DIFFERENTIAL MECHANISM-BASED INHIBITION OF CYP3A4 AND CYP3A5 BY VERAPAMIL

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ABSTRACT:

The genetic basis for polymorphic expression of CYP3A5 has been recently identified, but the significance of CYP3A5 expression is unclear. The purpose of this study is to quantify the capability of verapamil, a mechanism-based inhibitor of CYP3A, and its metabolites to inhibit the activities of CYP3A4 and CYP3A5, and to determine whether CYP3A5 expression in human liver microsomes alters the inhibitory potency of verapamil. Testosterone 6β-hydroxylation or midazolam 1′-hydroxylation was used to quantify CYP3A activity. The possibility that verapamil and its metabolites form metabolic-intermediate complex (MIC) with CYP3A was assessed using dual beam spectrophotometry. Verapamil and N-desalkylverapamil (D617) were found to have little inhibitory effect on cDNA-expressed CYP3A5 activity and did not form a MIC with cDNA-expressed CYP3A5 as indicated by the appearance of the characteristic peak at 455 nm. At 50 μM, norverapamil showed time-dependent inhibition of CYP3A5 (30%), but to a much lesser extent compared with that of CYP3A4 (80%). The estimated values of the inactivation parameters $k_{inact}$ and $K_i$ of norverapamil were 4.53 μM and 0.07 min$^{-1}$ for cDNA-expressed CYP3A5, and 10.3 μM and 0.30 min$^{-1}$ for cDNA-expressed CYP3A4. Human liver microsomes that expressed CYP3A5 were less inhibited by both verapamil and norverapamil. The inactivation efficiency of verapamil and norverapamil was 30 times and 45 times lower, respectively, for CYP3A5-expressing microsomes compared with CYP3A5-non-expressing microsomes. These findings indicate that the presence of variable CYP3A5/CYP3A4 expression in the liver may contribute to the interindividual variability associated with verapamil-mediated drug interactions.

CYP3A4 and CYP3A5 are the most abundant CYP3A enzymes in adult human liver and small intestine, and they share 83% identity in amino acid sequence (Aoyama et al., 1989). These two CYP3A enzymes have similar substrate specificity and are responsible for metabolism of more than 50% of administered drugs that are eliminated through the liver (Thummel and Wilkinson, 1998). CYP3A substrates show large interindividual variations in oral bioavailability and systemic clearance. These variations are thought to be contributed by interindividual differences in CYP3A activities and their expression.

In addition to inducers and inhibitors, genetic variation is considered to be one of the major factors that contribute to interindividual variability (Ozdemir et al., 2000). CYP3A5 has been found in approximately 10 to 30% of livers from white adults, and the genetic basis for CYP3A5 polymorphic expression has been identified (Wrighton et al., 1989; Kuehl et al., 2001). An A→G substitution within intron 3 of the CYP3A5 gene accounts for much of the variability in CYP3A5 content found in the human liver and intestine (Kuehl et al., 2001). Individuals who have at least one CYP3A5*1 allele, the wild-type allele, produce high levels of CYP3A5 protein in their livers and intestines, whereas those who are homozygous for the CYP3A5*3 allele, the variant allele, produce very low or no CYP3A5 protein. The significance of CYP3A5 phenotypes is unclear because CYP3A4 and CYP3A5 have similar substrate specificity and no CYP3A5-specific probe substrates or inhibitors are available. One study found that, for individuals who have at least one CYP3A5*1 allele, CYP3A5 represents at least 50% of the total hepatic CYP3A content, whereas others found that CYP3A5 represents only 15 to 30% of the total CYP3A protein (Wrighton et al., 1989; Lin et al., 2002; Westland-Johnsson et al., 2003). It has been suggested that individual differences in CYP3A5 may contribute to the interindividual variability in CYP3A activity and their susceptibility to drug interactions (Gibbs et al., 1999; Xie et al., 2004).

