COMPARATIVE METABOLIC CAPABILITIES OF CYP3A4, CYP3A5, AND CYP3A7

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ABSTRACT:
The human cytochromes P450 (P450) CYP3A contribute to the biotransformation of 50% of oxidatively metabolized drugs. The predominant hepatic form is CYP3A4, but recent evidence indicates that CYP3A5 contributes more significantly to the total liver CYP3A than was originally thought. CYP3A7 is the major fetal form and is rarely expressed in adults. To compare the metabolic capabilities of CYP3A forms for 10 substrates, incubations were performed using a consistent molar ratio (1:7:9) of recombinant CYP3A, P450 reductase, and cytochrome b5. A wide range of substrate concentrations was examined to determine the best fit to kinetic models for metabolite formation. In general, Km or S50 values for the substrates were 3 to 4 times lower for CYP3A4 than for CYP3A5 or CYP3A7. For a more direct comparison of these P450 forms, clearance to the metabolites was determined as a linear relationship of rate of metabolite formation for the lowest substrate concentrations examined. The clearance for 1'-hydroxy midazolam formation at low substrate concentrations was similar for CYP3A4 and CYP3A5. For CYP3A5 versus CYP3A4, clearance values at low substrate concentrations were 2 to 20 times lower for the other biotransformations. The clearance values for CYP3A7-catalyzed metabolite formation at low substrate concentrations were substantially lower than for CYP3A4 or CYP3A5, except for clarithromycin, 4-OH triazolam, and N-desmethyl diltiazem (CYP3A5 ~ CYP3A7). The CYP3A forms demonstrated regioselective differences in some of the biotransformations. These results demonstrate an equal or reduced metabolic capability for CYP3A5 compared with CYP3A4 and a significantly lower capability for CYP3A7.

Cytochromes P450 (P450) in the CYP3A subfamily are estimated to participate in the biotransformation of 50% of drugs known to undergo oxidative metabolism (Benet, 1996). Four members of the CYP3A subfamily have been described in humans: CYP3A4, CYP3A5, CYP3A7 (Nelson et al., 1996), and CYP3A43 (Domanski et al., 2001a). CYP3A4 is the most abundant hepatic and intestinal form. Initial data suggested that CYP3A5 accounted for only a small proportion of the total hepatic CYP3A content in only about 20% of samples (Wrighton et al., 1989). However, recent evidence indicates that CYP3A5 may represent more than 50% of the total CYP3A in some individuals (Kuehl et al., 2001). Furthermore, CYP3A5 is expressed in one-third of Caucasian livers and over one-half of African-American livers examined (Kuehl et al., 2001). High levels of CYP3A5 expression are related to possession of the CYP3A5*1 allele, whereas those individuals that carry the CYP3A5*3 and CYP3A5*6 alleles express insignificant levels (Kuehl et al., 2001). Expression of CYP3A7 protein is mainly confined to the fetal liver, although in rare cases CYP3A7 mRNA has been detected in adults (Schuetz et al., 1994). The highest level of transcript expression of the recently discovered CYP3A43 gene is in the prostate, whereas hepatic mRNA level is only 0.2 to 5% that of CYP3A4 (Gellner et al., 2001).

Metabolite formation by CYP3A forms in some cases demonstrates atypical kinetic behavior where a plot of substrate concentration versus activity curve does not follow a Michaelis-Menten-described hyperbola. The types of nonhyperbolic kinetics observed for some CYP3A substrates include autoactivation and substrate inhibition. It has been hypothesized that this nonhyperbolic behavior reflects the binding of two substrate molecules simultaneously in the CYP3A4 active site (Korzekwa et al., 1998). Site-directed mutagenesis of substrate recognition residues also suggests that multiple substrate molecules can bind within the CYP3A4 active site (Domanski et al., 2001b). It has been proposed that at least three subpockets may exist for substrate binding to the active site including one allosteric “effector” site too distal from the heme for metabolism to occur (Domanski et al., 2001). An alternative hypothesis is that multiple conformations of CYP3A4 exist (Koley et al., 1997). Finally, 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). In attempting to accommodate the atypical behavior of CYP3A when scaling from in vitro models to the in vivo situation, investigators have developed empirical descriptors to describe the kinetics. For example maximal clearance, Clmax, for autoactivation kinetics has been used to estimate maximal clearance for use in...
scaling from in vitro to in vivo (Houston and Kenworthy, 2000). It is interesting to note that observations of activation of CYP3A metabolism in hepatocytes (Maenpaa et al., 1998) and in monkeys in vivo (Tang et al., 1999) lend credence to the clinical relevance of the observations of atypical kinetics for CYP3A-catalyzed reactions in vitro.

