IN VITRO METABOLISM OF MIDAZOLAM, TRIAZOLAM, NIFEDIPINE, AND 
TESTOSTERONE BY HUMAN LIVER MICROSONES AND RECOMBINANT 
CYTOCHROMES P450: ROLE OF CYP3A4 AND CYP3A5 

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(Received July 15, 2002; accepted March 24, 2003)

This article is available online at http://dmd.aspetjournals.org

ABSTRACT:
Midazolam, triazolam (TRZ), testosterone, and nifedipine have all been widely used as probes for in vitro metabolism of CYP3A. We used these four substrates to assess the contributions of CYP3A4 and CYP3A5 to in vitro biotransformation in human liver microsomes (HLMs) and in recombinant enzymes. Recombinant CYP3A4 and CYP3A5 (rCYP3A4 and rCYP3A5) both produced 1-OH and 4-OH metabolites from midazolam and triazolam, 6β-hydroxytestosterone from testosterone, and oxidized nifedipine from nifedipine. Overall, the metabolic activity of CYP3A5 was less than that of CYP3A4. Ketoconazole potently inhibited midazolam, triazolam, testosterone, and nifedipine metabolite formation in HLMs and in rCYP3A4. The inhibitory potency of ketoconazole in rCYP3A5 was about 5- to 19-fold less than rCYP3A4 for all four substrates. In testosterone interaction studies, testosterone inhibited 1-OH-TRZ formation, but significantly activated 4-OH-TRZ formation in HLMs and rCYP3A4 but not in rCYP3A5. Oxidized nifedipine formation was inhibited by testosterone in rCYP3A4. However, in rCYP3A5, testosterone slightly activated oxidized nifedipine formation at lower concentrations, followed by inhibition. Thus, CYP3A4 and CYP3A5 both contribute to midazolam, triazolam, testosterone, and nifedipine biotransformation in HLMs, with CYP3A5 being metabolically less active than CYP3A4 in general. Because the inhibitory potency of ketoconazole in rCYP3A5 is substantially less than in rCYP3A4 and HLMs, CYP3A5 is probably less important than CYP3A4 in drug-drug interactions involving ketoconazole and CYP3A substrates.

In humans, CYP3A represents one of the most important subfamilies of the P450 superfamily. The CYP3A subfamily is the most abundantly expressed P450 in human liver, and CYP3A is involved in the biotransformation of approximately 50% of drugs that are metabolized (Komori et al., 1990). As a result, drug-drug interactions associated with modulation of CYP3A-mediated metabolism can be of substantial clinical importance. CYP3A5 is present in significant quantities in 20 to 60% of human liver and is reported to account for at least 50% of total CYP3A in those individuals (Wrighton et al., 1989, 1990; Kuehl et al., 2001, Lin et al., 2002). When the expression of CYP3A5 was examined in different age groups, it was observed that CYP3A5 was detected in a significantly higher percentage of children and adolescents (19 years old or under) as compared with the remaining population (47% versus 24%, respectively) (Wrighton et al., 1990). CYP3A5 demonstrates 84% amino acid sequence homology to CYP3A4, and there is substantial overlap between substrate specificities of CYP3A4 and CYP3A5 (Wrighton and Stevens, 1992). Hepatic CYP3A5 content ranges from 2 to 202 pmol/mg of microsomal protein and shows large interindividual variations (Aoyama et al., 1989; Kuehl et al., 2001). The content of CYP3A4 in liver ranges from 47 to 523 pmol/mg of microsomal protein, and also shows large interindividual variations (Wandel et al., 1998; Hirota et al., 2001). Thus, CYP3A5 could contribute considerably to the total metabolic clearance of the many CYP3A substrates in people polymorphically expressing CYP3A5. A recent study by Williams et al. (2002) demonstrated an equal or reduced metabolic capability for CYP3A5 compared with CYP3A4.

Midazolam (MDZ) and triazolam (TRZ) hydroxylation, yielding their respective 1-OH and 4-OH metabolites, testosterone (TST) hydroxylation, yielding the 6β-hydroxy metabolite (6β-HST), and nifedipine, yielding the oxidized nifedipine metabolite, are considered to be relatively specific index reactions for human CYP3A-mediated metabolism, both clinically and in vitro (Guengerich et al., 1986; Kronbach et al., 1989; Gorski et al., 1994; von Moltke et al., 1996a,b; Perloff et al., 2000; Kenworthy et al., 2001; Venkatakrishnan et al., 2001). Kenworthy et al. (1999), using coefficients of determination and cluster analysis, classified CYP3A substrates into three groups. In our study, we used one or more substrates from each group.

