INHIBITION OF CYTOCHROME P-450 3A (CYP3A) IN HUMAN INTESTINAL AND LIVER MICROSONES: COMPARISON OF $K_i$ VALUES AND IMPACT OF CYP3A5 EXPRESSION

MEGAN A. GIBBS, KENNETH E. THUMMEL, DANNY D. SHEN, AND KENT L. KUNZE

Departments of Medicinal Chemistry (K.L.K.) and Pharmaceutics (M.A.G., K.E.T., D.D.S), School of Pharmacy, University of Washington, Seattle, Washington

(Received July 10, 1997; accepted September 2, 1998)

This paper is available online at http://www.dmd.org

ABSTRACT:

The purpose of this study was to compare the kinetics of intestinal and hepatic cytochrome P-450 3A (CYP3A) inhibition by using microsomal midazolam 1'-hydroxylation as a marker of enzyme activity. The effect of two antifungal agents commonly implicated in CYP3A drug-drug interactions was examined. Inhibition type and affinities were determined for human liver and intestinal microsomes screened for the presence or absence of CYP3A4 and CYP3A5, as well as for cDNA-expressed CYP3A4 and CYP3A5 microsomes. Ketoconazole and fluconazole were found to be non-competitive inhibitors of both enzymes. Ketoconazole exhibited a $K_i$ for cDNA-expressed CYP3A4 of 26.7 ± 1.71 nM, whereas the $K_i$ for cDNA expressed CYP3A5 was 109 ± 19.7 nM. Corresponding $K_i$ values for fluconazole were 9.21 ± 0.51 µM and 84.6 ± 12.9 µM. For liver and intestinal microsomes that contained only CYP3A4, the average ketoconazole $K_i$ was found to be 14.9 ± 6.7 nM and 17.0 ± 7.9 nM, respectively, whereas fluconazole yielded mean respective $K_i$ values of 10.7 ± 4.2 µM and 10.4 ± 2.9 µM. Liver and intestinal microsomes that contained an equal or greater amount of CYP3A5, in addition to CYP3A4, were less susceptible to inhibition by both ketoconazole and fluconazole. These findings suggest that there can be significant differences in the affinity of these two enzymes for inhibitors. This may further broaden interindividual variability with respect to the magnitude of in vivo drug-drug interactions. We also conclude that there is no significant difference in inhibition type and affinity of ketoconazole and fluconazole for hepatic versus intestinal CYP3A4.

Many substrates for the cytochrome P-450 3A (CYP3A) enzymes display low and variable bioavailability after oral administration, including felodipine (Edgar et al., 1985), verapamil (McTavish and Sorkin, 1989), saquinavir (Williams et al., 1992), cyclosporine (Fahr, 1993), midazolam (Smith et al., 1981), and terfenadine (Lalonde et al., 1996). First-pass metabolism is a major factor contributing to the poor bioavailability of these and other drugs. CYP3A4 is the dominant P450 enzyme in both the liver and mucosa of the gastrointestinal tract (Watkins et al., 1987; DeWaziers et al., 1990; Shimada et al., 1994; Paine et al., 1997). Thus, because of anatomical location with respect to the delivery of drug from the intestinal lumen to the systemic circulation, both liver and intestinal CYP3A4 can contribute to the first-pass effect (Kolara et al., 1991; Paine et al., 1996).

The oral bioavailability of CYP3A4 substrates can be profoundly altered by modulators of enzyme catalytic activity (Thummel and Wilkinson, 1998). Results from in vivo interaction studies suggest that both hepatic and intestinal metabolic extraction are sensitive to the effect of known CYP3A inducers, such as rifampin and phenytoin (Hebert et al., 1992; Fromm et al., 1996; Holibecher et al., 1996), and the CYP3A inhibitor, ketoconazole (Gomez et al., 1995; Fleishaker et al., 1996). However, the effect of a coadministered modulator of CYP3A function might be expected to be more pronounced at the level of intestine, compared to the liver, based on presumed local concentration differences during the period of modulator absorption. In some circumstances, it is even conceivable that only the small intestine would be subject to the enzyme-altering effects of the xenobiotic. For example, ingestion of grapefruit juice causes an increase in midazolam and cyclosporine area under the blood concentration-time curve (and a presumed reduction in intestinal first-pass metabolism) after oral administration of the drugs, but not after i.v. administration (Ducharme et al., 1995; Kupferschmidt et al., 1995).