The contribution of CYP3A5 to the total metabolic clearance of CYP3A substrates in the liver in vivo has not been well characterized. In general, the CYP3A forms are believed to have similar substrate specificity. Furthermore, CYP3A5-specific probe substrates/inhibitors have not been identified, although it has been possible to get some indication of CYP3A5 levels in microsomes using midazolam (MDZ) hydroxylase since the 1'-hydroxy (OH) to 4-OH ratio is higher for CYP3A5 than for CYP3A4 (Gorski et al., 1994; Kuehl et al., 2001). It is also possible to immunoquantify liver microsomal CYP3A5 in vitro using specific antibodies (Kuehl et al., 2001), but the value of protein levels is limited without an idea of the comparative metabolic capabilities of the CYP3A forms. The aim of the current study was to provide information on the relative metabolic capabilities of the hepatic CYP3A forms by conducting an in vitro comparison of the capabilities of CYP3A4, CYP3A5, and CYP3A7 to metabolize a structurally diverse set of molecules. It is now well established that the activity of CYP3A enzymes and the kinetics of substrate metabolism are sensitive to assay constituents including accessory enzymes and components of the buffer (Maenpaa et al., 1998; Schrag and Wienkers, 2000). Therefore, in designing the current study, simple assay conditions and a consistent molar ratio of P450 to reductase to cytochrome b5 were maintained so that any observed kinetic differences for each substrate may be attributed to inherent differences in the activity of the CYP3A enzymes.

Materials and Methods

Chemicals. All chemicals were from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. Midazolam (MDZ) was obtained from F. Hoffman-La Roche (Nutley, NJ). 1'-OH and 4-OH-MDZ were obtained from Ultrafine (Manchester, UK). Hydroxylated alprazolam (APZ) and triazolam (TZ) metabolites were gifts from Pharmacia & Upjohn Diagnostics (Kalamazoo, MI), Clarithromycin (CLR) and metabolites were obtained from Abbott Laboratories (Abbott Park, IL). Tamofoxifen (TAM) and N-desmethyl TAM were obtained from AstraZeneca Pharmaceuticals LP (Wilmington, DE). N-Desmethyldiltiazem (DTZ) was a gift from Tanabe Seiyaku Co. (Osaka, Japan). Oxidized nifedipine (NIF); 7-benzyloxy-4-(trifluoromethyl)-coumarin (BFC); 7-hydroxy-4-(DTZ) was a gift from Tanabe Seiyaku Co. (Osaka, Japan). Oxidized nifedipine (NIF); 7-benzyloxy-4-(trifluoromethyl)-coumarin (BFC); 7-hydroxy-4-trifluoromethylocoumarin (HFC); and microsomes prepared from insect cells containing baculovirus-expressed CYP3A4, CYP3A5, and CYP3A7 (Supersomes) were obtained from BD Gentest Corporation (Woburn, MA). The quality control procedures from Gentest for Supersomes activity were as follows. In brief, standard operating procedures were used (BD Gentest) in which the activity of each CYP3A Supersomes lot was compared with a lot of similar material that has a known level of activity. The activity of the reference lot of material must be within 20% of the expected value of the reference lot activity for the lot to be accepted (BD Gentest). NADPH cytochrome P450 reductase and cytochrome b5 were obtained from PanVera Corporation (Madison, WI).