CYP3A4 has been reported to be stimulated by cytochrome b5 (b5) (Yamazaki et al., 1996, 1999), with the effects of b5 dependent on the
specific substrate. The effects of addition of b5 differ between recombinant CYP3A4 and recombinant CYP3A5 (Lee et al., 1995; Hirota et al., 2001; Nakajima et al., 2002; Yamazaki et al., 2002). Hence, in our study we used recombinant CYP3A4 and recombinant CYP3A5, both without b5. These recombinant enzymes had comparable ratios of oxidoreductase to cytochrome P450.

Metabolism studies and molecular modeling suggest that the active site of CYP3A4 has the capacity to accommodate more than one substrate (Shou et al., 1994; Domanski et al., 2000). A recent study by Khan et al. (2002), using MDZ as a CYP3A substrate, suggests that the two MDZ metabolites result from the binding of MDZ at two separate sites and also proposes that the two binding sites may be partially overlapping.

We evaluated four established human CYP3A index substrates (MDZ, TRZ, TST, and nifedipine) in human liver microsomes (HLMs), heterologously expressed CYP3A4 (rCYP3A4), and heterologously expressed CYP3A5 (rCYP3A5), using in vitro metabolism to assess the role of both CYP3A4 and CYP3A5 in the metabolism of these four substrates. This study utilizes inhibition studies with ketoconazole for all four substrates and also evaluates the effect of TST on TRZ and nifedipine metabolism in HLMs, in rCYP3A4, and in rCYP3A5 to understand the drug-drug interaction (DDI) potential of CYP3A5 in comparison with CYP3A4.

Materials and Methods

Materials. 1-OH-TRZ (1-hydroxy-triazolam) and 4-OH-TRZ, as well as MDZ and its metabolites, were kindly provided by their pharmaceutical manufacturers or purchased from Ultrafine Chemicals (Oxford, UK). TST and 6b-HST (6b-hydroxytestosterone) were purchased from Sigma-Aldrich (St. Louis, MO). Ketoconazole was a gift from Janssen Pharmaceutica N.V. (Beerse, Belgium). Reaction cofactors (NADP+ and Mg2+ solutions) were obtained from Sigma-Aldrich. Recombinant CYP3A4 and CYP3A5, which are expressed from human CYP3A4 and CYP3A5 cDNA using a baculovirus expression system, were purchased from BD Gentest (Woburn, MA). These microsomes also contain cDNA-expressed human P450 reductase, but not human cytochrome b5. Sample protein concentration and P450 content were determined using methods described previously (from Molnke et al., 1993). In brief, microsomes were prepared through ultracentrifugation; microsomal pellets were resuspended in 0.1 M potassium phosphate buffer containing 20% glycerol and stored at 80°C until use. Total protein concentration was determined by a bicinchoninic acid assay.

Microsomal Preparation. Liver samples from human donors with no known liver disease were provided by the International Institute for the Advancement of Medicine (Exton, PA), the Liver Tissue Procurement and Distribution System (University of Minnesota, Minneapolis), or the National Disease Research Interchange (Philadelphia, PA). Twelve human livers, characterized as relatively high CYP3A metabolizers from a library of livers, were provided by the International Institute for the Disease Research Interchange (Philadelphia, PA). Twelve human livers, characterized as relatively high CYP3A metabolizers from a library of livers, were provided by the International Institute for the Disease Research Interchange (Philadelphia, PA). Twelve human livers, characterized as relatively high CYP3A metabolizers from a library of livers, were provided by the International Institute for the Disease Research Interchange (Philadelphia, PA).

Incubation. Incubation mixtures contained 50 mM phosphate buffer, 5 mM Mg2+, 0.5 mM NADP+, and an isocitrate/isocitric dehydrogenase regenerating system. Incubations were performed at 37°C, 0 to 400 μM MDZ, 0 to 1500 μM TRZ, 0 to 750 μM TST, and 0 to 20 μM nifedipine, to establish kinetic parameters for metabolite formation in human liver microsomes or heterologously expressed cytochromes (von Molnke et al., 1996a,b). Organic solvents were completely evaporated to dryness prior to the incubations. Reaction mixtures with human microsomes were incubated at 0.25 mg/ml of microsomal protein for all four substrates. The volume of incubation reactions was 250 μl. Formation of metabolites with human liver microsomes was linear with respect to incubation time and microsomal protein concentration over ranges relevant to this study. Incubations were initiated by the addition of microsomal protein. After 5 min (MDZ), 20 min (TRZ), 15 min (TST), or 10 min (nifedipine) at 37°C, reactions were stopped by cooling on ice and the addition of acetonitrile. Phenacetin was added as the internal standard for MDZ and TRZ, androstenedione for TST, and diazepam for nifedipine. The incubation mixture was centrifuged, and the supernatant was transferred to an autosampling vial for HPLC analysis. All samples were processed in duplicate. The formation rate of individual metabolites in reaction mixtures was determined based on calibrations constructed from a series of standards containing varying known amounts of metabolite standards together with internal standard. Reaction velocities were calculated in units of nanomoles of product formed per minute per milligram of microsomal protein. For the heterologously expressed P450s, reaction velocities were calculated in units of nanomoles of product formed per minute per microme of P450. Control incubations with no cofactor, no protein, and/or no substrate were performed concurrently to validate P450-dependent metabolism. The identity of metabolites was verified by comparing HPLC retention time with authenticated standards. The analytical assays used gave a coefficient of variation (n = 3, below 10%). Calibration curves were linear and passed through the origin. We did not evaluate recovery because no extraction was performed. The lowest point in the calibration curve corresponded to the following concentrations: 7.8 ng/ml for 1-OH-MDZ and 4-OH-MDZ, 25 ng/ml for 1-OH-TRZ and 4-OH-TRZ, 10 ng/ml for 6b-HST, and 100 ng/ml for oxidized nifedipine.