Central to discussions of in vivo hepatic and intestinal CYP3A inhibition is the assumption that the enzyme(s) behave(s) identically with respect to the mechanism of inhibition and inhibitor binding affinity, despite the different cellular origin. This hypothesis, however, has not been fully tested. Previously, investigators evaluated the kinetics of inhibition of CYP3A activity in pig intestinal and human liver microsomes (Lampen et al., 1995). Their results showed that the $K_i$ for several drugs was lower with respect to human hepatic metabolism when compared with pig intestinal metabolism, whereas for other drugs including ketoconazole, the pig intestinal $K_i$ was lower than the human hepatic $K_i$. Because of the study design, the authors were not able to conclude whether the observed differences in $K_i$ were species-specific or organ-specific.

In vitro microsomal experiments are well suited for characterization...
of enzyme inhibition kinetics. One purpose of this study was to determine whether there is a difference in the kinetics of human intestinal and hepatic CYP3A4 inhibition, by using microsomal midazolam 1'-hydroxylation as a prototypical enzyme-selective reaction. A second complicating feature of CYP3A-dependent metabolism, relevant to inhibition studies in microsomal preparations, is the contribution of CYP3A5 to the metabolism of drugs. Because this closely related enzyme is variably expressed in both tissues, the contribution of this enzyme to product formation may confound interpretation of inhibition studies by single-enzyme kinetic models. The second purpose of this paper was to examine the impact of CYP3A5 on inhibition kinetics. To this end, the inhibitory effect of two antifungal agents commonly implicated in drug-drug interactions, fluconazole and ketoconazole, was examined in typed human liver and intestinal microsomes as well as in cDNA-expressed CYP3A4 and CYP3A5 microsomal preparations.

Materials and Methods

Materials. NADPH was obtained from Sigma Chemical Company (St. Louis, MO). Ketoconazole was acquired from Research Diagnostics, Inc. (Flanders, NJ). Fluconazole was a gift from Pfizer, Inc. (Groton, CT). Midazolam, 1'-hydroxymidazolam and 1'-[1H2]hydroxymidazolam were kindly provided by Roche Laboratories (Nutley, NJ). Acetonitrile, acetone, and ethyl acetate were purchased from Fisher Scientific (Santa Clara, CA). N-Methyl-N,N,N-(2-buty1-dimethylamlyl)trifluoroacetamide was purchased from Pierce Chemical (Rockford, IL). SDS-polyacrylamide gel electrophoresis reagents (acylamide, ammonium persulfate, N,N,N',N'-tetra-methyl-ethylene diamine) were purchased from Bio-Rad (Hercules, CA). Nitrocellulose was purchased from (Flanders, NJ). Fluconazole was a gift from Pfizer, Inc. (Groton, CT). Mida-
zolam 1'-hydroxymidazolam was purchased from Roche Laboratories (Nutley, NJ). Ketoconazole was acquired from Research Diagnostics, Inc. (Rockford, IL). SDS-polyacrylamide gel electrophoresis reagents (acrylamide, ammonium persulfate, N,N,N',N'-tetra-methyl-ethylene diamine) were purchased from Bio-Rad (Hercules, CA). Nitrocellulose was purchased from Schleicher & Schuell (Keene, NH) and 5-bromo-4-chloro-3-indol phosphate and nitroblue tetrazolium from Kirkegaard & Perry (Gaithersburg, MD). Cell microsomes containing cDNA-expressed CYP3A4 or CYP3A5 and P450 reductase (P207 and P235) were purchased from Gentest (Woburn, MA).

Tissue Collection. Human liver and small intestine were obtained through the Solid Organ Transplant Program at the University of Washington Medical Center and the Northwest Organ Procurement Agency (Seattle, WA). Liver and intestinal mucosal microsomes were prepared as described elsewhere and stored at −80°C (Paine et al., 1997). Protein concentrations were determined by the method of Lowry et al. (1951). Microsomal preparations from 13 livers and 8 intestines were used in these studies.

