INHIBITION OF CYTOCHROMES P450 BY ANTIFUNGAL IMIDAZOLE DERIVATIVES

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ABSTRACT:

The interactions of a panel of antifungal agents with cytochromes P450 (P450s), as a means of predicting potential drug-drug interactions, have not yet been investigated. The objective of this study was to evaluate the specificity and selectivity of five antifungal agents using selective probe reactions for each of the eight major P450s. The index reactions used were phenacetin O-deethylation (for CYP1A2), coumarin 7-hydroxylation (CYP2A6), diclofenac 4'-hydroxylation (CYP2C9), omeprazole 5-hydroxylation (CYP2C19), dextromethorphan O-demethylation (CYP2D6), 7-ethoxy-4-trifluoromethylcoumarin deethylation (CYP2B6), chlorozoxazone 6-hydroxylation (CYP2E1), and omeprazole sulfonation (CYP3A4). Five antifungal agents that include an imidazole moiety ( clotrimazole, miconazole, sulconazole, tioconazole, and ketoconazole) were examined in cDNA-expressing microsomes from human lymphoblast cells or human liver microsomes. All inhibitors studied demonstrated nonselective inhibition of P450s. Ketoconazole seemed to be the most selective for CYP3A4, although it also inhibited CYP2C9. High-affinity inhibition was seen for CYP1A2 (sulconazole and tioconazole K_i, 0.4 μM), CYP2B6 (miconazole K_i, 0.05 μM; sulconeazole K_i, 0.04 μM), CYP2C19 (miconazole K_i, 0.05 μM; sulconeazole K_i, 0.008 μM; tioconazole K_i, 0.04 μM), CYP2C9 (sulconeazole K_i, 0.01 μM), CYP2D6 (miconazole K_i, 0.70 μM; sulconeazole K_i, 0.40 μM), CYP2E1 (tioconazole K_i, 0.4 μM), and CYP3A4 (clotrimazole K_i, 0.02 μM; miconazole K_i, 0.03 μM; tioconazole K_i, 0.02 μM). Therefore, this class of compounds is likely to result in significant drug-drug interactions in vivo.

Hepatic cytochrome P450 enzymes (P450s) constitute a superfamily of hemoproteins that play a major role in the metabolism of endogenous compounds and in the detoxification of xenobiotic molecules. CYP1A2, 2A6, 2B6, 2C9, 2C19, 2D6, 2E1, and 3A4 are the most important forms in humans, mediating the metabolism of about 70% of therapeutic drugs and endogenous compounds (Shimada et al., 1994). The interactions of any substance being considered for therapeutic use must first be evaluated with the major P450s to fully understand its efficacy and potential adverse reactions, such as drug-drug interactions.

Antifungal imidazole derivatives are frequently used both systemically and topically (depending on the particular agent) in the treatment of systemic candidal infections and mycoses. These derivatives, including ketoconazole (KET), miconazole (MIC), tioconazole (TIO), clotrimazole (CLO), and sulconazole (SUL), are recognized as potent ligands of the heme iron atom of P450s (Fig. 1) (Sheets et al., 1986; Maurice et al., 1992; Katz, 1999). The interactions of antifungal imidazole derivatives with P450 enzymes have been studied to an extent, with information on all of the major P450s and some newer antifungals lacking. KET is frequently used as a CYP3A-selective inhibitor in in vitro P450 identification studies. KET has shown to be a selective inhibitor up to 10 times its K_i value in human liver microsomes (Bourrie et al., 1996). However, another study using cDNA-expressing microsomes demonstrated 90% inhibition of CYP1A1-catalyzed 7-ethoxy-coumarin N-deethylation at 5 μM KET with an IC_50 value of 0.33 μM, lower than the IC_50 value obtained with CYP3A4 (0.44 μM). CYP2C8/9/19 were also inhibited when the KET concentration was increased (Sai et al., 2000). Using midazolam as a substrate in human liver microsomes, CLO was shown to inhibit CYP3A4 with a K_i value of 0.25 nM (Gibbs et al., 1999). Although specific, its selectivity for CYP3A4 seems poor. In another study in human liver microsomes, CLO was shown to potently inhibit CYP2A6-mediated coumarin 7-hydroxylation (K_i, 0.42 μM) (Draper et al., 1997). In keeping with the lack of CYP3A selectivity of CLO, MIC has been shown to strongly inhibit both CYP3A and CYP2A6 in vitro (Maurice et al., 1992; Draper et al., 1997). Moreover, in vivo both flucnazole and MIC have shown to potently inhibit CYP2C9, as demonstrated by clinically significant drug interactions observed in the presence of concomitant CYP2C9 substrates, including warfarin and phenytoin (Blum et al., 1991; Black et al., 1996; Laine et al., 2000; Venkataraman et al., 2000). Little information exists on the P450 interaction profile of TIO. Experiments conducted in vitro in mouse hepatic P450s demonstrated TIO inhibition of CYP3A-dependent drug metabolism.
testosterone hydroxylation with IC_{50} values ranging from 0.18 to 3.3 μM (Ballard et al., 1988). The P450 interaction profile of SUL is largely unknown, with further studies necessary.