Antibodies and Quantitative Western Blotting. Amounts of CYP3A4 and CYP3A5 in human liver microsomal preparations from 12 livers were determined by quantitative Western blotting as described previously (Perloff et al., 2000). Microsomal protein (varying amounts of recombinant P450 standards and an optimal amount of liver microsomal protein) was denatured for 5 min at 100°C in 100 mM Tris buffer (pH 6.8) containing 10% glycerol, 2% β-mercaptoethanol, 2% SDS, and 5 mg/ml pyronin Y. Recombinant CYP3A4 and CYP3A5 (BD Gentest) were used to generate calibration standards in concentrations ranging from 0.07 to 0.25 pmol/mg and 0.039 to 5 pmol/mg, respectively. Protein was separated by SDS-polyacrylamide gel electrophoresis in precast 7.5% polyacrylamide gels (Bio-Rad, Hercules, CA) in 25 mM Tris/0.192 M glycine/0.1% SDS running buffer (pH 8.3) and transferred to Immobilon-P paper (0.45-mm pore size; Millipore, Bedford, MA) by electroblotting at 100 V for 1 h in 25 mM Tris/0.192 M glycine/20% methanol transfer buffer. Blots were blocked, incubated with primary antibody for 1 h, washed, incubated with HRP-labeled secondary antibody for CYP3A4 and peroxidase-conjugated anti-rabbit secondary antibody for CYP3A5 for 1 h, and washed again; and the bound HRP signal was activated by enhanced chemiluminescence (ECL) using the Super Signal Cl-HRP substrate system (Pierce Chemical). All postantibody washings were done three times (5 min each) in TBS (0.15 M NaCl, 0.04 M Tris Cl, pH 7.7) containing 0.1% Tween 20 (TBS-Tween). Blots were exposed to film, developed, and quantified by computer-aided densitometry (NIH Image 1.62 image analysis software; National Institutes of Health, Bethesda, MD). A calibration curve of integrated band intensity (the product of band area and band intensity; Y) versus the quantity of P450 standard in picomoles was as follows: Y = m (log x + b) for CYP3A4 and Y = mx + b for CYP3A4; where m and b are slope and intercept terms, respectively. Integrated band densities of liver microsomal samples were used to determine the concentrations of P450 per milligram of microsomal protein relative to the calibration curve. Antibodies used for CYP3A4 and CYP3A5 were specific, with no cross-reactivity, based on data from their manufacturers.

Testosterone-Triazolam Interaction. For evaluating the effect of testosterone on triazolam metabolism, fixed concentrations of TRZ (10 μM) were incubated with a range of TST concentrations (0–100 μM). Metabolite formation was expressed as a percentage of control with no inhibitor, based on metabolite to internal standard peak height ratio. Briefly, reaction mixtures were incubated with human microsomes (0.25 mg/ml) or rCYP3A4 or rCYP3A5. After 20 min at 37°C, reactions were stopped by cooling on ice and by the addition of acetonitrile. Phenacetin was added as the internal standard for both TST and TRZ. The incubation mixture was centrifuged, and the supernatant was transferred to an autosampling vial for HPLC analysis. All samples were processed in duplicate. The identity of metabolites was verified by comparing HPLC retention time with authenticated standards.

Testosterone-Nifedipine Interaction. For evaluating the effect of testosterone on nifedipine metabolism, fixed concentrations of nifedipine (10 μM)
were incubated with a range of TST concentrations (0–100 μM). Metabolite formation was expressed as a percentage of control with no inhibitor, based on metabolite to internal standard peak height ratio. Briefly, reaction mixtures were incubated with human microsomes (0.25 mg/ml) or rCYP3A4 or rCYP3A5. After 10 min at 37°C, reactions were stopped by cooling on ice and by the addition of acetonitrile. Diazepam was added as the internal standard for nifedipine. The incubation mixture was centrifuged, and the supernatant was transferred to an autosampling vial for HPLC analysis. All samples were processed in duplicate. The identity of metabolites was verified by comparing HPLC retention time with authenticated standards.