Western Blot Analysis. Immunounquantification of CYP3A4 and CYP3A5 content in microsomal preparations was performed as described previously with purified CYP3A4 as the reference standard (Paine et al., 1997). Because the antibody used was prepared against purified CYP3A4 (Kharasch and Thummel, 1993), the relative immunoreactivity for an equimolar amount of cDNA-expressed CYP3A5 versus cDNA-expressed CYP3A4 was determined in a separate analysis. An integrated optical density for each nitrocellulose-bound protein band was obtained with a Bioimage II scanner (Bedford, MA).

Kinetic Protocols. All incubations were performed in duplicate in solutions containing 0.1 M potassium phosphate, pH 7.4, and 1 mM EDTA. Ketoconazole dissolved in acetone was placed directly into designated incubation tubes; the solvent was allowed to evaporate over 30 min before addition of other components. Midazolam and fluconazole (where indicated) were added to the reaction mixture in buffer. Substrate, inhibitor, and enzyme were preincubated at 37°C for 5 min before addition of NADPH (1 mM final concentration). Reactions were terminated by the addition of 0.1 M Na2CO3, pH ~11 (1 ml), and 1'-hydroxymidazolam to the incubates and associated standard curve samples were measured by negative ion chemical ionization gas chromatography-mass spectroscopy, as described previously (Paine et al., 1997).

1'-Hydroxymidazolam Formation versus Time and Protein. Incubations (15 ml) with midazolam (8 μM) were performed with cDNA-expressed CYP3A4 or CYP3A5 (10 pmol/ml), human liver microsomes that contained only CYP3A4 (HI-149, 35 μg of protein/ml), or intestinal microsomes that contained only CYP3A4 (HI-37, 70 μg of protein/ml). One-milliliter aliquots were transferred to tubes containing 1 ml of 0.1 M Na2CO3, pH 11, at 0 (before addition of NADPH), 0.5, 0.75, 1, 2, 3, and 4 min after the addition of NADPH.

The effect of liver microsomal protein concentration (25, 50, or 100 μg/ml HL-151) on the percentage of inhibition of midazolam (4 μM) 1'-hydroxylation by fluconazole (30 μM) or ketoconazole (50 nM) was also determined. Product formation was measured after a 4-min incubation with NADPH.

Ketoconazole and fluconazole inhibition kinetics. All kinetic studies were conducted over an interval of 4 min in a 1-ml incubation volume. Incubations to determine the K_i for ketoconazole and fluconazole were carried out with 35 and 70 μg of liver and intestinal microsomal protein or 10 pmol of the expressed enzyme. Three liver (HL-129, -131, and -149) and three intestinal (HI-17, -21, and -22) preparations that contained only CYP3A4, as determined by Western blot analysis, were examined. An additional two livers (HL-127 and -141) that also contained appreciable amounts of CYP3A5 were studied. Final concentrations of ketoconazole were 0, 10, 50, and 100 nM; final concentrations of fluconazole were 0, 10, 30, 60, and 90 μM. Midazolam concentrations were 1, 2, 4, and 8 μM for all K_i determinations yielding a 4 × 4 (ketoconazole) or a 4 × 5 (fluconazole) matrix. For CYP3A5 incubations only, the matrix was expanded by an additional concentration of ketoconazole (200 nM) or fluconazole (120 μM).

Relationship between percentage of inhibition and CYP3A5 content. Fractional inhibition was also determined for a set of liver (35 μg/ml) or intestinal microsomes (70 μg/ml) incubated for 4 min with midazolam (4 μM) and fluconazole (30 μM) or ketoconazole (50 nM). Eight microsomal preparations were positive for CYP3A5 (five livers and three intestines), as assessed by Western blot analysis, and another eight were negative (five livers and three intestines). Microsomes from all CYP3A5-positive tissues that were available from our banks of human liver and intestine were included in the experiment. The eight CYP3A5-negative microsomal preparations were prepared from a larger set of donated tissues obtained over the same period of time as the CYP3A5-positive tissues. In this case, selection for the experiment was made without regard to the specific CYP3A4 content (pmol/mg microsomal protein).

