Inhibition of Human CYP3A Catalyzed 1'-Hydroxy Midazolam Formation by Ketoconazole, Nifedipine, Erythromycin, Cimetidine, and Nizatidine

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INTRODUCTION

The human CYP3A subfamily of cytochromes P450 contains perhaps the most important cytochromes P450 involved in drug metabolism. The human CYP3A subfamily has been shown to be composed of 4 genes (1). Two of the cytochromes P450 encoded for by these genes are so highly related that they can not be differentiated and are thus referred to as CYP3A3/4 (1,2). These cytochromes P450 have been shown through a variety of techniques to be responsible for the metabolism of a large number of structurally diverse endogenous agents and xenobiotics including cortisol, erythromycin, nifedipine, midazolam, and terfenadine (2). The other two members of the CYP3A subfamily are CYP3A5 and CYP3A7 (1,2). Little is known about the catalytic activity of CYP3A5 which has been detected in only about 25% of the adult human liver samples examined (2). Similarly, the catalytic activity of CYP3A7 is largely unknown, however, it is the major cytochrome P450 present in the human fetal liver (2).

Because of the large number of compounds metabolized by the members of the CYP3A subfamily the potential for drug-drug interactions to occur is substantial. The result of drug-drug interactions can be of great clinical significance. For example, the inhibition of CYP3A-mediated terfenadine metabolism by ketoconazole (3) or erythromycin (4) results in plasma levels of terfenadine that can be cardiotoxic and potentially life threatening. Due to the potential of an interaction with terfenadine, the question arose concerning the ability of the H_2-receptor antagonist nizatidine to inhibit the CYP3A-mediated metabolism. Therefore, the goal of the current studies was to use a form-selective catalytic marker of CYP3A, 1'-hydroxy midazolam formation, to model the inhibition kinetics of CYP3A using two clinically relevant inhibitors, erythromycin and ketoconazole; a substrate of CYP3A, nifedipine; and two H_2-receptor antagonists, cimetidine and nizatidine.

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MATERIALS AND METHODS

Midazolam, 1'-hydroxy midazolam and 4-hydroxy midazolam were gifts from Hoffmann La Roche (Nutley, NJ). Nizatidine was synthesized at Eli Lilly and Co. (Indianapolis, IN). Ketoconazole was a gift from Janssen Pharmaceutica (Piscataway, NJ). Cimetidine, erythromycin, nifedipine, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, β-NADPH, and flunitrazepam were obtained from Sigma (St. Louis, MO).

Human liver sample HL-O was obtained under a protocol approved by the Human Research Committee of the Medical College of Virginia (Richmond, VA). Microsomes were prepared by differential centrifugation as described (5) and were found to contain 0.21 nmol of CYP3A3/4/mg protein with no detectable CYP3A5 (2).

Microsomal incubations were carried out as described by Kronbach et al. (6) with slight modification. Incubation mixtures contained 0.1 mg protein, 66 mM Tris-HCl buffer (pH 7.4), NADPH generating system (0.4 U glucose-6-phosphate dehydrogenase; 1 mM NADPH; 1 mM glucose 6-phosphate; 5 mM $MgCl_2$), and midazolam with or without inhibitor in a final volume of 200 μl. The formation of the metabolites of midazolam under the conditions described was linear for 5 minutes. The reaction was stopped with 200 μl of either methanol or cold methanol: acetonitrile (35:21; v:v) with the latter resulting in a more consistent precipitation of the denatured protein. Internal standard, flunitrazepam, in methanol was added to the tubes. The denatured protein was removed by centrifugation of the tubes for 10 min at 3000 × g and 50–100 μl of supernatant subjected to HPLC analysis.

The HPLC analysis of midazolam, 4-hydroxy midazolam and 1'-hydroxy midazolam, was carried out by modifications of the method of Kronbach et al. (6). A Hewlett-Packard series 1050 HPLC system was used with the UV variable wavelength detector operated at 220 nm. A Dupont Zorbax RX-C18 column (Mac-Mod Analytical, Inc., Chadds Ford, PA), 4.6 × 250 mm was used with a precolumn 0.2 mm frit or a Zorbax RX-C18 guard column. The isocratic mobile phase of 10 mM potassium phosphate, pH 7.4:Methanol:Acetonitrile (44:35:21; v:v:v) was delivered at 1.0 ml/min. Under these conditions, flunitrazepam, 4-hydroxy midazolam, 1'-hydroxy midazolam, and midazolam eluted at respectively, 7.5, 8.5, 10.3, and 17.5 min. Chromatographic data were collected through a Model 941 analog/digital interface (PE Nelson Systems Inc., Cupertino, CA) and were analyzed with the software package, Access*Chrom, version 1.8 (PE Nelson).