Although data exist on the interactions of selected P450s with various imidazole derivatives, the interactions of a panel of these compounds with all of the major P450s in vitro has not yet been performed. Moreover, pharmacokinetic information on some antifungals (TIO, SUL) is lacking. Our study was therefore conducted to evaluate the selectivity of several N-substituted imidazole derivatives currently used as antifungal or antibacterial agents [clotrimazole, miconazole, sulconazole, tioconazole, and ketoconazole (Fig. 1)] toward the major cDNA-expressing P450s from human lymphoblast cells. These studies will shed further light on likely in vivo drug-drug interactions of this class of compounds, which is important given the high likelihood of concomitant drug therapy.

Materials and Methods

Chemicals and Reagents. Budipine, chloroxazone, clotrimazole, cotinine, coumarin, dextromethorphan hydrobromide, dextrophan, diclofenac sodium, diethyldithiocarbamate, 7-ethoxy-4-trifluoromethylcoumarin (7-ETC), 7-hydroxycoumarin, 7-hydroxy-4-trifluoro-methylcoumarin, ketoconazole, miconazole, α-naphthoflavone, reduced NADPH, nicotine, orphenadrine, phenacetin, sulconazole, sulfaphenazole, and tranylcypromine were purchased from Sigma Chemical (St. Louis, MO). Fluconazole was extracted from Diflucan (Pfizer, Montreal, PQ, Canada) using ethyl acetate. Tioconazole was extracted from GyneCare (Pfizer) also using ethyl acetate. Omeprazole, omeprazole sulfone, and 5-hydroxymeproprazole were generously donated by Astra Hassle (Moldnal, Sweden). 6-Hydroxychloroxazone and 4'-hydroxyclofencos sodium were purchased from GENTEST (Woburn, MA). All other chemicals and reagents used were of the highest commercially available quality.

cDNA-Expressing P450s and Human Liver Microsomes. cDNA-expressing P450s (1A2, 2A6, 2B6, 2C9, 2C19, 2D6, 2E1, and 3A4) from human lymphoblasts and baculovirus insect cell systems were purchased from GENTEST. Pooled liver microsomes from human liver samples were prepared according to standard procedures (Tyndale et al., 1989).

P450 Index Reaction Assays. K_{m} and V_{max} values were determined in human liver microsomes for the index reactions used for which incubation conditions and method references are listed in Table 1. The index reaction metabolite for each P450 was quantified by interpolating peak area ratios of the respective metabolite and the internal standard from a standard curve of known metabolite concentrations. For each substrate (probe drug), preliminary experiments were performed to determine whether metabolite formation was linear with respect to time, NADPH, and microsomal protein concentrations. The percent conversion of all metabolites never exceeded 15% of the total substrate added. Assay variation ranged from 1.3 to 7.2% based on results from two different days; detection limits ranged from 0.025 to 0.05 μM for the different probe assays. The analytical system used was a Hewlett-Packard (HP) 1100 series UV-liquid chromatographic system (Palo Alto, CA).

Index reactions for CYP2B6 were a modification of that of Ekins et al. (1997). After a 15-min incubation at 37°C of 7-ETC (0.25–100 μM) with human liver or cDNA-expressing microsomes (final concentration, 0.2 mg/ml) in the presence of 1 mM NADPH (as was used for all assays), trioxalen was added as the internal standard, and the mixture was extracted with ethyl acetate. The organic phase was evaporated to dryness and reconstituted into 200 μl of mobile phase before high-performance liquid chromatography analysis [HP Spherisorb ODS2 column; UV, 280 nm; acetonitrile–H₂O/acid (45:55:0.1) at 1 ml/min].

Index reactions for CYP2C9 were a modification of that of Leemann et al. (1993). After a 20-min incubation of microsomes (0.1 mg/ml) with diclofenac (0.2–40 μM), coumarin was added as the internal standard, and the mixture was extracted with ethyl acetate. The organic phase was evaporated to dryness and reconstituted in 200 μl of 20% acetic acid before high-performance liquid chromatography analysis [HP Spherisorb ODS2 column; UV, 280 nm; acetonitrile–H₂O/acid (40:60:0.25) at 1 ml/min].

Index reactions for CYP1A2, 2A6, 2C19, 2E1, and 3A4 were described by the corresponding references listed in Table 1. Conditions were the same in both human liver (KET experiments) and cDNA-expressing microsomes.