Data Analysis. Kinetic data sets were evaluated for type of inhibition by graphical analysis with Lineweaver-Burk and Dixon plots. Fits to competitive, noncompetitive, or mixed type inhibition models (Segel, 1975) were carried out by unweighted nonlinear regression analysis (Systat, Evanston, IL). Parameters are reported with asymptotic standard errors. Criteria used to determine the inhibition type and K_i included visual inspection of Lineweaver-Burk plots, how well the K_m and V_max estimates obtained from the full inhibitor-substrate matrix of incubations agreed with those parameters generated from substrate incubations performed in the absence of inhibitor, a normal distribution of residuals, and the F ratio test to determine whether there was a statistical difference in the residual sum of squared errors for each model fit.

A series of simulations was also performed to predict product formation velocities at midazolam concentrations of 1, 2, 4, and 8 μM and inhibitor concentrations of 0, 10, 30, 60, and 120 μM fluconazole or 0, 10, 50, and 100 nM ketoconazole. Predicted velocities (constant total CYP3A) were calculated based on the following noncompetitive model of inhibition for a two-enzyme system (eq. 4A5, the CYP3A5 mole fraction, varied from 0 to 1) and experimentally determined K_m, K_a, and K_i values obtained from individual cDNA-expressed CYP3A4 and CYP3A5 incubations:

\[
\frac{V}{S} = \frac{(1 - f_{A5}) \cdot k_{cat}^{3A4}}{K_{m}^{3A4} \cdot \left(1 + \frac{I}{K_{i}^{A5}}\right) + \left(1 + \frac{I}{K_{i}^{A4}}\right)} + \frac{f_{A5} \cdot k_{cat}^{3A5}}{K_{m}^{3A5} \cdot \left(1 + \frac{I}{K_{i}^{A5}}\right) + \left(1 + \frac{I}{K_{i}^{A4}}\right)}.
\]

Simulated velocities obtained above were fit to the single-enzyme, noncompetitive model (eq. 2) with unweighted nonlinear regression to assess the
impact of multi-enzyme inhibition kinetics on the estimation of $K_i$ if it is erroneously assumed that only a single enzyme is involved in the reaction:

$$v = \frac{V_{\text{max,app}}}{S} \left( \frac{K_{\text{m,app}}}{1 + \frac{I}{K_{\text{i,app}}}} \right).$$

For this exercise, $V_{\text{max,app}}$, $K_{\text{m,app}}$, and $K_{\text{i,app}}$ are all “apparent” kinetic parameters because product formation data generated from a two-enzyme system are forced to fit a single-enzyme model.

**Results**

CYP3A4 was detected by Western blot analysis in all human liver and intestinal microsomal samples tested. The highly related protein, CYP3A5, was found in some but not all of these same samples. A representative Western blot of human liver and intestinal microsomes, and authentic CYP3A standards, is shown in Fig. 1. CYP3A5, the immunoreactive protein with the slowest electrophoretic mobility (Wrighton et al., 1990), was found in 5 of the 13 liver and 3 of the 8 intestinal microsomal preparations. It is possible that more livers and intestines contained CYP3A5 protein below our detection limits (Joulnäidi et al., 1996). Comparison of the integrated band density of equimolar amounts of cDNA-expressed CYP3A4 and CYP3A5 standards indicated that the anti-CYP3A4 antibody used exhibited 1.8-fold greater immunoreactivity for CYP3A4 than for CYP3A5. Thus, liver HL-127 appeared, by this criterion, to contain more CYP3A5 than CYP3A4, whereas comparable amounts of the two isozymes were present in HL-141. For both CYP3A5-positive intestines, the amount of CYP3A5 was less than the amount of CYP3A4.

The time course of 1'-hydroxymidazolam formation was measured for incubations of 8 µM midazolam with liver or intestinal microsomes, or cDNA-expressed CYP3A4 or CYP3A5. As seen in Fig. 2, the accumulation of 1'-hydroxymidazolam between 0.5 and 4 min was proportional to the incubation time with HL-149 or HI-37 microsomes. Similar results were found for cDNA-expressed CYP3A4 and CYP3A5 (data not shown). However, the presence of a slight positive intercept to the linear regression line suggested that there might have been a small burst of product formation immediately after the addition of NADPH. The kinetic consequence of this phenomenon was thought to be limited because the rates of 1'-hydroxymidazolam formation calculated from 0.5- to 4-min- and 0- to 4-min-interval data were very similar: for HL-149, 28.4 pmol/min and 29.7 pmol/min, respectively; for HI-37, 12.0 and 12.4 pmol/min, respectively. Incubation of 8 µM midazolam with human liver and intestinal microsomes or cDNA-expressed CYP3A enzymes for periods longer than 4 min resulted in a gradual decline in metabolite formation rates (data not shown). A similar finding of short-term (0–5 min) 1'-hydroxymidazolam formation linearity with human liver microsomes was reported recently (Ghosal et al., 1996).

