 (Fig. 4), formation of nor-VER from R(+)- and S(−)-VER also exhibited the substrate inhibition kinetic profiles as observed with CYP3A4. As in the case of CYP3A4, formation rates of nor-VER were analyzed according to a biphasic substrate inhibition model, since the monophasic substrate inhibition model could not fit...
The data reasonably. The $a$ and $b$ values of two enantiomers are smaller than 1, and the $c$ and $d$ values are greater than 190 for both enantiomers. These results again suggest that the substrate binding in one site leads to a smaller nor-VER formation rate and to lower substrate binding affinity in the other site. The $b$ value of nor-VER formed from $S$-VER was less than 0.01 in the initial fitting, and the value was set to zero in the final fitting. In contrast to CYP3A4, formation of the D617 metabolite showed a simple biphasic kinetic profile without substrate inhibition over the substrate concentration range tested in the study. Formation of all O-demethylated metabolites (M1, M2, M3, and M4) from both enantiomers showed biphasic kinetic profiles as observed with CYP3A4 (Fig. 4). The formation rates for D617, M1, M2, M3, and M4 were analyzed by both a biphasic substrate inhibition model (eq. 3) and a simple two-site binding model using eq. 4. Based on the initial analysis, the simple two-site binding model (eq. 4) was chosen for final fit of the data (Table 2).

Formation of nor-VER from $R$-($\pm$)- or $S$-($\pm$)-VER with CYP3A7 (Fig. 5) also exhibited a substrate inhibition kinetic profile as observed with CYP3A4 and CYP3A5. However, unlike CYP3A4 or CYP3A5,
the monophasic substrate inhibition model (eq. 2) showed reasonably good fit. Furthermore, formation of D617 and O-demethylated products, M1, M2, M3, and M4 from both VER enantiomers showed typical Michaelis-Menten kinetics (Fig. 5; Table 3).

Metabolism and Enantioselectivity. The metabolic rates of VER to nor-VER and D617 were much greater than those to any O-demethylated metabolites for both VER enantiomers, regardless of CYP3A isoforms. These results indicate that N-dealkylated products were the major metabolites of both R- and S-VER by all three human CYP3A enzymes (Figs. 3–5). However, CYP3A4 was much more active than CYP3A5 and CYP3A7, which is consistent with previous findings (Williams et al., 2002). The apparent $K_{m}$ and $V_{max}$ values for each metabolite formation were determined (data not shown), and enantioselectivity in the VER metabolism was examined based on the apparent $V_{max}$. With CYP3A4, S-(-)-VER was more extensively metabolized to nor-VER, M1, and M2 metabolites than R-(+)-VER,
whereas R- (H11001)-VER was more extensively metabolized to D617, M3, and M4 (Table 4). With CYP3A5, R- (H11001)-VER was more extensively metabolized to nor-VER, D617, and M2 metabolites than S- (H11002)-VER, whereas S- (H11002)-VER was more extensively metabolized to M1, M3, and M4. With CYP3A7, S- (H11002)-VER was more extensively metabolized to nor-VER, D617, M1, and M2 metabolites than R- (H11001)-VER, whereas R- (H11001)-VER was more extensively metabolized to M3 and M4.

Metabolism Inhibition. Figure 6 shows formation rates of metabolites after R- (H11001)- or S- (H11002)-VER (5 M) was incubated with CYP3A4 (0.2 mg/ml) in the presence of midazolam, testosterone, nifedipine, or anti-CYP3A4 antibody. Midazolam inhibited the formation of nor-VER, D617, M2, M3, and M4 in a concentration-dependent manner while it activated formation of M1 for both VER enantiomers. The formation rates of nor-VER, D617, M2, M3, and M4 decreased to 10 to 50% of the uninhibited rate with midazolam at 20.6 M, and the degree of inhibition was substantially different depending on the metabolite. The increase in the formation rate of M1 was highest (about 1.5-fold) at 5 M midazolam for both VER enantiomers. In contrast to midazolam, testosterone activated formation of the M2 metabolite and inhibited the formation of all the other

TABLE 4

<table>
<thead>
<tr>
<th>CYP</th>
<th>Metabolite</th>
<th>NV</th>
<th>D617</th>
<th>M1</th>
<th>M2</th>
<th>M3</th>
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R and S represent more extensively metabolized VER enantiomer.

whereas R- (H11001)-VER was more extensively metabolized to D617, M3, and M4 (Table 4). With CYP3A5, R- (H11001)-VER was more extensively metabolized to nor-VER, D617, and M2 metabolites than S- (H11002)-VER, whereas S- (H11002)-VER was more extensively metabolized to M1, M3, and M4. With CYP3A7, S- (H11002)-VER was more extensively metabolized to nor-VER, D617, M1, and M2 metabolites than R- (H11001)-VER, whereas R- (H11001)-VER was more extensively metabolized to M3 and M4.