Chemical Inhibition. For inhibition studies, MDZ, TRZ, or TST in methanol solution was aliquoted into incubation tubes to yield final concentrations of 50, 250, and 75 μM, respectively. Nifedipine in acetone solution was aliquoted into incubation tubes to yield a final concentration of 10 μM. Inhibition studies with ketoconazole (0–10 μM) were performed by coaddition to MDZ, TRZ, TST, or nifedipine incubates. Controls with no inhibitor were performed at the beginning and end of each inhibition experiment. Metabolite formation was expressed as a percentage of control without inhibitor.

HPLC Analysis. For MDZ and its metabolites, the HPLC mobile phase consisted of 20%/35%/45% acetonitrile/methanol/10 mM phosphate buffer, pH adjusted to 7.4 with NaOH, with a flow rate of 1.4 ml/min. For TRZ and its metabolites, HPLC mobile phase consisted of 70%/20%/10% 50 mM phosphate buffer/acetonitrile/methanol, with a flow rate of 1.5 ml/min. For the TST and its metabolite, and nifedipine and its metabolite, HPLC mobile phase consisted of 55%/45% methanol/water, with a flow rate of 1.0 ml/min. For the TST-TRZ interaction, HPLC mobile phase and flow rate were similar to those used for TRZ and its metabolites. For TST-nifedipine interaction, the HPLC mobile phase and flow rate were similar to those used for TST and its metabolism and nifedipine and its metabolite. The analytical column (3.9 × 150 mm) was a stainless steel reverse phase C-18 Nova-Pak (Waters, Milford, MA). Column effluent was monitored by ultraviolet absorbance at 220 nm for MDZ and TRZ, 254 nm for TST, 270 nm for nifedipine and the TST-nifedipine interaction, and 220 nm for the TST-TRZ interaction.

Data Analysis. Kinetic parameters for MDZ, TRZ, TST, and nifedipine biotransformation without the addition of inhibitors were determined through nonlinear least-squares regression analysis of untransformed data. Model selection was based on empiric goodness of fit criteria. 1-OH-MDZ and 4-OH-MDZ formation data for MDZ were fit to a Michaelis-Menten model. For triazolam 1-OH-TRZ formation, data were fit to a model incorporating Michaelis-Menten kinetics with uncompetitive substrate inhibition, and 4-OH-TRZ formation data for TRZ were fit to a Michaelis-Menten model (von Moltke et al., 1996b; Perloff et al., 2000; Venkatakrishnan et al., 2001). For TST (6β-HST formation), data were fit to a substrate activation (Hill) model. For nifedipine (oxidized nifedipine formation), data were fit to a Michaelis-Menten model. The parameters that were estimated are: \( V_{\text{max}} \), the maximum reaction velocity (uninhibited); \( K_s \), the uncompetitive substrate inhibition constant; and \( a \), the Hill coefficient. When the biotransformation profile was consistent with a sigmoidal (Hill) model, the \( V_{\text{max}}/K_s \) ratio was used as an approximate estimate of intrinsic clearance (Houston and Kenworthy, 2000). \( I_{50} \) (the inhibitor concentration corresponding to a 50% reduction in metabolite formation velocity) values were determined through nonlinear regression of relative reaction velocities at a single substrate concentration in the presence of varying inhibitor concentrations (von Moltke et al., 1998).

Results

Quantitative Western Blotting. Wide variability in CYP3A4 expression was noted, with levels ranging from 9 to 829 pmol/mg of microsomal protein. In our samples, the contribution of CYP3A5 was up to 25% of CYP3A4 content (Table 1). CYP3A5 was detected in 83% (10 of 12) of the liver samples (Table 1). However, 6 of the 10 livers had CYP3A5 less than 0.25 pmol/mg of microsomal protein.

MDZ Biotransformation in Vitro. Mean kinetic parameters for MDZ hydroxylation in HLMs (Fig. 1A) are shown in Table 2. MDZ hydroxylation by rCYP3A4 and rCYP3A5 are also shown (Table 2). MDZ 1-hydroxylation accounted for a greater proportion of estimated intrinsic clearance than did 4-hydroxylation in HLMs, rCYP3A4, and rCYP3A5. This indicates that 1-OH-MDZ formation is the principal MDZ clearance pathway. Total estimated intrinsic clearance (EIC) was higher with rCYP3A4 as compared with rCYP3A5.