The mechanism of inhibition of CYP3A4 activity by ketoconazole and fluconazole was determined in liver and intestinal microsomes that contained CYP3A4 and no detectable amounts of CYP3A5 (CYP3A4-only). Representative Lineweaver-Burk plots for the inhibition of liver and intestinal microsomal CYP3A4-catalyzed midazolam 1'-hydroxylation are depicted in Fig. 3. Both ketoconazole and fluconazole were found to be noncompetitive inhibitors. Shown in Fig. 4 are representative Dixon plots for the inhibition of CYP3A4 in human liver and intestinal microsomal incubations by fluconazole. Similar plots were obtained for ketoconazole. For both inhibitors, the linear nature of the reciprocal plots was consistent with a single-enzyme (i.e., CYP3A4) catalytic system. Nonlinear regression estimates of the $K_i$ for three liver and three intestinal microsomes are summarized in Table 1. Ketoconazole was a very potent inhibitor of
CYP3A4 with an average $K_i$ of $14.9 \pm 6.7 \text{nM}$ and $17.0 \pm 7.7 \text{nM}$ for liver and intestinal microsomes, respectively. Fluconazole was much less potent than ketoconazole, with an average $K_i$ of $10.7 \pm 4.2 \text{mM}$ and $10.4 \pm 2.9 \text{mM}$ for liver and intestinal microsomes, respectively. A two-tailed $t$ test of mean $K_i$ values, assuming equal variance, revealed no significant interorgan differences for ketoconazole ($P = .74$) or fluconazole ($P = .93$) inhibition of hepatic versus intestinal CYP3A4.

Experiments were conducted to ensure that inhibited rates of midazolam (4 $\mu$M) metabolism (in the presence of 50 nM ketoconazole or 30 $\mu$M fluconazole) over the 4-min incubation interval were independent of microsomal protein concentration. For ketoconazole, the rates were 53.2, 51.9, and 57.2 pmol/min/mg protein for incubations with 25, 50, and 100 $\mu$g of protein, respectively; for fluconazole, the rates were 156, 138, and 125 pmol/min/mg for incubations with 25, 50, and 100 $\mu$g of protein, respectively.

Lineweaver-Burk and Dixon plots for the effect of fluconazole on midazolam 1'-hydroxylation catalyzed by a representative liver (HL-127) containing CYP3A4/5 are depicted in Fig. 5. Visual inspection of the Dixon plot for this and a second CYP3A4/5 liver (HL-141) indicated a slight nonlinearity in the transformed data. If this information was ignored and the data fit to a single-enzyme noncompetitive inhibition model, the fluconazole $K_{i,\text{app}}$ was calculated to be 63 $\mu$M (HL-127) and 76 $\mu$M (HL-141). These two values were more than 6-fold greater than the highest $K_i$ value obtained with CYP3A4-only livers (Table 1). There was a similar discrepancy between the ketoconazole $K_i$ values computed for CYP3A4/5 versus CYP3A4-only livers. Ignoring the nonlinearity in the Dixon plot transformations for the two CYP3A4/5 livers, the ketoconazole $K_{i,\text{app}}$ values were determined to be 53.9 and 52.1 nM. Again, these values were more than 3-fold greater than the highest $K_i$ value obtained with CYP3A4-only liver microsomes.

The disagreement between the observed data points for a CYP3A4/5 liver (HL-127) and a CYP3A4-only liver (HL-129) and the idealized regression lines obtained for a single-enzyme, noncompetitive inhibition model is depicted in Fig. 6. For HL-127, but not HL-129, there was a consistent deviation from the regression lines for the two lowest concentrations of substrate. In addition, the residuals from the nonlinear fit of HL-127 were not normally distributed (not shown).