The kinetic parameters for 1'hydroxy midazolam formation were determined assuming the involvement of a single catalytic site, by nonlinear regression analysis using PCNONLIN v.4.0 (Statistical Consultants Inc., Lexington, KY). The data was fitted using conventional relationships for competitive, non-competitive, uncompetitive, and mixed-type inhibition (7). Type of inhibition was determined by goodness of fit based on standard error of the parameter estimate, the residual sum of squares, the distribution of residuals, and visual inspection of the double reciprocal plots of the data.

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RESULTS AND DISCUSSION

The formation of 1′-hydroxy midazolam has been demonstrated to be a form-selective catalytic activity of human CYP3A (2,6). Using a preparation of human liver microsomes containing relatively high levels of CYP3A3/4 (0.21 nmol CYP3A3/4/mg of protein), the kinetics of 1′-hydroxy midazolam formation were determined. The formation of 1′-hydroxy midazolam was observed to follow simple Michaelis-Menten kinetics. The kinetic parameters of apparent Km, 9.7 ± 0.5 μM, and Vmax, 2212 ± 27 pmol formed/min/mg protein, were estimated by nonlinear regression analysis.

Ketoconazole, erythromycin, nifedipine, cimetidine, and nizatidine were examined for their ability to inhibit the formation of 1′-hydroxy midazolam. The rates of formation of 1′-hydroxy midazolam were modeled using nonlinear regression analysis for competitive, non-competitive, uncompetitive, and mixed inhibition relationships. The kinetics of the inhibition of CYP3A was visualized by fitting the curves obtained by nonlinear regression analyses to the Lineweaver-Burk equation (Figure 1). Ketoconazole was demonstrated to be a high affinity, Ki = 0.11 μM, non-competitive inhibitor of CYP3A catalytic activity (Table 1, Figure 1). Erythromycin (Ki = 194 μM), nifedipine (Ki = 22 μM), and cimetidine (Ki = 268 μM) were all shown to be competitive inhibitors of CYP3A (Table 1, Figure 1). Nizatidine was found to be an extremely poor non-competitive inhibitor (Ki = 2860 μM) of midazolam 1′-hydroxylation (Table 1, Figure 1).

In vitro models have been developed to predict the clinical significance of drug-drug interactions (8,9). In the extrapolation of *in vitro* results to the clinic several factors should be taken into account. First, the degree to which the clearance of the drug is dependent upon the route of metabolism being inhibited is important. Second, the relative binding affinities (ratio of Ki to Km) of the inhibitor and the drug for the enzyme can be useful in predicting the clinical significance of inhibition. Finally, the concentrations of the drug and inhibitor at the site of metabolism is important. *In vitro* experiments can be readily performed that determine the enzymes responsible for the formation of the metabolites of a drug that are important for the clearance of the drug. Furthermore, the results reported here and elsewhere demonstrate (8,9) that *in vitro* experiments can be used to determine the relative binding affinities (Ki/Km) of the inhibitor and substrate for the enzyme. However, the concentration of the inhibitor and substrate at the site of the enzyme in the liver cannot be determined with microsomal studies, although it may be possible to use human hepatocytes as a model.

Of the compounds examined in this study, ketoconazole was found to be the most potent inhibitor of CYP3A. Specifically, ketoconazole was a high affinity non-competitive inhibitor of CYP3A. Its low Ki (0.11 μM) in comparison to the Km (~10 μM) for the formation of 1′-hydroxy midazolam (Ki/Km ≅ 0.01) indicates that ketoconazole is a potent inhibitor of CYP3A. The Km obtained for 1′-hydroxy midazolam formation indicates that midazolam has a relatively high affinity for CYP3A. Therefore, ketoconazole, which reaches a peak plasma concentration of ~10 μM (10), would be expected to be a potent inhibitor for all CYP3A substrates.