Enzyme Incubations. The metabolic capabilities of CYP3A4, CYP3A5, and CYP3A7 were compared using a consistent ratio of CYP3A forms to accessory enzymes, specifically 1 mol CYP3A to 6.6 mol reductase to 9 mol cytochrome b5. CYP3A4 Supersomes were supplied with the indicated ratio. Therefore, CYP3A5 and CYP3A7 Supersomes were supplemented with P450 reductase and cytochrome b5 before the start of each experiment to match this ratio. P450 reductase and cytochrome b5 additions were performed according to a previously published method (Evert et al., 1997). Specifically, after addition, the mixtures were vortexed and left to stand for 15 min at room temperature before initiation of metabolism experiments. Experiments with midazolam confirmed that the rates and regioselectivity of metabolism occurred as expected, which provides supporting evidence for the appropriate behavior of the three CYP3A enzymes under these assay conditions. Pilot experiments were performed with each biotransformation and CYP3A form to ensure that comparisons of the metabolite formation by CYP3A4, CYP3A5, and CYP3A7 were determined under linear rate conditions. Incubation concentrations were set to make up a total volume of 190 μl were made in the following order: sodium phosphate buffer (100 mM, pH 7.4), NADPH (final concentration 2 mM), and substrate dissolved in methanol, except for BFC which was dissolved in acetonitrile (final concentration 0.5% v/v) to prevent oxidation of the catechol estrogen metabolites. The reactions were initiated by addition of cold recombinant CYP3A enzyme (10 μl), except for BFC, when the enzyme was prewarmed for 6 min before addition to the incubation mix. TEST was added first as a methanolic solution (200 μl) that was evaporated under nitrogen. After dissolution of TEST in buffer and addition of NADPH, there was a 6-min preincubation period before addition of enzyme to start the reaction (final volume 200 μl). For each substrate, 10 to 12 concentrations were examined over the following ranges: 1 to 1000 μM for MDZ, 1 to 2000 μM for APZ, 1 to 500 μM for TZ, 3.12 to 1000 μM for DTZ, 1.56 to 300 μM for TEST and E2, 1 to 150 μM for CLR, 0.62 to 200 μM for NIF, 0.78 to 200 μM for BFC, and 5 to 1000 μM for TAM. All reactions were stopped by the addition of ice-cold methanol (200 μl), except for DTZ (ice-cold methanol/acetonitrile 50:50) and BFC (acetonitrile at room temperature). Internal standard was added (CLR and TAM reaction mixtures were first made basic with sodium hydroxide), except for BFC, and incubations were left on ice for 5 min. BFC, DTZ, and E2 samples were analyzed immediately. CLR and TAM metabolites were subjected to an extraction procedure (see below). All other samples were stored at −80°C before analysis.

Analysis of Metabolites. Metabolites of APZ, MDZ, TZ, and CLR were determined by liquid chromatography/mass spectrometry/mass spectrometry detection. Metabolites for APZ and MDZ were extracted from the microsomal media using Waters Oasis HLB 3cc 60 mg cartridges (Waters Corp., Milford, MA). The cartridges were conditioned sequentially with methanol (2 ml) and deionized water (2 ml). The sample was loaded, washed with deionized water, and metabolites were eluted with methanol (2 ml). Samples were dried at 45°C under nitrogen, reconstituted in methanol (10% v/v, 200 μl), and injected onto the HPLC column. Analysis of the metabolites for TZ were conducted without extraction by diluting the microsomal mixture 4-fold into 10% methanol. Separation of the hydroxylated metabolites was achieved on a YMC AQ 2 mm × 100 mm column (Waters Corp.) following gradient elution using methanol and ammonium acetate (50 mM) buffer. The methanol concentration was increased from 60 to 90% in 4 min and held at 90% for 0.5 min. Metabolites for APZ and MDZ were detected using a Micromass Quattro (Manchester, UK) mass spectrometer monitoring the appropriate transitions in multiple reaction monitoring mode. The internal standard was α-OH TZ. Metabolites for TZ were detected using a Sciex API 3000 mass spectrometer (PerkinElmerSciex Instruments; Boston, MA) in the multiple reaction monitoring mode using 1'-OH MDZ as the internal standard. Oxidized NIF was determined by diluting the microsomal mix 4-fold with methanol (10%) containing the internal standard metabolite. Samples were injected onto a monochrome 5 μ, 30 × 2 mm column (MetaChem Technologies Inc., Torrance, CA), and the metabolite and internal standard were eluted starting with a mobile phase of formic acid (0.2%), isopropanol (5%), and ammonium formate (2 mM) and moving in a linear gradient to 95% methanol in formic acid (0.2%), isopropanol (5%), and ammonium formate (2 mM) in 1.5 min. The metabolite and internal standard were detected using a Micromass Quattro mass spectrometer monitoring the transition for each component in multiple reaction-monitoring mode. For analysis of CLR metabolites, sodium hydroxide was added to the reaction mixture, rifampin (internal standard) was added and then extracted into ethyl acetate/hexane (50:50). The organic phase was then dried under vacuum and reconstituted with HPLC buffer consisting of 10 mM ammonium acetate/methanol (20:80). CLR metabolites were analyzed using a Finnegan Navigator (Thermo Finnegan, San Jose, CA) mass spectrometer. Analysis of TEST 6β-hydroxylation was carried by HPLC with UV detection (237 nm). The internal standard was estradiol. N-Desmethyld TAM concentrations were measured by C18 reverse phase chromatography with UV detection. Briefly,
sodium hydroxide was added to the reaction mixture, imipramine (internal standard) was added then extracted into ethyl acetate/hexane (50:50, v/v). The resultant mixture was then dried under vacuum and reconstituted with HPLC buffer consisting of 10 mM ammonium acetate, pH 8.5/methanol (25:75), and the eluent was monitored at 265 nm. Analysis of E2 hydroxylation was performed by HPLC with electrochemical detection at 400 mV. The analytes and internal standard (2,2,5,7,8-pentamethyl-6-chromanol) were separated with a Zorbax SB-CN 3.5/H9262, 4.6/H11003, 150 mm column (MAC-MOD Analytical Inc., Ford, PA) and a mobile phase of acetonitrile/100 mM potassium phosphate pH 3 (40:60). The mobile phase flow rate was 1.5 ml/min, and column was maintained at 25 °C. BFC metabolism HFC was measured by fluorimetry (excitation wavelength, 405 nm; emission wavelength, 540 nm). Background fluorescence was measured by adding enzyme after the stop solution. Production of metabolite was quantified using a standard curve of authentic HFC.