Although the rate data generated from CYP3A4/5 livers suggested a composite effect of inhibitor on two kinetically distinct enzymes, the data were insufficient to allow numerical identification of $K_m$, $V_{\text{max}}$, and $K_i$ for the two enzymes (eq. 1). Therefore, incubations were performed with cDNA-expressed CYP3A4 or CYP3A5 microsomes for a separate determination of $K_m$ and $K_i$ values. As seen in Table 2, there was a slightly higher $K_m$ value for CYP3A5- than for CYP3A4-catalyzed midazolam 1'-hydroxylation. However, both $K_m$ values...
were within the range of values measured for human liver and intestinal microsomes (Paine et al., 1997). Noncompetitive inhibition of midazolam 1'-hydroxylation was observed for both ketoconazole and fluconazole. The $K_i$ for incubation of ketoconazole with CYP3A4 was 26.7 ± 1.71 nM. This value was similar to, but slightly higher than, the $K_i$ values obtained from incubations of ketoconazole with human liver and intestinal microsomes that contained only CYP3A4 (14.9 and 17.0 nM, respectively). In contrast, the $K_i$ value for ketoconazole with cDNA-expressed CYP3A5 was approximately 4-fold higher (104 ± 23.3 nM) than either cDNA-expressed CYP3A4 or CYP3A4-only liver or intestinal microsomes. A similar differential pattern of inhibition was seen from incubations with fluconazole. The $K_i$ values for fluconazole's inhibitory effect on cDNA-expressed CYP3A4 were comparable to the $K_i$ values obtained from incubations with liver or intestinal microsomes containing only CYP3A4 (9.2 μM versus 10.4 and 10.7 μM, respectively), whereas the $K_i$ value for fluconazole and cDNA-expressed CYP3A5 was approximately 9-fold higher (85 μM) than either cDNA-expressed CYP3A4 or CYP3A4-only liver or intestinal microsomal $K_i$ parameters.

Because the incubation of midazolam for more than 4 min will result in a decline in the rate of 1'-hydroxymidazolam formation, ketoconazole and fluconazole $K_i$ parameters were also determined for cDNA-expressed CYP3A4 and CYP3A5 microsomes, with product formation rates measured over a 1 min period of incubation. The $K_i$ values obtained for both ketoconazole and fluconazole were somewhat lower than those generated from the 4 min incubation interval (Table 2); ketoconazole, 13.3 ± 1.87 nM and 142 ± 25.6 nM for CYP3A4 and CYP3A5, respectively; fluconazole, 22.1 ± 3.03 μM and 148 ± 25.6 μM for CYP3A4 and CYP3A5, respectively. The difference between 4-min and 1-min values ($\sim$2-fold) was attributed to a greater inherent time-dependent and analytical error in the determination of 1-min rate values. Nonetheless, the differences between $K_i$ values for CYP3A4 and CYP3A5 were of comparable or greater magnitude when 1-min data were used for the analysis instead of 4-min rate data.

Because the $K_i$ for the effect of inhibitor on the CYP3A5-catalyzed reaction was found to be much greater than its corresponding effect on CYP3A4, we conducted a simulation to compute the apparent microsomal $K_i$ values (calculated from eq. 2) that would be expected for fluconazole and ketoconazole on the basis of varying amounts of CYP3A5 as a percentage of total CYP3A. Simulated reaction velocities were calculated for the same substrate and inhibitor concentration matrix used in our kinetic studies. Individual enzyme kinetic parameters from Table 2 were used in the simulations. As predicted from experimental data, an increasing fraction of CYP3A5 resulted in an increase in apparent $K_i$ for both ketoconazole and fluconazole (Fig. 7). The degree of inhibition observed in eight CYP3A5-positive and eight CYP3A5-negative liver or intestinal microsomal incubations, for a single concentration of midazolam (4 μM) and inhibitor (ketocon-
azole, 50 nM; fluconazole, 30 μM), is shown in Fig. 8. Both ketoconazole and fluconazole were less potent inhibitors of midazolam 1′-hydroxylation with liver and intestinal microsomes that contained the greatest amounts of CYP3A5 (CYP3A4/CYP3A5 microsomal content ratio, 0.5) than with those that did not. This general trend was predicted by the simulations shown in Fig. 7. The mean percent inhibition for ketoconazole was 16 and 20% lower, respectively, when a detectable amount of CYP3A5 was present in liver and intestinal microsomal incubations, than when it was absent. The difference was slightly higher for fluconazole, with an average reduction of 23% in liver microsomes and 24% in intestinal microsomes. The difference in the percentage of inhibition for CYP3A5-positive livers, when compared to livers containing only CYP3A4, was significant for both ketoconazole (P < .05) and fluconazole (P < .005).