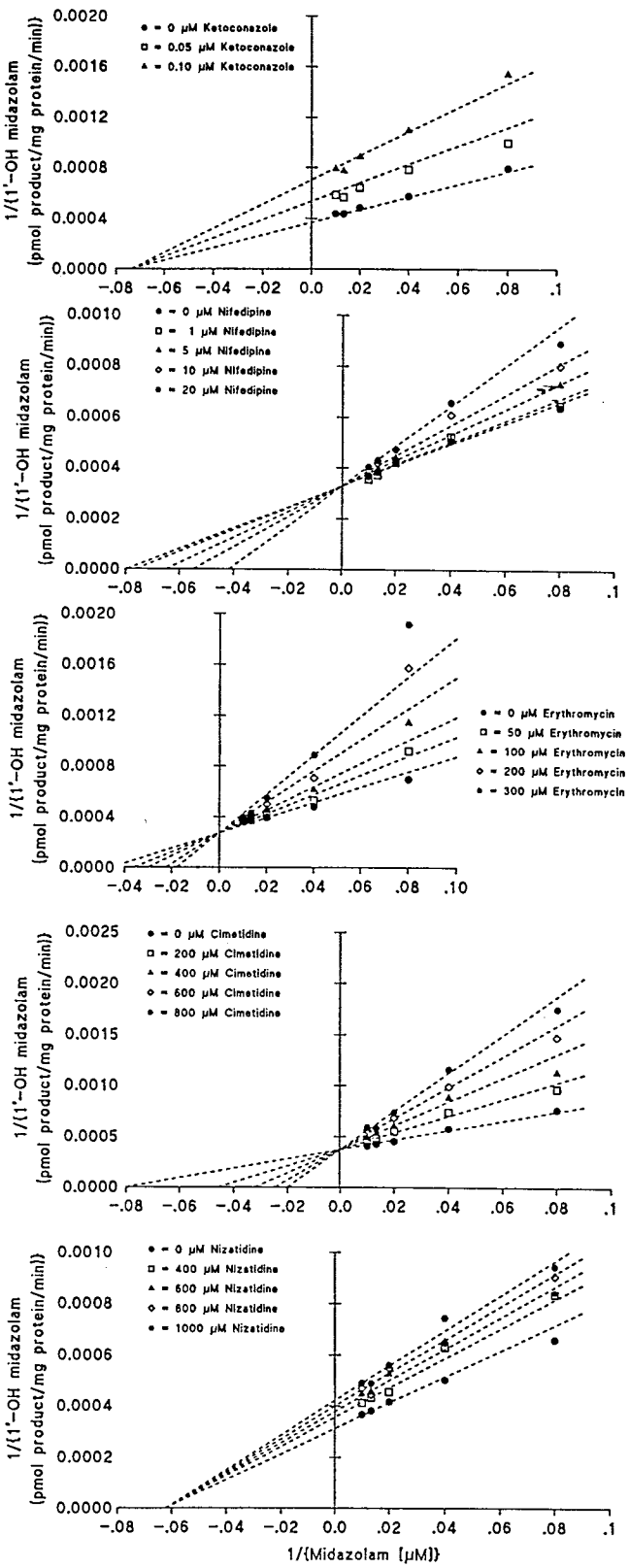

Fig. 1. The effect of (from top to bottom) ketoconazole, nifedipine, erythromycin, cimetidine, and nizatidine on the 1′-hydroxylation of midazolam by human liver microsomes. The data points represent the average of duplicate determinations. The type of inhibition kinetics was determined by nonlinear regression analyses and are visualized by fitting the results of the analyses to the Lineweaver-Burk equation.

Table I. Characteristics of the inhibition 1'-hydroxy midazolam formation. Incubations, in duplicate, contained midazolam at one of five concentrations (12.5, 25, 50, 75, or 100 μM) and carrier or one of four concentrations of the various inhibitors. The concentrations of the inhibitors were: ketoconazole, 0.05, 0.1, 0.5, and 1.0 μM; nifedipine, 1, 5, 10, and 20 μM; erythromycin, 50, 100, 200, and 300 μM; cimetidine 200, 400, 600, and 800 μM; and nizatidine, 400, 600, 800, 1000 μM.