Verapamil is a calcium channel blocker widely used in the treatment of angina pectoris, coronary artery disease, cardiac arrhythmias, and hypertension (McTavish and Sorkin, 1989). O-Demethylation and N-dealkylation are two major metabolic pathways of verapamil. N-Dealkylated products, norverapamil and desalkylverapamil (D617), are the major metabolites of both R- and S-verapamil (Kroemer et al., 1992; Shen et al., 2004) formed by both CYP3A4 and CYP3A5. CYP3A4, however, was shown to be much more active than CYP3A5, and the two enzymes exhibited different enantiomeric selectivity in verapamil metabolism, based on their apparent $V_{max}$ (Shen et al., 2004). For example, with CYP3A4, S-verapamil was more extensively metabolized to norverapamil and O-demethylated metabolites, M1 [2-(3,4-dimethoxyphenyl)-8-4-hydroxy-3-methoxyphenyl)-6-methyl-2-isopropyl-6-azaocatinitrile] and M2 [2-(3,4-dimethoxyphenyl)-8-(3-hydroxy-4-methoxyphenyl)-6-methyl-2-isopropyl-6-azaocatinitrile], than R-verapamil. With CYP3A5, S-verapamil was more extensively metabolized to M1, M3 [2-(4-hydroxy-3-methoxyphenyl)-
8-(3,4-dimethoxyphenyl)-6-methyl-2-isopropyl-6-azaoctanitrile] and M4 [2-(3-hydroxy-4-methoxyphenyl)-8-(3,4-dimethoxyphenyl)-6-methyl-2-isopropyl-6-azaoctanitrile] than R-verapamil (Shen et al., 2004). The differences in verapamil metabolism by CYP3A4 and CYP3A5 likely reflect differences in the active site structure and conformation.

Verapamil is a CYP3A inhibitor (Backman et al., 1994), and one study using cDNA-expressed CYP3A4 and CYP3A5 showed that the relative mean IC_{50} value of verapamil for CYP3A5 was about 16-fold less than that for CYP3A4, indicating that CYP3A4 and CYP3A5 were differentially inhibited by verapamil (D. M. Stresser, S. D. Turner, T. Ho, and C. L. Crespi (2004) Gentest poster, http://wwwbdbiosciences.com/discovery_labware/gentest/products/pdf/S01T055R1.pdf, accessed on September 27, 2004.). We and others have previously shown that verapamil inhibited CYP3A4 activity through a metabolic-intermediate complex (MIC) formation (Ma et al., 2000; Wang et al., 2004), in which an amine functional group undergoes a series of oxidation steps to form a nitrosoalkane that complexes with reduced heme in the CYP3A4 molecule. The inhibition effect of the complex persists even after the inhibitor is eliminated from the body because the complex does not break down under physiological conditions. The objectives of this study were to quantify the mechanism-based inactivation of CYP3A4 and CYP3A5 by verapamil and its N-dealkylated metabolites and to determine whether expression of CYP3A5 in human liver microsomes alters the inactivation efficiency of verapamil.

**Materials and Methods**

**Chemicals.** R-Verapamil, S-verapamil, norverapamil, testosterone, 6βH4-dihydroxytestosterone, midazolam, and desmethyldiazepam were purchased from Sigma-Aldrich (St. Louis, MO). D617 was generously supplied by Abbott GmbH and Co. (Ludwigshafen, Germany). NADPH (98%) was purchased from Roche Diagnostics (Indianapolis, IN). 1’-Hydroxydiazepam was purchased from Ultrafine (Manchester, UK). All other reagents were of HPLC grade and were purchased from Fisher Scientific Co. (Pittsburgh, PA). The CYP3A5-specific polyclonal antibody was a generous gift from Dr. Stephen Leeder (Mercy Children’s Hospital, Kansas City, MO). The CYP3A4-specific antibody was purchased from BD Gentest (Woburn, MA).

**Human Liver Microsomes (HLMs) and cDNA-Expressed Human P450s.** The HLMs were prepared from five human liver tissues obtained at surgery in accordance with protocols approved by the Institutional Review Board of Indiana University-Purdue University at Indianapolis (Gorski et al., 1994). Microsomal fractions were prepared and pellets were suspended in a buffer to a protein concentration of 20 mg/ml and were kept at −80 °C (Gorski et al., 1994). Cytochrome P450 was quantified by the method of Omura and Sato (1964). Protein concentration of the HLMs was assayed using the Lowry method (Gorski et al., 1994). The CYP3A5 genotype of the human livers was assessed by real-time reverse transcriptase-polymerase chain reaction as described previously (Le Corre et al., 2004). Preparations (Supersomes) containing cDNA-expressed CYP3A4 (Cat. 465207) or CYP3A5 (Cat. 465235) without supplemented cytochrome b_{5} were purchased from BD Gentest. The P450 reductase activity (610 and 620 nmol/min/pmol P450 for CYP3A4 and CYP3A5, respectively), microsomal protein concentrations, and P450 content were provided by the manufacturer. Where indicated, cDNA-expressed CYP3A4 and CYP3A5 were supplemented with cytochrome b_{5} (Invitrogen, Carlsbad, CA) at a molar ratio of 3:1 (cytochrome b_{5}/CYP3A; McConnaughay et al., 2004).