Statistical Analysis.

Duplicate values for rate of metabolite formation for each substrate concentration were fit to equations describing hyperbolic (Michaelis-Menten, eq. 1; Segel, 1975), sigmoidal (Hill, eq. 2; Segel, 1975), or substrate inhibition (eq. 3; Copeland, 1996) relationships using WinNonlin software (Pharsight Corporation, Mountain View, CA). The best fit of the data to a relationship was determined according to established criteria (Ring et al., 1994).

\[ v = \frac{V_{\text{max}}[S]}{K_m + [S]} \]  
\[ v = \frac{V_{\text{max}}[S]^{n_1}}{[S_0]^{n_2} + [S]^{n_3}} \]  
\[ v = \frac{V_{\text{max}}}{1 + K_s/[S] + [S]/K_m} \]  

\( K_m \) values were derived from the Michaelis-Menten or substrate inhibition equations and \( S_50 \) values were derived from the Hill equation. Clearance values for comparison of metabolite formation at low substrate concentrations were determined for each enzyme/metabolite combination by calculating the slope at the lowest linear portion (2–6 substrate concentrations plus the origin) of the velocity/substrate concentration curve.

Metabolite Intermediate Complex Formation with DTZ. Metabolite intermediate complex (MIC) formation by DTZ with CYP3A4, CYP3A5, and CYP3A7 was performed according to Jones et al. (1999). In brief, the sample cuvette contained CYP3A4, CYP3A5, or CYP3A7 with the indicated levels of P450 reductase and cytochrome b5, DTZ (100 μM), and NADPH (2 mM) in a 1 ml volume whereas the reference cuvette contained equivalent amounts of reconstituted CYP3A enzyme, methanol, and NADPH. As a result of the form in which the P450s were obtained, no more than 100 pmol CYP3A4 or CYP3A7 could be used for MIC formation, but up to 500 pmol CYP3A5 could be used without making the incubation solution too turbid for accurate MIC measurement. MIC formation was measured by dual beam spectroscopy by scanning the cuvette from 380 to 500 nm maintained at 37°C to monitor the formation of an absorbance maximum at 455 nm. Readings were taken at 0, 5, 10, 15, 20, 25, 30, 35, 45, and 60 min.

Results

Determination of Enzyme Kinetic Parameters. Figure 1 shows structures of the compounds examined and the sites of metabolism monitored in this study. Table 1 indicates the kinetic model that best
fit the data and estimated kinetic parameters for each metabolite formed, CYP3A4, CYP3A5, and CYP3A7. In human liver microsomes, CYP3A-mediated metabolism of MDZ resulted in the production of a major metabolite at the 1′-OH position and a minor metabolite at the 4-OH position (Gorski et al., 1994). The data and best fit kinetic relationships for MDZ 1′-hydroxylation by CYP3A4, CYP3A5, and CYP3A7 are shown in Fig. 2. For CYP3A4, the results obtained over a broad concentration range (1–1000 µM) best fit a substrate inhibition model with a $K_m$ value of 5 µM and a $K_i$ value of 2438 ± 855 µM (Fig. 2a and Table 1). However, when the results obtained with substrate concentrations below 50 µM MDZ were examined, the best fit model was the Michaelis-Menten equation yielding a $K_m$ value of 6 µM (Table 1, Fig. 2b). For CYP3A5 (Fig. 2c) and CYP3A7 (Fig. 2d), the kinetics of formation of MDZ 1′-OH best fit the Michaelis-Menten equation (Table 1).

The major pathway of APZ metabolism by CYP3A in human liver microsomes has been reported to be via 4-hydroxylation (Gorski et al., 1999). In agreement with that work, 4-OH APZ was the major metabolite observed with each CYP3A form. Interestingly, the kinetics of 4-OH APZ formation best fit the Hill equation for CYP3A4, CYP3A5, and CYP3A7 yielding Hill coefficient values of 1.4 or greater. However, formation of the minor metabolite, 1-OH APZ, best fit the Michaelis-Menten equation for CYP3A4, CYP3A5, and CYP3A7 (Table 1).