Metabolism Inhibition. Figure 6 shows formation rates of metabolites after R- (H11001)- or S- (H11002)-VER (5 M) was incubated with CYP3A4 (0.2 mg/ml) in the presence of midazolam, testosterone, nifedipine, or anti-CYP3A4 antibody. Midazolam inhibited the formation of nor-VER, D617, M2, M3, and M4 in a concentration-dependent manner while it activated formation of M1 for both VER enantiomers. The formation rates of nor-VER, D617, M2, M3, and M4 decreased to 10 to 50% of the uninhibited rate with midazolam at 20.6 M, and the degree of inhibition was substantially different depending on the metabolite. The increase in the formation rate of M1 was highest (about 1.5-fold) at 5 M midazolam for both VER enantiomers. In contrast to midazolam, testosterone activated formation of the M2 metabolite and inhibited the formation of all the other

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metabolites for R(+)-VER. The greatest increase (1.3-fold) in the formation rate of M2 was observed at 13 μM testosterone. The formation rates of nor-VER, D617, M1, M3, and M4 metabolites decreased to 35 to 70% of the uninhibited rate at 35 μM testosterone, and the degrees of inhibition varied among the metabolites. Unlike the R-enantiomer, testosterone inhibited the formation of all metabolites from S(-)-VER and no activation was observed. The formation rates of each metabolite decreased to 40 to 80% of the uninhibited rate at 35 μM testosterone, and the degree of inhibition was also different for each metabolite. Nifedipine selectively inhibited formation of nor-VER, D617, M1, M2, M3, and M4 for both enantiomers. However, unlike midazolam or testosterone, lower concentrations of nifedipine activated M1 formation, whereas higher concentrations inhibited M1 formation for both VER enantiomers. The greatest increase (1.4-fold for R(+)-enantiomer, 1.2-fold for S(-)-enantiomer) in the formation of M1 was observed at 7 μM nifedipine. The formation of nor-VER, D617, M1, M3, and M4 decreased to 20 to 50% of the uninhibited rate at 36 μM nifedipine. When R(+)-VER or S(-)-VER was incubated with CYP3A4 in the presence of anti-CYP3A4 monoclonal antibody, the formation of all metabolites was inhibited in a concentration-depen-
dent manner. However, the degree of inhibition was similar among all metabolites demonstrating product-independent inhibition of VER metabolism. When R(+)- and S(-)-VER were incubated with midazolam, testosterone, nifedipine, or anti-CYP3A4 antibody at a lower concentration of CYP3A4 (0.05 mg/ml), the same pattern of activation and inhibition was observed (data not shown).

When R(+)-VER or S(-)-VER (5 µM) was incubated with CYP3A5 (0.44 mg/ml) in the presence of various concentrations of midazolam, midazolam selectively inhibited nor-VER, D617, M2, M3, and M4 formation while activating M1 formation for both VER enantiomers (Fig. 7). The greatest increase (1.4-fold) in the formation of M1 was observed at 10 µM midazolam for both VER enantiomers. The formation rates of nor-VER, D617, M2, M3, and M4 decreased to 10 to 50% of the uninhibited rate at 20.6 µM midazolam. Testosterone selectively inhibited formation of nor-VER, D617, M2, M3, and M4 by CYP3A5 while activating M1 formation for R(+)-VER (Fig. 7). The greatest increase (1.2-fold) in the formation of M1 was observed at 13 µM testosterone. The formation rates of nor-VER, D617, M2, M3, and M4 decreased to 60 to 80% of the uninhibited rate at 35 µM testosterone. Unlike the R-enantiomer, testosterone inhibited the formation of all metabolites of S(-)-VER by CYP3A5, and the formation rate of the metabolites decreased to 55 to 75% of the uninhibited rate at 35 µM testosterone. Nifedipine inhibited the formation of nor-VER, D617, M2, M3, and M4 by CYP3A5 while activating M1 formation at lower concentrations of CYP3A5.
nifedipine concentration and inhibiting (or having no effect on) M1 formation at higher nifedipine concentration for both VER enantiomers. The formation rates of nor-VER, D617, M2, M3, and M4 decreased to 20 to 50% of the uninhibited rate at 36 μM nifedipine depending on the metabolite. When R- or S-VER was incubated with CYP3A5 in the presence of CYP3A4 monoclonal antibody, the formation of all metabolites was inhibited in a concentration-dependent manner, and the inhibition was product-independent. When R- and S-VER were incubated with midazolam, testosterone, nifedipine, or anti-CYP3A4 antibody at a lower concentration of CYP3A5 (0.11 mg/ml), the same results were observed (data not shown).