TRZ Biotransformation in Vitro. Mean kinetic parameters for TRZ hydroxylation in HLMs (Fig. 1B) are shown in Table 3. TRZ hydroxylation by rCYP3A4 and rCYP3A5 are also shown (Table 3). 1-OH-TRZ formation demonstrated substrate inhibition in HLMs (mean \( K_s = 5395 \mu M \)) as well as in rCYP3A4 and rCYP3A5. TRZ 1-hydroxylation accounted for a greater proportion of EIC than did 4-hydroxylation in rCYP3A4 and rCYP3A5, but not in HLMs (Table 3). Total EIC was higher with rCYP3A4, compared with rCYP3A5.

TST Biotransformation in Vitro. Mean kinetic parameters for TST hydroxylation in HLMs (Fig. 1C) are shown in Table 4. TST hydroxylation by rCYP3A4 and rCYP3A5 are also shown (Table 4). \( K_m \) values for 6β-HST using rCYP3A4 were about 3 times lower than those with rCYP3A5. EIC for rCYP3A5 was much lower than rCYP3A4 (Table 4).

Nifedipine Biotransformation in Vitro. Mean kinetic parameters for nifedipine oxidation in HLMs (Fig. 1D) are shown in Table 5. Nifedipine oxidation by rCYP3A4 and rCYP3A5 are also shown (Table 5). \( K_m \) values for oxidized nifedipine using rCYP3A4 were about 5 times lower than those with rCYP3A5. EIC for rCYP3A5 was much lower than that for rCYP3A4.

Inhibition of MDZ, TRZ, TST, and Nifedipine Biotransformation in Vitro with Ketoconazole. Ketoconazole was a potent inhibitor of both 1-OH and 4-OH formation of MDZ and TRZ in HLMs and rCYP3A4. Using rCYP3A4, IC_{50} values were 0.044 and 0.053 μM for 1-OH- and 4-OH-MDZ formation, and 0.05 and 0.049 μM for 1-OH- and 4-OH-TRZ formation, respectively (Fig. 2, Tables 2 and 3). However, for rCYP3A5, IC_{50} values for 4-OH and 1-OH formation for MDZ and TRZ were around 12 to 18 times higher than those for heterologously expressed CYP3A4 (Fig. 2, Tables 2 and 3). Ketoconazole was also a potent inhibitor of both 6β-HST formation from TST and oxidized nifedipine formation from nifedipine in heterologously expressed CYP3A4, with IC_{50} values of 0.045 and 0.024 μM, respectively (Tables 4 and 5). However, for rCYP3A5, IC_{50} values for formation of 6β-HST from TST and oxidized nifedipine formation from nifedipine were about 6 to 8 times higher than those for rCYP3A4 (Tables 4 and 5).

TST-TRZ Interactions. The effect of TST on TRZ metabolism was evaluated at low concentrations of TRZ (10 μM) and at varying concentrations of TST (0–100 μM) using HLMs, rCYP3A4, and rCYP3A5. Using rCYP3A4, TST inhibited 1-OH-TRZ formation (down to 10% of control), whereas 4-OH-TRZ formation was significantly activated (up to 173% of control) (Fig. 3). In HLMs the pattern was similar except that 4-OH-TRZ formation was activated to an even

<table>
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<th>HLMs</th>
<th>CYP3A4</th>
<th>CYP3A5</th>
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<tr>
<td>pmol/mg protein</td>
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<tr>
<td>L1</td>
<td>25.2</td>
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<tr>
<td>L2</td>
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<td>2.20</td>
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TABLE 1

Immunquantification of HLMs used in our study
greater degree (Fig. 3). In contrast, for rCYP3A5, TST only slightly activated both 1-OH- and 4-OH-TRZ formation (Fig. 4).

TST-Nifedipine. The effect of TST on nifedipine metabolism was evaluated at low concentrations of nifedipine (10 μM) and at varying concentrations of TST (0–100 μM) in HLMs, rCYP3A4, and rCYP3A5. Using rCYP3A4, oxidized nifedipine formation was inhibited (down to 60% of control values). In contrast, for rCYP3A5, TST slightly activated oxidized nifedipine formation (up to 115% of control values) at lower TST concentration, followed by inhibition. In HLMs, TST caused activation of oxidized nifedipine (up to 160% of control values) (Fig. 5).