Autoactivation kinetics were observed for 1′-OH TZ formation for CYP3A4 (Hill coefficient, $n = 1.3$), CYP3A5 ($n = 1.9$), and CYP3A7 ($n = 1.4$) enzymes (Table 1). This was the major metabolite of TZ formed for CYP3A4 and CYP3A5 below 100 µM substrate concentration, although the difference between the formation rates of the two metabolites was relatively small (e.g., approximately 2 to 1 ratio for 4-OH to 1′-OH TZ at 12.5 µM, and 1.2 to 1 at 100 µM). Autoactivation kinetics were observed for CYP3A5- and CYP3A7-catalyzed TZ 4-OH formation and best fit the Hill equation whereas CYP3A4-catalyzed TZ 4-OH formation best fit the Michaelis-Menten equation (Table 1).

Formation of 6β-OH TEST by CYP3A4 and CYP3A7 demonstrated autoactivation and best fit the Hill equation (Table 1) yielding Hill coefficients of 1.2 and 1.4, respectively. Formation of 6β-OH TEST by CYP3A5 best fit the Michaelis-Menten equation. Interestingly, in addition to 6β-OH TEST, 2α-OH TEST was a major TEST metabolite produced by CYP3A7 (50% 6β-OH TEST response, data not shown).

CLAR N-demethylation and hydroxylation followed Michaelis-Menten kinetics after incubation with CYP3A4 and CYP3A7. The rates of metabolite formation were linear for CYP3A5 up to the highest concentration of CLAR tested (150 µM). Thus, it was not appropriate to fit the data with CYP3A5.

Hydroxylation of E2 followed Michaelis-Menten kinetics for 2-OH and 4-OH formation. 2-OH E2 was the major metabolite formed by all three CYP3A forms (Table 1). 16α-OH E2 was not produced in detectable amounts by any CYP3A enzyme (see Materials and Methods). DTZ N-demethylation catalyzed by CYP3A5 followed autoactivation kinetics and best fit the Hill equation ($n = 1.3$), whereas N-demethylation of DTZ by CYP3A4 and CYP3A5 best fit Michaelis-Menten kinetics (Table 1). NIF oxidation catalyzed by CYP3A4 best fit the Hill equation (Table 1). Formation of oxidized NIF catalyzed by CYP3A5 and CYP3A7 fit the Michaelis-Menten equation best. HFC formation from BFC and N-desmethyl TAM formation from TAM followed Michaelis-Menten kinetics for all three CYP3A enzymes (Table 1).

**Comparison of $V_{max}$, $K_m$/S$_{50}$ and $n$ Values.** Table 1 lists where appropriate the $V_{max}$, $K_m$, $S_{50}$, and the Hill coefficient, $n$, values for each enzyme/substrate/metabolite combination. Between all biotransformations, $V_{max}$ values varied 1054-fold for CYP3A4 (between N-desmethyl CLAR and BFC), 13,500-fold for CYP3A5 (between 4-OH APZ and BFC), and 200-fold for CYP3A7 (between 1′-OH MDZ and BFC). The $V_{max}$ values were greatest for CYP3A4 except for N-desmethyl DTZ and the major benzodiazepine metabolites 4-OH APZ and 1′-OH MDZ for which CYP3A5 demonstrated the greatest values. In general, the $K_m$ or $S_{50}$ values for CYP3A4-catalyzed metabolism of each substrate were 2 to 20 times lower than for CYP3A5 or CYP3A7 (Table 1). For those substrates showing autoactivation kinetics, the ranges for Hill coefficient values were 1.2 to 1.5 for CYP3A4, 1.3 to 1.9 for CYP3A5, and 1.1 to 1.9 for CYP3A7.

**Comparison of Metabolite Formation by the CYP3A Forms at Low Substrate Concentrations.** The observed nonhyperbolic kinetics often occurred at substrate concentrations substantially higher than would be expected in tissues or plasma. Furthermore, due to the mixture of different kinetic fits that were observed for the formation of the various metabolites, direct comparisons of kinetic parameters, such as intrinsic clearance ($V_{max}/K_m$), between biotransformations was not appropriate. Therefore clearance by the CYP3A enzymes to the various metabolites were compared at low substrate concentrations by determining the slope at the lowest linear portion of the velocity/substrate concentration relationship (Table 2). For sigmoidal plots, this portion of the curve would be in what appears as the early “lag phase” of the curve. A comparison of these clearance values between CYP3A4 and CYP3A5 indicates similar values for the formation of 1′-OH MDZ (3.34 ml/min/mmol for CYP3A4 versus 3.31 ml/min/mmol for CYP3A5). For 4-OH MDZ, N-desmethyl DTZ, N-desmethyl TAM, and both metabolites of APZ and TZ, clearance by CYP3A5 was somewhat lower (2–4 times) compared with CYP3A4. Compared with CYP3A4, clearance values to the other metabolites investigated were 12 to 50 times lower for CYP3A5 (Table 2). The clearance values for CYP3A7 were 4-fold (for N-desmethyl DTZ) to 500-fold (for 4-OH E2) lower compared with CYP3A4. Except for both metabolites of CLAR, 4-OH TZ and N-desmethyl DTZ, for which CYP3A7 clearance values at low substrate concentrations were similar to those observed for CYP3A5, the rate of metabolite formation by CYP3A7 was significantly lower than that for CYP3A4 and CYP3A5.