**Quantitation of P450 CYP3A Inactivation.** Testosterone 6β-hydroxylase and midazolam 1’-hydroxylation were used as a marker to quantify CYP3A activity. HLMs (0.8 mg/ml) or cDNA-expressed human CYP3A5s (40 pmol) were preincubated with various concentrations of verapamil enantiomers or their metabolites, respectively, in the presence of NADPH at 37°C for various times. Concentrations of verapamil and their metabolites were chosen based on the maximal rate of inactivation determined previously (Wang et al., 2004). Fifty microliters of preincubation mixture was transferred into a tube containing 250 μM testosterone or 20 μM midazolam and 1 mM NADPH in 0.1 M sodium phosphate buffer (950 μl). A saturating concentration of testosterone or midazolam was used to measure the remaining catalytically active CYP3A. Testosterone 6β-hydroxylation was determined at 37°C for 10 min and terminated by adding 1 ml of cold acetonitrile. Midazolam 1’-hydroxylation was determined at 37°C for 4 min and terminated by adding 1 ml of sodium hydroxide-glycine (pH 11.3).

**HPLC Assay of 6β-Hydroxytestosterone and 1’-Hydroxymidazolam.** 6β-Hydroxytestosterone concentration was determined as previously described (Zhou et al., 2002). The HPLC system used a 5-μm C18 column (Luna; Phenomenex, Torrance, CA) and a mobile phase of methanol/20 mM sodium phosphate buffer (60:40, v/v), containing 0.1% (v/v) triethylamine adjusted to pH 6.3 with orthophosphoric acid, pumped at a flow rate of 1 ml/min, and ultraviolet detection at a wavelength of 254 nm. Standard curves ranged from 25 ng/ml (limit of quantification) to 4000 ng/ml. Quality control samples (n = 2; concentrations of 150 and 1500 ng/ml) were run with each set of unknowns. Standard curves were deemed acceptable if the quality control concentrations estimated from the standard curve were within 10% of their prepared concentrations. If the estimates were >10%, then the standard curve and unknown samples were discarded and repeated.

For 1’-hydroxymidazolam, the reaction mixture was extracted with 5 ml of cyclohexane/methylene chloride (70:30). The mobile phase consisted of a mixture of 40:20:40 v/v of acetonitrile/methanol/ammonium acetate (pH 7.4) when verapamil was used as an inactivator. When norverapamil was used, the mobile phase composition was 35:20:45 v/v to avoid interference. Detection was by UV absorbance at a wavelength of 230 nm.

**Formation of P450-Iron(II)-Metabolic Intermediate Complex.** The cDNA-expressed CYP3A4 or CYP3A5 was used to characterize MIC formation associated with the metabolism of verapamil or norverapamil. MIC was measured as a function of time in samples containing R-verapamil, S-verapamil, or norverapamil at 10 μM in a microsomal buffer (100 mM sodium phosphate buffer, 5 mM magnesium chloride, pH 7.4). Spectral differences between the reference and sample cuvettes were obtained with dual-beam spectroscopy (Uvikon 933 double-beam UV/visible spectrophotometer; Research Instruments International, San Diego, CA) by scanning from 400 to 500 nm and monitoring every 5 min for up to 60 min as previously described (Jones et al., 1999). In each case, the sample cuvette contained 200 pmol of CYP3A4 or 400 pmol of CYP3A5, inactivator, and 1 mM NADPH. The contents of the reference cuvette were the same as the sample cuvette except that no inactivator was added. All MIC formation experiments were maintained at 37°C and initiated by the addition of NADPH in a final incubation volume of 1 ml. The extent of MIC formation was quantified based on the previously reported extinction coefficient (455–490 nm) value of 65 M^{-1}cm^{-1} (Pershing and Franklin, 1982).

**Western Blotting Analysis.** Proteins in human liver microsomes (100 μg) were resolved on a 7.5% Tris-HCl polyacrylamide gel (Ready Gel; Bio-Rad, Hercules, CA), then transferred to a polyvinylidene difluoride membrane (Amersham Biosciences UK Ltd., Little Chalfont, Buckinghamshire, UK), and blotted with a CYP3A5- or CYP3A4-specific antibody. The cDNA-expressed CYP3A4 and CYP3A5 were used as controls. The immunoreactive bands were detected with an enhanced chemiluminescence kit (Amersham Biosciences UK Ltd.). Optical densities of the bands on the film were converted to quantitative numbers by using the Kodak electrophoresis documentation and analysis system 290 (EDAS 290) and Kodak 1D image analysis software (Eastman Kodak, Rochester, NY). For each liver sample, the amount of CYP3A4 and CYP3A5 per milligram of microsomal protein was estimated by comparing the band densities with the standard curve of the cDNA-expressed CYP3A4 and CYP3A5 within the linear range. The standards were run on the same gel as the liver samples.