The changes in metabolite formation after incubation of R- or S-VER (5 μM) were also examined with CYP3A7 (0.7 mg/ml) in the presence of midazolam, testosterone, or nifedipine. With increasing concentration of inhibitor, midazolam, testosterone, nifedipine, or CYP3A4 antibody at a lower concentration of CYP3A5 (0.11 mg/ml), the same results were observed with midazolam, testosterone, and nifedipine (data not shown).

Discussion

In the present study, metabolic characteristics of CYP3A isoforms were compared using VER as a probe substrate. The major metabolic pathways of VER by these enzymes were N-demethylation and N-dealkylation to produce nor-VER and D617, respectively, regardless of isoforms. However, CYP3A4 was much more active than CYP3A5 and CYP3A7. Furthermore, when enantioselective metabolism was compared, only two metabolites had the same direction in the selectivity between CYP3A4 and CYP3A5 (Table 4). In contrast, the pattern of CYP3A7 enantioselectivity was quite similar to that of CYP3A4, and five metabolites have the same direction in the selectivity between these two isoforms. When CYP3A5 and CYP3A7 were compared, only M1 metabolite had same direction of enantioselectivity. Interestingly, similarity in the direction of enantioselectivity coincides with the degree of homology of amino acid sequences at the SRS and also the degree of homology of total amino acid sequences of these enzymes. The homology of amino acids at the putative SRS of CYP3A4 (Szklarz and Halpert, 1997; Harlow and Halpert, 1998; Xue et al., 2003) compared with those of CYP3A7 is 88.2% (Table 5). This value is substantially higher than homology between CYP3A4 and CYP3A5 (78.9%) and also between CYP3A5 and CYP3A7.
TABLE 5

<p>| Amino acids (AA) reported to be involved in the active site structure, six putative substrate recognition sites (SRS) of CYP3A4, and their corresponding amino acids of CYP3A5 and CYP3A7 |
|-------|--------|---------------------------------|</p>
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<td>3A7</td>
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</tbody>
</table>

Bolded letters show different amino acids between CYP3A4 and CYP3A5 and also between CYP3A4 and CYP3A7. Underlined letters show different amino acids between CYP3A5 and CYP3A7.

(76.3%) at the active site. The homology in total amino acid sequences between CYP3A4 and CYP3A7 (88.1%) is also higher than that between CYP3A4 and CYP3A5 (83.9%) and between CYP3A5 and CYP3A7 (81.7%) (Person et al., 1997).

In addition to differential enantioselectivity among the CYP3A isoforms, substantially different kinetic characteristics such as substrate inhibition and biphasic kinetics were also observed in the in vitro metabolism of VER (Figs. 3–5). More interestingly, in vitro metabolism of VER in the presence of effectors (midazolam, testosterone, and nifedipine) exhibited differential activation or inhibition, which was dependent upon enantiomers, metabolites, effectors, and CYP3A isoforms (Figs. 6–8). The CYP3A-mediated metabolism of midazolam has been reported to show atypical kinetics (Kronbach et al., 1989; Gorski et al., 1994; Perloff et al., 2000). For example, testosterone inhibited 1'-hydroxymidazolam formation and activated the formation of 4-hydroxymidazolam (Wang et al., 2000; Schrag and Wienkers, 2001). To explain the unusual kinetic phenomena, several kinetic models involving multiple substrate binding in CYP3A4 have been proposed. Korzekwa et al. (1998) suggested a two-site (or multiple-site) model in which the enzyme can bind two molecules of one substrate, one molecule each of the two substrates, or one molecule each of the substrate and effector. Many atypical CYP3A4 kinetics like activation, autoactivation, partial inhibition, and substrate inhibition can be described by this two-site model. Shou et al. (1999) also proposed a model that assumed two cooperative binding sites. Both models (Korzekwa and Shou models) yield an equation very similar to eq. 2 to describe substrate inhibition or activation. However, substrate inhibition data of VER obtained from CYP3A4 and CYP3A5 were not reasonably fit using this equation. Instead, the data were better fit to the newly generated equation (eq. 3) based on the proposed scheme (Fig. 2). Furthermore, a model for two distinct noninteracting binding sites could not explain VER kinetics in the presence of effectors. For example, with CYP3A4, midazolam activated M1 formation only and inhibited all other metabolites for both enantiomers, whereas testosterone activated formation of M2 metabolite only for R-VER and inhibited formation of other metabolites for both R- and S-enantiomers. This is a surprising finding because if there were only two binding sites and high-affinity binding sites of midazolam and testosterone are different, activation and inhibition by midazolam are expected to be completely opposite to those by testosterone. Therefore, these atypical kinetic data of VER may be better explained by introducing the concept of steric effect into the popular two-site model. It has been generally accepted that formation of different metabolites is due to different substrate orientation, and both sites (in the two binding-site model) are involved in the formation of each metabolite. The substrate in one site may or may not impose a steric effect on the substrate in the other site depending on the orientation of the two substrates or one substrate-one effector. Based on this proposed model, a kinetic scheme was generated as shown in Fig. 2. M1, M2, M3, and M4 formation in substrate-substrate interaction for CYP3A4, and D617, M1, M2, M3, and M4 formation for CYP3A5 displayed simple biphasic kinetics without proposed steric effects. Nor-VER and D617 formation for CYP3A4 and nor-VER for CYP3A5 displayed biphasic substrate inhibition with the proposed steric effects. The smaller-than-1 a and b values and greater-than-40 c and d values for nor-VER and D617 formation in CYP3A4 and nor-VER in CYP3A5 are consistent with this substrate inhibition effect. After one site was occupied by one substrate, the substrate binding in the other site displayed not only a smaller binding affinity but also a smaller product formation rate. In contrast, in the interaction study with midazolam, the M1 formation may have been promoted by the lack of steric hindrance from midazolam toward the VER orientation leading to M1 formation, whereas nor-VER, D617, M2, M3, and M4 formation may have been inhibited. This type of promotion can be illustrated by enhancement of probability of orientation leading to M1 formation while decreasing probability of orientations leading to other five metabolites’ formation. Similarly, in the VER-testosterone interaction, M2 formation from only R-VER may have been promoted by the lack of a steric effect from testosterone. In VER-nifedipine interaction, although nor-VER, D617, M2, M3, and M4 formation is inhibited, the M1 formation is promoted at low nifedipine concentration due to the lack of a steric effect from nifedipine. The decrease in the promotion effect of M1 formation at high nifedipine concentration may be explained by an increase in inhibition effect, when more nifedipine molecules occupied both binding sites. Therefore, using the two-site model with the concept of steric effect, the substrate inhibition and biphasic kinetics in substrate-substrate interaction and differential inhibition/activation in substrate-effector interaction could be adequately explained for CYP3A4 and CYP3A5.