Discussion

It was recently reported that abundance of CYP3A5 in human liver is greater than thought previously, especially in African Americans (Kuehl et al., 2001). Additionally, CYP3A5 is believed to have substrate specificity similar to that of CYP3A4. In earlier reports, reconstituted CYP3A5 showed comparable if not greater catalytic activity toward most CYP3A4 substrates (Gillam et al., 1995). A recent study by Williams et al. (2002) demonstrated an equal or reduced metabolic capability for CYP3A5 compared with CYP3A4 using 10 CYP3A substrates. In a study by Jounaidi et al. (1996), CYP3A5 protein was detected in 74% of white subjects. The authors believe that this greater frequency as compared with the previous reports (Aoyama et al., 1989; Kuehl et al., 2001) may be due to the greater sensitivity of the method of detection (ECL) used. The minimum amount of CYP3A5 protein reported by Hirota et al. (2001) and by Lin et al. (2002) was 1 pmol/mg of protein and 0.25 pmol/mg of protein, respectively. In our study, CYP3A5 was detected in 83% (10 of 12) of the liver samples, although 6 of the 10 livers had CYP3A5 less than 0.25 pmol/mg of microsomal protein. One liver sample (L2) that had the highest CYP3A5 content was from an African-American donor. The low content of CYP3A5 detected in our samples suggests that a contribution of CYP3A5 to net catalytic activity is unlikely. This may not be the case for sample L2, in which CYP3A5 content was 25% of CYP3A4 content. This sample had an

![Fig. 1. Metabolite formation rates (1-OH- and 4-OH-MDZ (A), 1-OH- and 4-OH-TRZ (B), 6β-HST (C), and oxidized nifedipine (D)) in relation to concentrations of the substrates MDZ, TRZ, TST, and nifedipine, respectively, by a representative preparation of HLMs. Lines represent functions determined by nonlinear least-squares regression analysis.](image-url)

![TABLE 2](table-url)

**TABLE 2**

<table>
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<tr>
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<th>HLMs (n = 6)</th>
<th>rCYP3A4</th>
<th>rCYP3A5</th>
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<td></td>
<td>1-OH-MDZ</td>
<td>4-OH-MDZ</td>
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<tr>
<td>V\text{\textsubscript{max}} (nmol/min/mg protein)</td>
<td>0.18 ± 0.03</td>
<td>0.42 ± 0.05</td>
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<td>K\text{\textsubscript{m}} (μM)</td>
<td>3.9 ± 0.9</td>
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<td>K\text{\textsubscript{m}}/V\text{\textsubscript{max}} (mM/nmol/min/pmol P450)</td>
<td>69 ± 20</td>
<td>6.1 ± 1.2</td>
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<td>IC\text{\textsubscript{50}} for ketoconazole (μM)</td>
<td>0.097 ± 0.03</td>
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* a Units are nmol/min/mg protein for HLMs and nmol/min/pmol P450 for rCYP3A4 and rCYP3A5.

* b For clearance (V\text{\textsubscript{max}}/K\text{\textsubscript{m}} ratio), units are μl/min/mg protein for HLMs and μl/min/pmol P450 for rCYP3A4 and rCYP3A5.
The presence or absence of b5. These recombinant enzymes had comparable ratios of OR to cytochrome P450.

For clearance (V_{max}/K_{m} ratio), units are μmol/min/mg protein for HLMs and μmol/min/pmol of P450 for rCYP3A4 and rCYP3A5.

### TABLE 3

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<th>HLMs (n = 6)</th>
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<td>1-OH-TRZ</td>
<td>4-OH-TRZ</td>
<td>1-OH-TRZ</td>
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<tr>
<td>V_{max} a</td>
<td>1.3 ± 0.3</td>
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<tr>
<td>K_{m} (μM)</td>
<td>104 ± 17</td>
<td>238 ± 21</td>
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<td>V_{max}/K_{m}</td>
<td>15.1 ± 3.4</td>
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<td>IC_{50} for ketoconazole (μM)</td>
<td>0.045 ± 0.004</td>
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a Units are nmol/min/mg protein for HLMs and nmol/min/pmol P450 for rCYP3A4 and rCYP3A5.

### TABLE 4

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<th>Oxidized Nifedipine</th>
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<th>rCYP3A4</th>
<th>rCYP3A5</th>
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<td>5.4 ± 1.6</td>
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<td>K_{m} (μM)</td>
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</tr>
<tr>
<td>V_{max}/K_{m} b</td>
<td>101 ± 23</td>
<td>147</td>
<td>23</td>
</tr>
<tr>
<td>Hill coefficient (a)</td>
<td>1.37 ± 0.08</td>
<td>1.17</td>
<td>1.26</td>
</tr>
<tr>
<td>IC_{50} for ketoconazole (μM)</td>
<td>0.044 ± 0.005</td>
<td>0.045</td>
<td>0.26</td>
</tr>
</tbody>
</table>

a Units are nmol/min/mg protein for HLMs and nmol/min/pmol P450 for rCYP3A4 and rCYP3A5.