**Estimation of Inactivation Constants.** For incubations with HLMs or the cDNA-expressed CYP3A5, the natural log of the percentage of the remaining activity was plotted against the preincubation time. The observed inactivation rate constants (k_{inact}) were determined from the slopes of the initial linear decline in activity (Silverman, 1995). The parameters k_{inact} and K_{I} were obtained from plotting k_{obs} against the inhibitor concentration using nonlinear regression (WinNonlin 4.0; Pharsight, Mountain View, CA) according to the following equation (Mayhew et al., 2000): k_{obs} = k_{inact} × [I] / (K_{I} + [I]), where k_{inact} is the rate constant that defines the maximal rate of inactive enzyme formation, f is the initial concentration of the inhibitor, and K_{I} is the inhibitor
concentration when \( k_{\text{obs}} = k_{\text{max}}/2 \). This approach assumes that there is negligible change of \( I \) in the incubation period and that loss of enzyme is solely due to inactivation by the inhibitor.

**Results**

**Inactivation of CYP3A4 and CYP3A5 by Verapamil Enantiomers and Their Metabolites.** To quantify the capability of verapamil enantiomers to irreversibly inhibit CYP3A5, 30 \( \mu \text{M} \) \( S \)- or \( R \)-verapamil was preincubated with the cDNA-expressed human CYP3A5 or CYP3A4, respectively. After preincubations (up to 10 min), the reaction mixture was diluted 20-fold to minimize reversible inhibition. The CYP3A5 or CYP3A4 activity remaining after preincubation was assessed from the rate of testosterone 6-\( \beta \)-hydroxylation. In the absence of substrate, the activity of HLMs, CYP3A4, and CYP3A5 declined by 19, 26, and 5\%, respectively, over 10 min under the same conditions. However, in the presence of 400 \( \mu \text{M} \) alprazolam, a low turnover substrate that does not form a MIC, approximately 3.5\% of CYP3A4 activity was lost under these conditions in 10 min (Mayhew et al., 2000). We have therefore assumed that negligible loss of CYP3A activity occurs in our incubations in the presence of substrate/inhibitor. The extent of CYP3A4 inhibition by \( S \)-verapamil increased as preincubation times increased, but \( R \)-verapamil inhibited CYP3A4 to a much lesser extent. CYP3A5 exhibited little time-dependent inhibition (Fig. 1A). Additional experiments were performed to assess the time-dependent inhibition of CYP3A4 and CYP3A5 after supplementation with cytochrome \( b_5 \) (cyt \( b_5 \)). In the absence of inhibitor, addition of cyt \( b_5 \) to the microsomal suspension increased the rate of testosterone 6\( \beta \)-hydroxylation of CYP3A4 by 355\% and CYP3A5 by 173\%. Preincubation experiments with \( S \)-verapamil (30 \( \mu \text{M} \)) and CYP3A4 or CYP3A5, with and without cyt \( b_5 \), showed that enzyme supplemented with cyt \( b_5 \) resulted in a greater inactivation compared with the enzyme that did not contain cyt \( b_5 \) based on the slopes of the initial linear decline in activity (\( k_{\text{obs}} \)). The \( k_{\text{obs}} \) of \( S \)-verapamil with CYP3A4 was 0.098 min\(^{-1}\) compared with 0.143 min\(^{-1}\) for CYP3A4 plus cyt \( b_5 \) (Fig. 1A). Similarly, the \( k_{\text{obs}} \) of \( S \)-verapamil with CYP3A5 was 0.022 min\(^{-1}\) compared with 0.046 min\(^{-1}\) with CYP3A5 plus cyt \( b_5 \) (Fig. 1A).