**MIC Formation of CYP3A Enzymes with DTZ.** Formation of a DTZ MIC was detectable with 100 pmol CYP3A4 but not for CYP3A5 at 100 pmol or 500 pmol enzyme or for CYP3A7 at 100 pmol. For CYP3A4, 45 pmol of DTZ MIC had formed in the first 5 min. The rate of MIC formation slowed after that time, reaching a maximum of 92 pmol after 60 min.

**Metabolite Ratios for the Benzodiazepines and E2.** Ratios of major to minor metabolite for MDZ, APZ, TZ, and E2 as a function of substrate concentration are shown graphically in Fig. 3. For MDZ the 1′-OH to 4-OH ratio was about 2-fold greater for CYP3A5 than for CYP3A4 between 1 and 50 µM MDZ. Since the $K_m$ values for MDZ 1′-hydroxylation by CYP3A4 and CYP3A5 are much lower than the $K_m$ values for MDZ 4-hydroxylation, a decrease in the 1′-OH to 4-OH ratio was to be expected as substrate concentration increased. This sharp decrease in ratios as substrate concentration increased was not observed for the other benzodiazepines. The major metabolite to minor metabolite ratio was much greater for APZ compared with MDZ over all the concentrations examined. At substrate concentrations to 2000 µM, the 4-OH to 1′-OH APZ ratio of about 150 for CYP3A5 was approximately 15 times greater than the same ratio for CYP3A4 and CYP3A7. For TZ, the 4-OH to 1′-OH ratio was about 4 times greater for CYP3A7 than for CYP3A4 or CYP3A5 at concentrations up to 500 µM. For E2, the 2-OH to 4-OH ratio was
TABLE 1
Comparison of enzyme kinetic parameters for CYP3A4-, CYP3A5- and CYP3A7-catalyzed metabolite formation

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>CYP3A4</th>
<th>CYP3A5</th>
<th>CYP3A7</th>
</tr>
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<tr>
<td></td>
<td>Best Fit to Model</td>
<td>$V_{\text{max}}$</td>
<td>$S_d/K_{\text{m}}$</td>
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<td></td>
<td>nmol/min/nmol P450</td>
<td>µM</td>
<td></td>
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<td>1'-OH Midazolam*</td>
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<td>MM</td>
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<td>59</td>
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<td>Hill</td>
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<td>55</td>
</tr>
<tr>
<td>4-OH Alprazolam*</td>
<td>Hill</td>
<td>7</td>
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<tr>
<td>N-Desmethyl tamoxifen</td>
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<td>10</td>
<td>50</td>
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</tbody>
</table>

SI, substrate inhibition equation; MM, Michaelis-Menten; Hill, Hill equation; N.A., not applicable.

* Major metabolite.

$^{a}$ $K_m$ or $S_50$ values in this Table are derived from the best fit model to either Michaelis-Menten or Hill equations respectively, except for CYP3A4-mediated 1'-OH midazolam formation, which best fit a substrate inhibition kinetic model ($K_i = 2438$ µM).

$^{b}$ Hill coefficient, indicating the degree of sigmoidicity, for data that fits the Hill equation best.

$^{c}$ Did not fit the Hill equation.

$^{d}$ Rates of metabolite formation were not saturated up to the highest substrate concentration tested.
approximately 10 for CYP3A4, 4 for CYP3A5, and 19 for CYP3A7 at concentrations up to 300 \(M\). The \(N\)-desmethyl/14-OH CLAR ratio at concentrations at and below 50 \(\mu M\) for CYP3A4, the kinetics of 1'-OH midazolam formation fitted the Michaelis-Menten equation best. MDZ 1'-hydroxylation by CYP3A5 (c) and CYP3A7 (d) best fit the Michaelis-Menten equation.