### TABLE 5

<table>
<thead>
<tr>
<th>Oxidized Nifedipine</th>
<th>HLMs (n = 4)</th>
<th>rCYP3A4</th>
<th>rCYP3A5</th>
</tr>
</thead>
<tbody>
<tr>
<td>V_{max} a</td>
<td>20.4 ± 8.3</td>
<td>9.9</td>
<td>10</td>
</tr>
<tr>
<td>K_{m} (μM)</td>
<td>25.3 ± 9</td>
<td>4.5</td>
<td>25.8</td>
</tr>
<tr>
<td>V_{max}/K_{m} b</td>
<td>767 ± 77</td>
<td>2200</td>
<td>388</td>
</tr>
<tr>
<td>IC_{50} for ketoconazole (μM)</td>
<td>0.15 ± 0.1</td>
<td>0.024</td>
<td>0.19</td>
</tr>
</tbody>
</table>

a Units are nmol/min/mg protein for HLMs and nmol/min/pmol P450 for rCYP3A4 and rCYP3A5.

A fixed concentration of TRZ (250 μM) was incubated with ketoconazole in concentrations ranging from 0 to 10 μM. Reaction velocities were expressed as a ratio (in percent) relative to the velocity with no ketoconazole present. Each point for HLMs represents the mean ± S.E. for four different liver preparations. Mean values of the ketoconazole IC_{50} are shown (see Table 3).

### FIG. 2.

Effect of ketoconazole on 1-OH-TRZ metabolite formation in HLMs, in rCYP3A4, and in rCYP3A5.

A fixed concentration of TRZ (250 μM) was incubated with ketoconazole in concentrations ranging from 0 to 10 μM. Reaction velocities were expressed as a ratio (in percent) relative to the velocity with no ketoconazole present. Each point for HLMs represents the mean ± S.E. for four different liver preparations. Mean values of the ketoconazole IC_{50} are shown (see Table 3).

IC_{50} value for ketoconazole versus 4-OH-MDZ formation that was 3 times higher than in other samples (1.7 μM versus 0.5 μM). For the same substrate, ketoconazole IC_{50} values versus 1-OH-MDZ, 1-OH-TRZ, and 4-OH-TRZ formation also were higher compared with other human liver samples.

Mammalian P450s may be expressed in a number of heterologous systems, including bacterial, yeast, insect, and mammalian cells (Gonzalez and Korzekwa 1995). Lymphoblast-expressed CYP3A4 is available commercially, but lymphoblast-expressed CYP3A5 is not. Other factors relevant to selection of a recombinant system include the activity of the expression system, its correlation with activity in HLMs and in vivo, the ratios of oxidoreductase (OR) to cytochrome P450, and the presence or absence of b5. CYP3A4 activity has been reported to be stimulated by b5 (Yamazaki et al., 1996, 1999). However, the effects are shown to be substrate-dependent. In a study by Lee et al. (1995), recombinant CYP3A4 plus OR was catalytically similar to HLMs for testosterone 6β-hydroxylation activity. The coexpression of recombinant cytochrome b5 with CYP3A4-OR did not result in an additional increase in activity (Lee et al., 1995). In a study by Hirota et al. (2001), the in vitro intrinsic clearance (CL_{int}) of alprazolam by the CYP3A4-cytochrome b5 coexpression system was overestimated as compared with that by HLMs.

Yamazaki et al. (2002) observed that the presence or absence of b5 had different effects on CYP3A4 and CYP3A5 activity. When b5 was added to Supersomes, the CYP3A4 and CYP3A5 catalytic activity of testosterone increased by 30% and 230% respectively, as compared with the activity without addition of b5. However Supersomes without b5 and HLMs had comparable catalytic activity for nifedipine oxidation as compared with that between Supersomes with b5 and HLMs. In a study by Nakajima et al. (2002), although there was up to a 9-fold difference in b5/P450 ratio and up to a 3-fold difference in b5 expression in the baculovirus-infected recombinant CYP3A4 (with OR and b5), there was not much difference in testosterone 6β-hydroxylation activity. In the same study, testosterone 6β-hydroxylation and midazolam hydroxylation showed that V_{max} and the clearance values for both substrates were much higher with recombinant CYP3A4 (with OR and b5) as compared with HLMs. These studies demonstrate the differential effects of b5 based on the specific substrate as well as differential effects of addition of b5 to recombinant CYP3A4 compared with recombinant CYP3A5. Hence, in our study, we used recombinant CYP3A4 and recombinant CYP3A5, both without b5. These recombinant enzymes had comparable ratios of OR to cytochrome P450.
Atypical kinetics involving CYP3A has been reported in various in vitro studies, including our study (von Moltke et al., 1993, 1996a; Shou et al., 1994; Houston and Kenworthy, 2000; Perloff et al., 2000). Hence the $K_m$ values obtained in our study represent $S_{50}$ values, and intrinsic clearance calculated under these conditions must be considered as approximate estimates. In our study, TRZ biotransformation by HLMs, rCYP3A4, and rCYP3A5 displayed the phenomenon of substrate inhibition. These findings are similar to previous reports (Perloff et al., 2000; Schrag et al., 2001). In our study, TRZ metabolite formation with HLMs was linear over the incubation time of 20 min. This has been previously shown by von Moltke et al. (1996a).