Norverapamil and D617, two major metabolites of verapamil, have previously been shown to inactivate CYP3A4 in vitro, and based on the ratio of \( k_{\text{max}} \) over \( K_I \), the inactivation efficiency of norverapamil is comparable with that of \( S \)-verapamil (Wang et al., 2004). To examine the capability of norverapamil and D617 to irreversibly inhibit CYP3A5, 50 \( \mu \text{M} \) norverapamil or D617 was preincubated with the cDNA-expressed CYP3A5 or CYP3A4. At 50 \( \mu \text{M} \), norverapamil inhibited CYP3A4 activity in a time-dependent manner (Fig. 1B). D617 was a modest inactivator of CYP3A4 and had little, if any, effect on CYP3A5 (Fig. 1B). Norverapamil was a strong inactivator of CYP3A4 but inhibited CYP3A5 activity to a much lesser extent (Fig. 1B). Preincubation experiments with norverapamil (50 \( \mu \text{M} \)) and CYP3A4 or CYP3A5, with and without cyt \( b_5 \), illustrated that enzyme supplemented with cyt \( b_5 \) resulted in a greater inactivation compared with the enzyme that did not contain cyt \( b_5 \) based on the slopes (\( k_{\text{obs}} \)) of the initial linear decline in activity (Fig. 1B). The \( k_{\text{obs}} \) of norverapamil with CYP3A4 was 0.146 min\(^{-1}\) compared with 0.167 min\(^{-1}\) for CYP3A4 plus cyt \( b_5 \) (Fig. 1B). In addition, the \( k_{\text{obs}} \) of norverapamil with CYP3A5 was 0.076 min\(^{-1}\) compared with 0.109 min\(^{-1}\) for CYP3A5 plus cyt \( b_5 \) (Fig. 1B). Figure 1C shows the testosterone 6\( \beta \)-hydrolyase activities of CYP3A5 at zero preincubation time under different concentrations of norverapamil. The comparable CYP3A5 activities suggest that there was no apparent competitive inhibition by norverapamil. The estimated \( K_I \) and \( k_{\text{max}} \) values of norverapamil are 4.53 \( \mu \text{M} \) and 0.07 min\(^{-1}\) for the cDNA-expressed CYP3A5 (Fig. 2, A and C), and 10.3 \( \mu \text{M} \) and 0.30 min\(^{-1}\) for the cDNA-expressed CYP3A4 (Fig. 2, B and D). The inactivation efficiency of norverapamil for the cDNA-expressed CYP3A5 and CYP3A4 was 15.45 min\(^{-1}\) \( \cdot \text{NM}^{-1} \) and 29.13 min\(^{-1}\) \( \cdot \text{NM}^{-1} \), respectively.

In previous studies, \( R \)- and \( S \)-verapamil and their metabolites formed a MIC with CYP3A4 when coexpressed with cytochrome \( b_5 \) (Wang et al., 2004). To quantify the capability of \( R \)- and \( S \)-verapamil to form a MIC with CYP3A4 and CYP3A5 without cytochrome \( b_5 \) supplementation, MIC formation was monitored spectrophotometrically at wavelengths between 400 and 500 nm. Figure 3 shows time-dependent MIC formation by CYP3A4 incubated with \( R \)-verapamil, \( S \)-verapamil, and norverapamil, respectively, with peak absorbance differences at a wavelength of 455 nm. The maximal MIC formation at 60 min was 35\% of the total CYP3A4 in the sample for \( R \)-verapamil, 52\% for \( S \)-verapamil, and 68\% for norverapamil. However, no characteristic peak was observed when verapamil enantiomers and norverapamil were incubated with CYP3A5 for 60 min (Fig. 3).

**Impact of CYP3A5 Expression on Verapamil Inhibitory Effect in HLMs.** In view of the selective inactivation of CYP3A4 by verapamil (vide supra), we examined the hypothesis that HLMs from CYP3A5 expressers may be less susceptible to inhibition by verapamil than nonexpressors. CYP3A5 and CYP3A4 expression in five HLMs was immunoquantified with specific antibodies. CYP3A5 was detected in two of the five liver samples (Fig. 4). IUL-32 had higher CYP3A5 protein content (3.5 pmol/mg microsomal protein) compared with IUL35 (1.5 pmol/mg microsomal protein). CYP3A4 was detected in all five microsomal samples and varied between 28.5 and 48.2 pmol/mg microsomal protein (Fig. 4). CYP3A5 genotypes of the five livers are shown in Fig. 4.

The five individual human liver microsome preparations (100 \( \mu \text{g} \) of microsomal protein) were incubated with 30 \( \mu \text{M} \) \( S \)-verapamil, and the residual CYP3A activity was quantified from the rate of testosterone 6\( \beta \)-hydroxylation. All five liver microsomes exhibited time-dependent inhibition when incubated with 30 \( \mu \text{M} \) \( S \)-verapamil (Fig. 5A). After a 10-min preincubation with \( S \)-verapamil, approximately 37\% of the CYP3A activity remained for the three human liver microsomes that contain CYP3A4 and have no detectable CYP3A5 (Fig. 5A). In contrast, under the same conditions 57\% of CYP3A activity remained for IUL35 and 76\% remained for IUL32, which had the highest expression of CYP3A5 (Fig. 5A). The corresponding uninhibited control activity, as reflected in testosterone 6\( \beta \)-hydroxylation, is shown in Fig. 5B.