**Discussion**

In comparison to its expression in other tissues of the body, CYP3A4 is expressed at relatively high levels in both the liver and the mucosal epithelium of the small intestine. Both hepatic and intestinal enzyme have been implicated in the first-pass metabolism of several drugs, including midazolam (Paine et al., 1996; Thummel et al., 1996), cyclosporine (Kolars et al., 1991; Hebert et al., 1992; Gomez et al., 1995), verapamil (Fromm et al., 1996), and nifedipine (Holtbecker et al., 1996). The in vitro catalytic function of CYP3A is reported to be quite sensitive to the chemical composition of its immediate (lipid and aqueous) environment (Imaoka et al., 1992; Yamazaki et al., 1995) and to the abundance of key coenzymes, cytochrome b5 and cytochrome P-450 reductase (Gillam et al., 1993; Buters et al., 1994; Gillam et al., 1995). Thus, it would not be unusual to find differences in the catalytic function of CYP3A4 when it is expressed in different organs of the body. However, we have shown previously that although the specific content of CYP3A in liver or intestinal microsomes can vary significantly from one donor preparation to the next, there is little interindividual and interorgan variability in the Km for the prototypical CYP3A-catalyzed reaction, midazolam 1′-hydroxylation (Paine et al., 1997). The exception is a higher Km for mucosal microsomes isolated from distal ileum. In this study, we report that the Ki for the inhibition of midazolam 1′-hydroxylation by fluconazole and ketoconazole, and the mechanism of inhibition, was also comparable for CYP3A4 localized in the liver and proximal small intestine. By these tests of enzyme function, intestinal and hepatic CYP3A4 appears to behave in an identical fashion.

From the perspective of total enzyme and total intrinsic clearance,

![Graph](image1.png)

**Fig. 6.** Goodness of fit of the fluconazole-midazolam interaction in liver microsomes containing CYP3A4-only or CYP3A4/5.

Regression lines were generated from a fit of a nonlinear equation for a single-enzyme catalyzed reaction and noncompetitive inhibition to product formation data for HL-129 (a) and HL-127 (b).

**TABLE 2**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Inhibition Type</th>
<th>( V_{\text{max}} ) pmol/min/pmol</th>
<th>( K_{m} ) μM</th>
<th>( K_{i} ) nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ketocanol</td>
<td>CYP3A4 Noncompetitive</td>
<td>5.57 ± 0.661</td>
<td>3.13 ± 0.194</td>
<td>26.7 ± 1.71 nM</td>
</tr>
<tr>
<td>Ketocanol</td>
<td>CYP3A5 Noncompetitive</td>
<td>4.62 ± 0.568</td>
<td>4.64 ± 1.15</td>
<td>109 ± 19.7 nM</td>
</tr>
<tr>
<td>Fluconol</td>
<td>CYP3A4 Noncompetitive</td>
<td>3.87 ± 0.137</td>
<td>2.36 ± 0.215</td>
<td>9.21 ± 0.51 μM</td>
</tr>
<tr>
<td>Fluconol</td>
<td>CYP3A5 Noncompetitive</td>
<td>4.76 ± 0.423</td>
<td>4.13 ± 0.739</td>
<td>84.6 ± 12.9 μM</td>
</tr>
</tbody>
</table>