Discussion

The CYP3A-catalyzed metabolism of 10 substrates (15 biotransformations) was examined in this study. The three benzodiazepines examined were MDZ, a commonly used CYP3A probe substrate, APZ, which has been shown to exhibit activation kinetics in human liver microsomes (Gorski et al., 1999), and TZ, which has been shown to exhibit substrate inhibition kinetics after incubation with recombinant CYP3A4 (Schrag and Wienkers, 2001). DTZ, a benzothiazepine, is \(N\)-demethylated by CYP3A enzymes and forms a MIC in human liver microsomes with CYP3A (Jones et al., 1999). TEST represents a second “class” of CYP3A substrates (Kenworthy et al., 1999) and is an endogenous substrate. CLAR is a large molecular weight CYP3A substrate, which along with erythromycin, also groups with testosterone in a second class of substrates (Kenworthy et al., 1999). E2 is an important endogenous substrate of CYP3A enzymes (Satoh et al., 2000; Lee et al., 2001). NIF exhibits unique characteristics as a substrate in that many CYP3A inhibitors influence NIF metabolism in a manner distinct to that observed for other CYP3A substrates (Ken-
CYP3A5 may also explain why the contribution of CYP3A5 to MDZ metabolism is reduced metabolic capability of CYP3A5 compared with CYP3A4, which is in close agreement with the ratios of 5.5 and 16.1 at 8 μM reported by Kuehl et al. (2001). The difference in metabolite ratios between recombinant CYP3A4 and CYP3A5 is significantly greater for APZ than for MDZ in the current study. Therefore, the metabolite ratio for APZ may be a more sensitive probe for CYP3A5 expression in human liver microsomes than the MDZ ratio. This is particularly the case at higher alprazolam concentrations (>100 μM), which is above the lag phase of the sigmoidal curve where there is minimal difference between the rates of CYP3A4- and CYP3A5-catalyzed 4-OH APZ formation (not shown).

Assessment of benzodiazepine metabolite ratios using the incubation conditions described supports the use of APZ and MDZ as substrate probes to determine whether significant CYP3A5 is present in liver microsomes. The 1'-OH to 4-OH MDZ ratio at 6.25 μM substrate concentration for the incubations reported here in microsomal incubations was 7.7 for CYP3A4 and 15.5 for CYP3A5, which is in close agreement with the ratios of 5.5 and 16.1 at 8 μM reported by Kuehl et al. (2001). The difference in metabolite ratios between recombinant CYP3A4 and CYP3A5 is significantly greater for APZ than for MDZ in the current study. Therefore, the metabolite ratio for APZ may be a more sensitive probe for CYP3A5 expression in human liver microsomes than the MDZ ratio. This is particularly the case at higher alprazolam concentrations (>100 μM), which is above the lag phase of the sigmoidal curve where there is minimal difference between the rates of CYP3A4- and CYP3A5-catalyzed 4-OH APZ formation (not shown).

CLAR differs from erythromycin only by possessing an O-methyl group at the 6-position, which is not a site of metabolism by CYP3A enzymes. A previous study using purified CYP3A5 from human liver microsomes showed no erythromycin-metabolizing activity (Withington et al., 1990). In a second study using a reconstituted system with recombinant CYP3A5 purified from Escherichia coli, CYP3A5 was shown to have a 4-fold higher erythromycin-metabolizing activity than CYP3A4 (Gillam et al., 1995). Since the findings of the current study suggest low activity of CYP3A5 relative to CYP3A4 for CLAR metabolism, it appears that CYP3A5 enzymes expressed in bacterial membranes, purified and reconstituted with associated steps necessary, may have significantly over-predicted macrolide antibiotic metabolism by CYP3A5.

Formation of a MIC from DTZ was observed for CYP3A4 but not for CYP3A5 or CYP3A7. Individuals expressing high levels of CYP3A5 may therefore have less significant drug interactions with DTZ than nonexpressors, especially when DTZ is coadministered with compounds such as the benzodiazepines that are highly metabolized by CYP3A5. The lack of MIC formation by CYP3A5 cannot be explained by the rate of N-desmethyl DTZ formation, since CYP3A4 and CYP3A5 metabolize DTZ at the same rate. Differences between 1) the orientation of DTZ or N-desmethyl DTZ in the active site, 2) differences in the orientation of the ultimate reactive metabolite in the active site, and/or 3) the dissociation of metabolite from the enzyme may influence MIC formation.