The capability of reversible inhibitors to modulate CYP3A activity can be substrate-dependent (Kenworthy et al., 1999). To examine whether the CYP3A5 dependent inhibition of CYP3A activity by verapamil was dependent on the probe substrate, we conducted further studies using midazolam 1'-hydroxylation rate as the index of catalytic activity. After a 20-min preincubation with \( S \)-verapamil, 40 to 60\% of the CYP3A activity remained for the three human liver microsomes that contain CYP3A4 and have no detectable CYP3A5 (Fig. 5C). In contrast, under the same conditions, 79\% of CYP3A activity remained for IUL35 and 87\% remained for IUL32 (Fig. 5C). The corresponding uninhibited control activity, as reflected in midazolam 1'-hydroxylation rate, is shown in Fig. 4D. These data suggest that human liver microsomes expressing CYP3A5 were less susceptible to verapamil inactivation using either testosterone or midazolam as CYP3A3 probes.

To examine the impact of CYP3A5 expression on CYP3A inhibition by norverapamil, the five human liver microsomes were preincubated with norverapamil and the remaining enzyme activity was quantified using midazolam as a probe substrate. All five microsomal...
FIG. 1. Differential mechanism-based inhibition of CYP3A by verapamil enantiomers, norverapamil, and D617 using cDNA-expressed CYP3A4 and CYP3A5 with and without cytochrome b₅. Testosterone 6β-hydroxylation was used as a marker of CYP3A activity. The inhibitor concentrations were 30 µM for R- and S-verapamil (A), and 50 µM for racemic norverapamil and D617 (B). For A and B, the points are means of duplicate experiments and the lines of best fit were determined by log-linear regression. C, CYP3A5 activities at zero preincubation time, when incubated with different concentrations of norverapamil, were assessed using the cDNA-expressed CYP3A5. The bars represent means of four experiments and the error bars are the standard deviations.
preparations displayed time-dependent inhibition when incubated with 50 μM norverapamil. After 20 min of preincubation with norverapamil, 6 to 12% of the CYP3A activity remained for microsomes that have no detectable CYP3A5, but 26% remained for IUL35 (*1/*3) and 44% remained for IUL32 (*1/*3) (Fig. 6). The inactivation parameters $k_{\text{inact}}$ and $K_I$ were estimated using IUL31 and IUL32 to quantify the impact of CYP3A5 expression in the human liver microsomes. The estimates obtained from IUL31 were close to those obtained from the pooled HLMs (Wang et al., 2004), which had comparable CYP3A4 expression and little CYP3A5 expression.

**FIG. 2.** Time-dependent inhibition of CYP3A4 and CYP3A5 by norverapamil. A and B, estimation of observed inactivation rate constant ($k_{\text{obs}}$) for CYP3A5 (A) and CYP3A4 (B) inactivation at different concentrations of norverapamil. C and D, determination of irreversible inhibition constants ($k_{\text{inact}}$ and $K_I$) for norverapamil, and CYP3A5 (C) and CYP3A4 (D). The $k_{\text{obs}}$ was plotted against different norverapamil concentrations. The curves represent the line of best fit.

**FIG. 3.** Metabolic-intermediate complex formation by R-verapamil, S-verapamil, and norverapamil at 10 μM using the cDNA-expressed CYP3A4 and CYP3A5 (without cyt b5 supplement).
(CYP3A5 cannot be detected by Western blot; data not shown). We assumed the estimates from IUL33 and 34 would be similar to that from IUL31 and were not tested. For IUL35, the extent of enzyme inactivation by S-verapamil was small and variable, and no clear concentration-dependent inactivation was observed. The inactivation rate constants \( k_{\text{inact}} \) and \( K_I \) could not be estimated from the enzyme inhibition data. If we assume the \( k_{\text{inact}} \) equals the \( k_{\text{obs}} \) estimated from the highest concentration of S-verapamil used, \( k_{\text{inact}} \) for IUL35 would be 0.06 min\(^{-1}\). The inactivation efficiency of verapamil and norverapamil estimated using IUL32 was substantially lower than that estimated using IUL31 (Table 1). These findings suggest that microsomes containing CYP3A5 are less susceptible to verapamil and norverapamil inhibition compared with microsomes that lack CYP3A5.