Chemical Inhibition Studies. Initial screening experiments were carried out using two concentrations of inhibitor (1 and 10 μM or 20 and 200 μM), and known selective P450 inhibitors were selected as controls according to previously published reports (Bourrie et al., 1996; Eagling et al., 1998; Hichman et al., 1998). The controls were α-naphthoflavone (CYP1A2), pilocarpine (CYP2A6), orphenadrine (CYP2B6), sulfaphenazole (CYP2C9), S(+)-methylenedioxytriphenylamine (CYP2C19), budipine (CYP2D6), and ketoconazole (CYP3A4). Of note, orphenadrine has not shown to be CYP2B6-selective in previous studies.

<table>
<thead>
<tr>
<th>P450</th>
<th>Index Reaction</th>
<th>Final Protein Concentration</th>
<th>Incubation Time</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A2</td>
<td>Phenacetin 0-deethylation</td>
<td>2.0</td>
<td>25</td>
<td>Rodrigues et al. (1997)</td>
</tr>
<tr>
<td>2A6</td>
<td>Coumarin 7-hydroxylation</td>
<td>0.2</td>
<td>10</td>
<td>Shumada et al. (1996)</td>
</tr>
<tr>
<td>2B6</td>
<td>7-ETC demethylation</td>
<td>0.2</td>
<td>15</td>
<td>Ekins et al. (1997)</td>
</tr>
<tr>
<td>2C9</td>
<td>Diclofenac 4’-hydroxylation</td>
<td>0.1</td>
<td>20</td>
<td>Leemann et al. (1993)</td>
</tr>
<tr>
<td>2C19</td>
<td>Omeprazole 5-hydroxylation</td>
<td>0.4</td>
<td>30</td>
<td>Yamazaki et al. (1997)</td>
</tr>
<tr>
<td>2D6</td>
<td>Dextromethorphan 0-demethylation</td>
<td>0.15</td>
<td>30</td>
<td>Rodrigues et al. (1996)</td>
</tr>
<tr>
<td>2E1</td>
<td>Chloroxazone 6-hydroxylation</td>
<td>0.4</td>
<td>25</td>
<td>Lucas et al. (1996)</td>
</tr>
<tr>
<td>3A4</td>
<td>Omeprazole sulfonation</td>
<td>0.4</td>
<td>30</td>
<td>Yamazaki et al. (1997)</td>
</tr>
</tbody>
</table>
(Guo et al., 1997; Sai et al., 2000); however, a more selective alternative CYP2B6 inhibitor is not presently available. Probe substrate concentrations used for each P450 were identical to $K_m$ values obtained in metabolic studies of these substrates in human liver microsomes. Subsequent to screening and to determine apparent $K_i$ values, probe-drug final concentrations equaled $1/2K_m$, $K_m$, and $2K_m$ for each index reaction. Inhibitor concentration equaled $1/4IC_{50}$, $1/2IC_{50}$, $IC_{50}$, and $2IC_{50}$, with $IC_{50}$ referring to the concentration of inhibitor required to inhibit 50% of substrate metabolism (at the $K_m$ concentration). In the case of inhibitors that have previously demonstrated mechanism-based inactivation of P450s [orphenadrine (Reidy et al., 1989)], the inhibitor was preincubated with microsomes and NADPH for 30 min before the addition of substrate.

Data Analysis. $K_m$ and $V_{max}$ values were determined by use of nonlinear regression analysis by Michaelis-Menten kinetics (rate of metabolite formation against substrate concentration) with Enzpack 3 software (Cambridge, UK). Inhibitory patterns were determined with Dixon and Cornish-Bowden plots. $K_i$ values for competitive inhibition were estimated through Dixon plots or by using Pharm/PCS software (Springer-Verlag, NY).

Results

Kinetic Studies of Index Reactions. Apparent $K_m$ and $V_{max}$ values and intrinsic clearances ($V_{max}/K_m$) for all substrates in human liver microsomes were in agreement with those found in previous studies (data not shown) (Leemann et al., 1993; Andersson et al., 1994; Bourrie et al., 1996; Li et al., 1997; Rendic and Di Carlo, 1997; von Moltke et al., 1997; Eagling et al., 1998). Kinetic analyses indicated that coumarin 7-hydroxylation, diclofenac 4'-hydroxylation, and omeprazole sulfoxidation were characterized by single-enzyme kinetics in human liver microsomes. However, phenacetin O-deethylation, 7-ETC O-deethylation, omeprazole 5-hydroxylation, dextromethor-
P450 inhibition by clotrimazole and its analogs in microsomes from human lymphoblast cells