Compound	Type of Inhibition	Ki (μM)
Ketoconazole	non-competitive	0.11 ± 0.01
Nifedipine	competitive	22.1 ± 2.4
Erythromycin	competitive	194 ± 23
Cimetidine	competitive	268 ± 20
Nizatidine	non-competitive	2860 ± 232

Nifedipine was demonstrated here to be a competitive inhibitor of 1'-hydroxy midazolam formation. However, in clinical use nifedipine with an effective plasma concentration of ~0.2 μM (11) would be predicted to have limited potential to inhibit the metabolism of midazolam and other high affinity substrates since its Ki of ~20 μM is greater than the Km (~10 μM) for 1'-hydroxy midazolam formation (Ki/Km ≅ 2).

Erythromycin is a substrate for CYP3A and has been used as an *in vitro* and *in vivo* probe of its activity (2). Erythromycin was found to be a relatively low affinity competitive inhibitor of CYP3A. In fact, based on only the competitive nature of the interaction between erythromycin and midazolam (Ki/Km ≅ 20), one can predict that clinically erythromycin, which reaches a peak plasma concentration of about 3 μM (10), is a poor inhibitor of the metabolism of high affinity CYP3A substrates. Thus, the above prediction based on the Ki to Km ratio does not explain the large number of interactions with erythromycin that have been reported. However, these interactions can be readily explained when the ability of erythromycin and certain other macrolide antibiotics, like triacetyloleandomycin, to form metabolic-intermediate complexes with CYP3A is taken into account (2). Triacetyloleandomycin, unlike erythromycin, rapidly complexes all the CYP3A present (12). Erythromycin does form a metabolic-intermediate complex but at a much reduced rate and extent compared to triacetyloleandomycin. Therefore, it often requires several days of treatment with erythromycin before a significant difference in the pharmacokinetics of a co-administered CYP3A substrate can be detected (4). Thus, the current data indicate that a single erythromycin dose has little effect on the metabolism of midazolam and other high and moderate affinity CYP3A substrates. However, upon repeated administration of erythromycin, a substantial amount of the metabolic-intermediate complex will be formed which should decrease the metabolism of all CYP3A substrates. It is interesting to note that the progressive inhibition of CYP3A by erythromycin described above was recently reported to result in an adverse drug-drug interaction between erythromycin and terfenadine (4).

Cimetidine is an imidazole-containing H_2-receptor antagonist which has shown to inhibit the metabolism of a large number of drugs (12,13). The current data indicates that cimetidine is a low affinity competitive inhibitor of 1'-hydroxy midazolam formation. With an Ki to Km ratio of about 25, the co-administration of cimetidine, which is effective at a concentration of about 4 μM (10), with midazolam would be expected to result in little competitive inhibition of midazolam metabolism or the metabolism of other high to moderate affinity CYP3A substrates. However, it should be noted that the imidazole group of cimetidine complexes with several cytochromes P450 and that with chronic cimetidine use it appears that multiple cytochromes P450 are inhibited (12,13).

Nizatidine is a H_2-receptor antagonist that does not contain an imidazole group and thus unlike cimetidine it is not generally associated with drug interactions caused by the inhibition of the cytochromes P450. Nizatidine was found to be an extremely poor inhibitor of 1'-hydroxy midazolam formation. The Ki/Km ratio obtained for the inhibition of 1'-hydroxy midazolam formation by nizatidine was about 280:1 which is nearly 30,000-fold higher than that obtained for ketoconazole. This very high Ki/Km ratio indicates that nizatidine even at its peak plasma concentration of about 10 μM (10) has virtually no potential to inhibit the metabolism of any CYP3A substrate.

In conclusion, the results presented here indicate that ketoconazole is a potent inhibitor of CYP3A, nifedipine is a moderate inhibitor of CYP3A, and nizatidine is essentially not an inhibitor of CYP3A. *In vitro* studies can be used to predict drug-drug interactions and to guide clinical trials as described by Peck, et al. (14). However, the current results with erythromycin and cimetidine indicate that in predicting drug-drug interactions, it is not always sufficient to examine the kinetics of the direct interaction since other factors like the ability of the compounds to form complexes with the cytochromes P450 may also play a role.

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