**Discussion**

The present study demonstrated that verapamil differentially inhibited CYP3A4 and CYP3A5 via mechanism-based inhibition. Both S- and R-verapamil inhibited CYP3A4 activity but had little effect on CYP3A5 activity in the absence of cytochrome \( b_5 \). Supplementation of cDNA-expressed enzymes with cytochrome \( b_5 \) modestly increased the maximal rate of inactivation of CYP3A4 and CYP3A5 by S-verapamil. However, the moderate reduction in the CYP3A4/CYP3A5 selectivity of inactivation from 4.5 to 3.1 indicates that the relative resistance of CYP3A5 to inactivation remains in the presence of cytochrome \( b_5 \). In contrast to CYP3A4, CYP3A5 did not exhibit a characteristic peak absorbance difference at 455 nm when incubated with verapamil, suggesting that CYP3A5 did not form a MIC with verapamil. The reasons for the different effects of verapamil on

![Western blot analysis of CYP3A5 and CYP3A4 protein expression in human liver microsomes using CYP3A5- and CYP3A4-specific antibodies.](image1)

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**Fig. 4.** Western blot analysis of CYP3A5 and CYP3A4 protein expression in human liver microsomes using CYP3A5- and CYP3A4-specific antibodies. cDNA-expressed (rcb) CYP3A4 and CYP3A5 were used as positive and negative controls. For CYP3A5 analysis, 100 \( \mu g \) of microsomal protein was loaded on the SDS-polyacrylamide gel. For CYP3A4 analysis, 20 \( \mu g \) of protein was loaded. The values for the imunoquantifications are shown in the table below the blots. The corresponding CYP3A5 genotype of each liver sample is shown above the blots.

**Fig. 5.** Impact of CYP3A5 expression in human liver microsomes on CYP3A inhibition by S-verapamil. Norverapamil (50 \( \mu M \)) was preincubated with the five human liver microsomes using midazolam as a substrate. All values are means of duplicate experiments.

![Impact of CYP3A5 expression in human liver microsomes on CYP3A inhibition by S-verapamil.](image2)

**Fig. 6.** Impact of CYP3A5 expression in human liver microsomes on CYP3A inhibition by norverapamil. Norverapamil (50 \( \mu M \)) was preincubated with the five human liver microsomes using midazolam as a substrate. All values are means of duplicate experiments.
CYP3A4 and CYP3A5 are unclear. CYP3A4 and CYP3A5 differ in 78 of 503 amino acids, 17 of which fall within the six putative substrate recognition site domains (Aoyama et al., 1989; Wang et al., 1998) shown to be responsible for the substrate specificity of CYP3A4 with a wide range of compounds (Domanski and Halpert, 2001). Several of these residues are crucial for conversion of regioselectivity of CYP3A4-catalyzed aflatoxin B1 oxidation to that of CYP3A5 (Wang et al., 1998). CYP3A4 and CYP3A5 have been shown to exhibit different enantioselectivity, and product-dependent activation and inhibition in the metabolism of verapamil (Shen et al., 2004). It is possible that the structure differences in the substrate or metabolic intermediate-binding pocket between CYP3A4 and CYP3A5 may determine the conformational states of the heme environment and the accessibility of the nitroso group in the metabolic intermediate to the heme. Norverapamil, one of the major metabolites of verapamil, displayed a time-dependent inhibitory effect on CYP3A5, but to a much smaller extent than CYP3A4. As with S-verapamil, cytochrome b5 supplementation modestly increased the maximal rate of inactivation of CYP3A4 and CYP3A5 by norverapamil but had little effect on the CYP3A4/CYP3A5 selectivity of inactivation (1.9 versus 1.5). The mechanism underlying the inhibition of CYP3A5 by norverapamil remains unclear because, as observed with R- and S-verapamil, norverapamil did not exhibit a distinct peak absorbance difference at 455 nm when incubated with CYP3A5, and the absorbance difference did not increase with preincubation times. The effect of CYP3A5 expression on total microsomal CYP3A-catalyzed metabolism was quantified in five human liver microsomes. The capability of verapamil and norverapamil to inhibit total CYP3A4 activity was reduced when CYP3A5 was present in the microsomes, regardless of whether midazolam or testosterone was used as a probe. The inactivation efficiency of verapamil was more than 30 times lower for the CYP3A5-expressing microsomes compared with the microsomes only expressing CYP3A4, and the inactivation efficiency of norverapamil was 45 times lower (Table 1). The catalytic activity of CYP3A4 is significantly affected by the presence of cytochrome b5 (Yamazaki et al., 1999). Moreover, the extent of enhancement of catalytic activity by cytochrome b5 may depend on whether cytochrome b5 is coexpressed with CYP3A5 and P450 reductase or is added to the incubation mixture with the CYP3A enzyme prior to measurements (Wang et al., 1998). We found that CYP3A5 had 1.6 to 2 times lower catalytic activity toward testosterone 6β-hydroxylation compared with CYP3A4 (data not shown), but that cytochrome b5 stimulated CYP3A5 by 173% and CYP3A4 by 355%. Several studies have reported that CYP3A4 and CYP3A5 exhibit comparable catalytic activity toward midazolam 1′-hydroxylation (Gibbs et al., 1999; Williams AJ et al., 2002). Consistent with these findings, the differences in the initial CYP3A activity between the CYP3A5-expressing human liver microsomes and the microsomes that only express CYP3A4 is greater when using testosterone 6β-hydroxylation as a substrate compared with using midazolam 1′-hydroxylation as a substrate.