For CYP3A7 substrate-substrate interaction, only nor-VER formation displayed monophasic substrate inhibition, whereas formation of the other five metabolites displayed typical Michaelis-Menten kinetics. This can also be explained using a two-site model with only one high-affinity binding site having access to the heme, whereas the other low-affinity binding site does not. When two VER molecules bind to the two separate sites, only one molecule can make a contribution to the metabolite formation, and the other molecule imposes a steric effect on the nor-VER formation and no effects on the orientations of other metabolites’ formation. This hypothesis is consistent with the findings that in the CYP3A7 substrate-inhibitor interaction studies, formation of all metabolites was inhibited by midazolam, testosterone, and nifedipine, for both VER enantiomers as the effector binding to the high-affinity site blocked the metabolism of VER. Although the model for two binding sites with the steric effects can explain VER kinetic data reasonably well, it should be also noted that there are other possibilities to explain the data. For example, in addition to two distinct, active sites, there may be a third binding site that is far from the heme but can exert allosteric effects to cause
atypical kinetics. Several authors have already suggested that CYP3A4 may be an allosteric protein, although the nature of the allosteric interaction is unclear (e.g., Lee et al., 1995). Another possibility is the interaction with or displacement of water molecules from the active site, which can cause a decrease in hydrogen bonding (Etkin et al., 1998). This may permit conformational changes, resulting in atypical kinetics depending on substrates or effectors.

Homology of total amino acid sequences and amino acids at the active sites is higher between CYP3A4 and CYP3A7 than between CYP3A4 and CYP3A5. However, the nature of atypical kinetics of VER was more similar between CYP3A4 and CYP3A5. Furthermore, metabolic rates with CYP3A7 were much lower compared with CYP3A4 regardless of metabolites. This is a surprising observation. However, based on these results, differences in specific amino acids (e.g., S116 of CYP3A4 which is different from N116 of CYP3A7 but the same as that of CYP3A5) at the SRS (see Table 5) appear to be more important for kinetics of VER metabolism than overall homology at the SRS or in total amino acid sequences.

In summary, in the in vitro metabolism of VER, N-dealkylated products, nor-VER and D617, were the major metabolites for all human CYP3A isozymes tested. Enantioselectivity in VER metabolism was more similar between CYP3A4 and CYP3A7 than between CYP3A4 and CYP3A5 or between CYP3A5 and CYP3A7. Formation of nor-VER and D617 with CYP3A4 and nor-VER with CYP3A5 displayed biphasic substrate inhibition for both VER enantiomers, and the remaining metabolites measured showed biphasic kinetics. With CYP3A7, monophasic substrate inhibition was observed for nor-VER, and typical Michaelis-Menten kinetics were evident for the other metabolites. When metabolic rates of VER were examined in the presence of effectors, there was activation, inhibition, or activation/inhibition depending upon the enantiomers, metabolites, effectors, and P450 isoforms. These phenomena could be adequately explained by introducing the concept of steric interaction into the popular two binding-site model.

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References