In our study, the $K_m$ values obtained using rP450s were lower than in HLMs for MDZ and TRZ, which is consistent with previous studies (Ghosal et al., 1996; von Moltke et al., 1996a,b; Hamaoka et al., 2001). Our results show that rCYP3A5 has greater catalytic activity toward hydroxylation in the 1 position for both MDZ and TRZ. Similarly, in a study by Gorski et al. (1994), HLMs containing CYP3A5 in addition to CYP3A4 exhibited significantly greater ratios of 1-OH- to 4-OH-MDZ metabolite formation. Also, in a study by Hirota et al. (2001), alprazolam catalytic activity, as well as intrinsic clearance for 1-hydroxylation, in HLMs expressing significant amounts of CYP3A5 was greater than in HLMs not expressing significant amount of CYP3A5. In our study, for both MDZ and TRZ, the total EIC was lower for rCYP3A5 compared with rCYP3A4, indicating a lower metabolic capability for CYP3A5 compared with CYP3A4.

Testosterone 6β-hydroxylation is catalyzed by both CYP3A4 and CYP3A5, and this accounts for 75 to 80% of all metabolites formed (Draper et al., 1998). In our study, in addition to 6β-hydroxysterone, small amounts of 15β- and 2β-hydroxysterone were also detected but could not be quantified over a wide enough substrate concentration range to characterize the kinetic profile. The intrinsic clearance for rCYP3A5 was much lower than that found with rCYP3A4, indicating a relatively minor contribution of CYP3A5 to testosterone 6β-hydroxylation in the present study. Similarly, for nifedipine, intrinsic clearance for CYP3A5 was much lower than that found with rCYP3A4. Overall, the catalytic capability of CYP3A5 was lower than that of CYP3A4 for all of the four substrates. These results are consistent with the findings by Williams et al. (2002).

The inhibitory potency of ketoconazole was different for rCYP3A4- and rCYP3A5-mediated reactions forming the 1-OH as well as the 4-OH metabolite of both MDZ and TRZ (Fig. 2, Tables 2 and 3). Gibbs et al. (1999) also found that fluconazole and ketoconazole were less potent inhibitors of CYP3A5 compared with CYP3A4. Similarly, for TST 6β-hydroxylation and nifedipine oxidation, IC₅₀ values using ketoconazole were different between rCYP3A4 and rCYP3A5 (Tables 4 and 5). Because CYP3A5 has a lower susceptibility to inhibition by ketoconazole, CYP3A5 may contribute to interindividual variability in magnitude of DDIs encountered in vivo in people expressing CYP3A5. Hence, use of HLMs with significant CYP3A5 (single or pooled) content may underpredict the extent of interaction for the majority of individuals who do not have substantial levels of hepatic CYP3A5.
An important feature of CYP3A4 is that certain agents have been shown to stimulate its catalytic activity in vitro (Shou et al., 1994). Endogenous substances like progestrone have also been shown to stimulate CYP3A-mediated reactions (Johnson et al., 1988). Such interactions are substrate-dependent (Kenworthy et al., 1999). Studies by Schrag and Winkens (2001) and by Maenpää et al. (1998) showed that TST stimulated CYP3A metabolism of TRZ and MDZ, respectively. However, in the study by Schrag and Winkens (2001), the individual roles of CYP3A4 and CYP3A5 were not evaluated. In the present study, we used TST over a range of concentrations (0–100 μM) and TRZ at low concentrations (10 μM) to study this effect using HLMs, rCYP3A4, and rCYP3A5. We found substantial inhibition of 1-OH-TRZ and pronounced activation of 4-OH pathway using HLMs as well as with rCYP3A4, but not with rCYP3A5. In a study by Wang et al. (2000), the effect of TST on nifedipine metabolism was evaluated in HLMs. In the present study we used TST over a range of concentrations (0–100 μM) and nifedipine at 10 μM concentration to study this effect using HLMs, rCYP3A4, and rCYP3A5. TST caused slight activation of nifedipine metabolism in HLMs, consistent with the findings by Wang et al. (2000). Furthermore, using rCYP3A4, oxidized nifedipine formation was inhibited. However, for rCYP3A5, TST slightly activated oxidized nifedipine formation at a lower TST concentration followed by inhibition at higher concentrations.

Our conclusions from the current study are that CYP3A4 and CYP3A5 both contribute to MDZ, TRZ, TST, and nifedipine biotransformation in HLMs, but not necessarily in proportion to their relative abundance. Overall, the catalytic activity of CYP3A5 is less than that of CYP3A4. Inhibitory potency of ketoconazole toward rCYP3A5 was about 5 to 19 times lower than rCYP3A4 and HLMs for all four substrates, suggesting a less important role of CYP3A5 compared with CYP3A4 in DDIs.