The debate on the significance of CYP3A5 expression to drug metabolism is that the abundance of CYP3A5 in human liver microsomes is low relative to CYP3A4. One group found that CYP3A5 protein expression levels in individuals who express CYP3A5 accounted for over 50% of total hepatic CYP3A protein (Kuehl et al., 2001; Lin et al., 2002), whereas others found that CYP3A5 represented 15 to 30% of the total CYP3A protein (Wrighton et al., 1990; Westlind-Johnsson et al., 2003). In this study, CYP3A5 contributed 5 to 10% of total immunorequantified CYP3A. The discrepancy among these studies may be due to random selection or may be due to the differences in the protein standards and the CYP3A5-specific antibodies used. Despite of the low percentage of CYP3A5 present in the microsomes, the results from this study suggest that individuals who express CYP3A5, in addition to CYP3A4, may be less susceptible to inhibition by verapamil (Figs. 5 and 6).

Our studies show that the expression of CYP3A5 in human liver microsomes can result in estimations of potency of inhibitors that are sample-selective. This selectivity in turn will contribute to significant variability in inhibition parameter estimates that may be used to make in vivo predictions of drug interaction potential. Therefore, in addition to pooled human liver microsomes, it is important to use individual microsome preparations with different levels of CYP3A5 expression to fully characterize an inhibitory interaction. One limitation of this

**TABLE 1**

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>HLMs</th>
<th>$k_{\text{max}}$</th>
<th>$K_1$</th>
<th>$k_{\text{max}}/K_1$</th>
</tr>
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<tr>
<td>S-Verapamil</td>
<td>IUL31</td>
<td>0.13 ± 0.01</td>
<td>0.87 ± 0.16</td>
<td>145.67</td>
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<tr>
<td></td>
<td>IUL32</td>
<td>0.03 ± 0.00</td>
<td>8.02 ± 3.65</td>
<td>11.1</td>
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<tr>
<td>R,S-Norverapamil</td>
<td>IUL31</td>
<td>0.42 ± 0.02</td>
<td>4.58 ± 0.38</td>
<td>90.99</td>
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<tr>
<td></td>
<td>IUL32</td>
<td>0.05 ± 0.01</td>
<td>37.92 ± 22.66</td>
<td>1.42</td>
</tr>
</tbody>
</table>

* Values are ± standard errors of the parameters estimated.
study is that only a small number of microsomes have been studied; thus, it is difficult to predict the full range of parameter estimates that may be encountered. The study of a larger number of microsomes with a wide range of CYP3A5 expression is needed to evaluate whether the extent of verapamil inhibition is correlated with the abundance of CYP3A5 in liver microsomes.

The selective inhibition of CYP3A enzymes by verapamil has two potential clinical implications. First, verapamil is a moderately strong CYP3A inhibitor in vivo, and, therefore, CYP3A5 expressers may exhibit a relative resistance to drug interactions between verapamil and CYP3A substrates, such as midazolam, cyclosporine, and statins (Robson et al., 1988; Backman et al., 1994; Kantola et al., 1998). This phenomenon may contribute significantly to the considerable interindividual variation in CYP3A-based drug interactions. Second, verapamil itself is a CYP3A substrate, and upon repeated dosing, autoinhibition results in increases in oral bioavailability and decreased systemic clearance (Freedman et al., 1981; Eichelbaum and Somogyi, 1984). Thus, clinical responses, both desirable (e.g., blood pressure lowering and heart rate control) and undesirable (e.g., hypotension and heart block), may differ between CYP3A expressers and non-expressers.

In summary, our studies demonstrated that verapamil differentially inhibited CYP3A4 and CYP3A5. The differential effects of verapamil on CYP3A4 and CYP3A5 were independent of substrates used. Microsomes expressing CYP3A5 were less susceptible to inhibition by verapamil, suggesting that CYP3A5 expression may contribute to interindividual variability in the magnitude of drug interactions and response to verapamil in vivo.

References

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