Two endogenous compounds, TEST and E2, were included in this study as CYP3A enzymes have been implicated in being involved in controlling systemic steroid hormone levels. CYP3A5 was about 40 times less capable of forming the major metabolite of E2 and TEST, versus CYP3A4. Since liver CYP3A4 plays a much greater role in

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**TABLE 2**

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>CYP3A4</th>
<th>CYP3A5</th>
<th>CYP3A7</th>
</tr>
</thead>
<tbody>
<tr>
<td>1'-OH Midazolam</td>
<td>3.34</td>
<td>3.31</td>
<td>0.02</td>
</tr>
<tr>
<td>4'-OH Midazolam</td>
<td>0.99</td>
<td>0.15</td>
<td>0.04</td>
</tr>
<tr>
<td>1'-OH Alprazolam</td>
<td>0.05</td>
<td>0.03</td>
<td>0.003</td>
</tr>
<tr>
<td>4'-OH Alprazolam</td>
<td>1.10</td>
<td>0.34</td>
<td>0.02</td>
</tr>
<tr>
<td>1'-OH Triazolam</td>
<td>0.54</td>
<td>0.14</td>
<td>0.004</td>
</tr>
<tr>
<td>4'-OH Triazolam</td>
<td>0.30</td>
<td>0.05</td>
<td>0.006</td>
</tr>
<tr>
<td>N-Desmethyl diltiazem</td>
<td>0.69</td>
<td>0.20</td>
<td>0.18</td>
</tr>
<tr>
<td>6p-OH Testosterone</td>
<td>2.34</td>
<td>0.06</td>
<td>0.02</td>
</tr>
<tr>
<td>N-Desmethyl clarithromycin</td>
<td>51.2</td>
<td>4.30</td>
<td>5.06</td>
</tr>
<tr>
<td>14'-OH Clarithromycin</td>
<td>7.03</td>
<td>0.23</td>
<td>0.53</td>
</tr>
<tr>
<td>2-OH Estradiol</td>
<td>0.44</td>
<td>0.01</td>
<td>0.005</td>
</tr>
<tr>
<td>4-OH Estradiol</td>
<td>0.10</td>
<td>0.006</td>
<td>0.0002</td>
</tr>
<tr>
<td>Oxidized nifedipine</td>
<td>5.0</td>
<td>0.24</td>
<td>0.08</td>
</tr>
<tr>
<td>7-Hydroxy-4-trifluoro</td>
<td>0.02</td>
<td>0.014</td>
<td>0.0006</td>
</tr>
<tr>
<td>methylcoumarin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-Desmethyl tamoxifen</td>
<td>0.28</td>
<td>0.13</td>
<td>0.045</td>
</tr>
</tbody>
</table>

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**Note:** The table provides in vitro clearance values for 10 substrates by CYP3A4, CYP3A5, and CYP3A7 at low substrate concentrations. The values are given in terms of micromoles per minute per nanomole P450.
TEST and E2 hydroxylation versus CYP3A5, it seems unlikely that hepatic hydroxylation of these hormones will be significantly affected by the expression of CYP3A5 in the liver. These results therefore disagree with a previous suggestion that CYP3A5 has a significant role in hepatic estradiol metabolism (Lee et al., 2001). It is more likely that CYP3A4 will have a much greater influence on circulating E2 levels and on the hepatic production of catechol estrogens, which are E2 metabolites implicated as potential genotoxins in breast cancer risk (Williams and Phillips, 2000). However, CYP3A5 is a major CYP3A expressed outside the liver and intestine, and thus may have a role in controlling local steroid hormones in the kidney, breast, and lung. CYP3A5 may also play a part in the (extra-hepatic) tissue-specific production of potentially genotoxic estrogen metabolites (Williams and Phillips, 2000).

Nifedipine and BFC were metabolized at much lower rates by CYP3A5 and CYP3A7 compared with CYP3A4. CYP3A4 is therefore likely to play the dominant role in CYP3A-catalyzed NIF clearance in vivo. The experiments in the current study suggest BFC is a poor substrate for CYP3A5 and CYP3A7. This may not have significant practical applications because BFC is routinely used with recombinant CYP3A4 enzymes rather than human liver microsomes that usually express CYP3A4 and CYP3A5.

For people possessing at least one CYP3A5*1 allele, hepatic levels of CYP3A5 may approach that of CYP3A4 (Kuehl et al., 2001). A
previous report predicted that the highest clearance of CYP3A substrates may occur in those people who are heterozygous or homozygous for CYP3A5*3 and therefore express CYP3A4 and CYP3A5 (Kuehl et al., 2001). The results of the current study support this prediction for MDZ and possibly for other benzodiazepines, benzo- thiiazepines, and compounds with similar structures to TAM. However, based on the majority of CYP3A substrates investigated in the current study, the level of hepatic CYP3A4, and not CYP3A5 or CYP3A7, appears to be the major determinant in the metabolism of CYP3A substrates.

References