*a For each inhibitor parameter estimates (± asymptotic S.E.) were obtained from a nonlinear regression fit of the complete data set.
enzymes differ on the basis of their interaction with ketoconazole and fluconazole is not inconsistent with an apparent, comparable affinity toward midazolam. Several groups of investigators (Kerr et al., 1994; Shou et al., 1994; Gallagher et al., 1996; Ueng et al., 1997), have presented evidence for simultaneous binding of two substrate molecules, or a substrate and enzyme activator, to a single CYP3A molecule. Because the mechanism of inhibition for ketoconazole and fluconazole was noncompetitive in this study (classically interpreted to result from coincident binding of substrate and inhibitor to the enzyme) for both enzymes, it is possible that CYP3A4 and CYP3A5 contain separate substrate and inhibitor enzyme-binding sites or, alternatively, a single active site that can accommodate midazolam and inhibitor simultaneously. That not all substrate-inhibitor pairs need necessarily bind to CYP3A simultaneously is evidenced by the fact that some inhibitors cause competitive inhibition of CYP3A. Because of the atypical nature of the CYP3A active site, an inhibitor might exhibit competitive inhibition toward one substrate and noncompetitive inhibition toward another. This appears to be the case for fluconazole-CYP3A interactions. Fluconazole displays predominantly competitive inhibition toward the CYP3A4-catalyzed 10-hydroxylation of (R)-warfarin (Kunze et al., 1995), but noncompetitive inhibition toward midazolam 1'-hydroxylation (Tables 1 and 2). Interestingly, despite a difference in inhibition mechanism, $K_i$ values from the two studies were similar (18 and 11 μM). This suggests a single fluconazole binding site that, when occupied, prevents (R)-warfarin but not midazolam access to the substrate binding site.

In the analysis of rate data for liver microsomes that contained both CYP3A4 and CYP3A5, neither the Edaie-Hofstee plot nor the Lineweaver-Burk plot provided an indication of the phenomenon. For two enzymes with similar $K_m$ and $V_{max}$ values for a given substrate, such as CYP3A4 and CYP3A5, the degree of nonlinearity observed in the Dixon plot will depend on the difference in $K_i$ values for each enzyme and the molar fraction of each enzyme as a percent of the total (Fig. 7). An increase in the molar fraction of CYP3A5 drives the apparent $K_i$ upward from the lower $K_i$ values associated with CYP3A4. Correspondingly, the percentage of inhibition observed with microsomes containing a greater amount of CYP3A5 than CYP3A4 was reduced well below that observed when CYP3A5 content in liver or intestinal microsomes was negligible (Fig. 8).

Although the percentage of inhibition that was observed with the hepatic and intestinal microsomes used in this study varied between 2- and 4-fold (Fig. 8), it is conceivable that, with a much larger population, differences could be even greater. The differences exposed in this study suggest that the variable presence of CYP3A5 for metabolic studies with any substrate-inhibitor pair may confound the interpretation of kinetic results in an unpredictable manner, leading to misleading conclusions about inhibitor potency and variability in $K_{i,app}$. More comprehensive studies with the individual enzymes and CYP3A5-deficient and -positive human tissue preparations may be necessary to fully characterize an inhibitory interaction. However, because of the acknowledged peculiarities associated with the CYP3A active site, it is possible that results obtained with midazolam may not apply to interactions between ketoconazole or fluconazole and other CYP3A substrates. Additional comparative (CYP3A4 versus CYP3A5) interaction studies with a broad spectrum of substrates are warranted.

Clinically, the presence of variable CYP3A5/CYP3A4 enzyme expression in the gut and liver may contribute significantly to the interindividual variability associated with ketoconazole- and flucon-
azolediazepam interactions. Furthermore, the noncompetitive mechanism of CYP3A inhibition by ketoconazole and fluconazole may have important implications for substrates like midazolam that undergo extensive first-pass metabolism. At the nonsaturating substrate concentrations that are likely to be encountered in systemic blood, a competitive and noncompetitive inhibitor with equivalent Ki values would exert comparable inhibitory effects at comparable inhibitor concentrations. In contrast, a noncompetitive inhibitor would cause a far greater reduction in first-pass product formation than a competitive inhibitor if saturating substrate concentrations were encountered in the enterocyte, and substrate and inhibitor were simultaneously administered.

In summary, our studies show that the mechanism and potency of ketoconazole and fluconazole inhibition of midazolam 1'-hydroxylation is the same for liver, intestinal, and cDNA-expressed microsomes that do not contain appreciable amounts of CYP3A5, relative to CYP3A4. This finding provides a necessary foundation for studies designed to expose factors that impinge on interorgan comparisons of the magnitude of drug-drug interactions. We have also shown that CYP3A5 was less susceptible to inhibition by ketoconazole and fluconazole and suggest that this may contribute to interindividual variability in the magnitude of a drug-drug interaction encountered in vivo.

References