<table>
<thead>
<tr>
<th>P450</th>
<th>Clotrimazole</th>
<th>Miconazole</th>
<th>Sulconazole</th>
<th>Tioconazole</th>
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<tbody>
<tr>
<td>1A2</td>
<td>7.9</td>
<td>3.2</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>2A6</td>
<td>2.7</td>
<td>2.2</td>
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<tr>
<td>2C9</td>
<td>12.0</td>
<td>1.2</td>
<td>0.010</td>
<td>0.70</td>
</tr>
<tr>
<td>2C19</td>
<td>1.4</td>
<td>0.010</td>
<td>0.008</td>
<td>0.040</td>
</tr>
<tr>
<td>2D6</td>
<td>13.0</td>
<td>0.70</td>
<td>0.40</td>
<td>1.0</td>
</tr>
<tr>
<td>2E1</td>
<td>67.0</td>
<td>7.3</td>
<td>3.5</td>
<td>0.38</td>
</tr>
<tr>
<td>3A4</td>
<td>0.018</td>
<td>0.028</td>
<td>0.10</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Our observation that KET is capable of inhibiting CYP3A4 and to a lesser extent 2C9 is in agreement with previous studies (Sai et al., 2000). CYP1A1 was not investigated in the present study because of its general low involvement in therapeutic drug metabolism. In addition to previous studies demonstrating inhibition of CYP3A4 and 2A6, CLO was shown to potently inhibit CYP2C19. The coadministration of CLO with low therapeutic index compounds in which clearance primarily depends on CYP2C19 may thus be potentially hazardous. Previous studies showed inhibitory interactions between MIC and CYP3A4, 2A6, and 2C9 in vitro and in vivo (Blum et al., 1991; Maurice et al., 1992; Black et al., 1996; Draper et al., 1997; Laine et al., 2000). Our data show that in addition to these, MIC potently inhibits CYP2C19, 2D6, 2B6, and less so CYP1A2 in vitro in cDNA-expressing microsomes. Therefore, one may expect significant in vivo inhibition of several of the major P450s, posing potential drug interactions with a number of drugs metabolized by these enzymes. Little information exists concerning the P450 interaction profiles of SUL and TIO. In the present study, we observed nonselectivity with both compounds, with low micromolar and submicromolar K_i values obtained with all P450s investigated (Table 2). For SUL in particular, one would expect significant inhibition of CYP3A4 and 2D6 with low micromolar K_i values. With TIO, potent inhibition was observed with all of these aforementioned P450s, as well as CYP2E1. Therefore, among all the antifungals tested, TIO and SUL demonstrated the greatest nonselectivity and should be highlighted as compounds where significant in vivo drug interactions may occur. The N-substituted group at the imidazole moiety seems to affect the inhibition of CYP3A4; the K_i value of sulconazole (100 nM) is about 5 times less potent than clotrimazole (18 nM), miconazole (28 nM), and tioconazole (20 nM). The lipophilicity of side chains is also a determining factor in the inhibitory potency for CYP3A4 (Smith et al., 1998), as it is with other P450s also. The effects of such structural relationships on the inhibition of CYP3A4 have been observed and discussed (Ballard et al., 1988; Maurice et al., 1992). The potency of P450 inhibition observed with CLO, MIO, SUL, and TIO suggests that the imidazole group is important to P450 inhibition in vitro. Such potency of enzyme inhibition has been previously observed with imidazole-substituted compounds (Ballard et al., 1988), perhaps through orientation of the imidazole in close proximity to the heme iron of the P450. Indeed, KET, which possesses a large N-substituted group not present in the other imidazole-containing compounds examined, demonstrated much greater CYP3A4 selectivity than all of the other compounds tested (Fig. 2). In addition, the inhibitory capability of these compounds depends on the P450; therefore, the active sites of P450s probably determine whether an N-substituted group at

Discussion

Relevant in vitro studies of the inhibition of cDNA-expressing P450s by a particular drug in microsomes can be useful in assessing its potential for interaction with other drugs in vivo (Crespi and Pennman, 1997). Our purpose was to ascertain the interactions of a panel of antifungal agents with an imidazole moiety toward the major P450s; to do this, we used reconstituted systems containing the respective purified enzyme proteins, expressing the major P450s involved in drug metabolism and their respective P450 probe reactions.
the imidazole moiety is able to facilitate the coordination of the imidazole nitrogen atom to the hemoprotein or hinder the interaction. Clearly, further detailed studies are necessary to determine the structure-function relationships of this class of compounds.

The purpose of this study was to investigate and compare the P450 interaction profiles of various antifungal imidazole derivatives. The results indicate that these compounds in general, with the exception of KET, lack P450 selectivity. The very low K<sub>i</sub> values observed across several P450s indicate that care should be taken when administering these compounds with other P450-interacting compounds, particularly those with low therapeutically indices. Further imidazole and nonimidazole containing antifungals should be studied to better understand differences in potency and selectivity of P450 inhibition within this class of compounds.

References


