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), chloroxazone 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; sulconazole K_i, 0.04 μM), CYP2C19 (miconazole K_i, 0.05 μM; sulconazole K_i, 0.008 μM; tioconazole K_i, 0.04 μM), CYP2C9 (sulconazole K_i, 0.01 μM), CYP2D6 (miconazole K_i, 0.70 μM; sulconazole K_i, 0.40 μM), CYP2E1 (tioconazole K_i, 0.40 μM), and CYP3A4 (clo-trimazole 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 vitro and so CYP3A4 identification studies. KET has shown 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; sulconazole K_i, 0.04 μM), CYP2C19 (miconazole K_i, 0.05 μM; sulconazole K_i, 0.008 μM; tioconazole K_i, 0.04 μM), CYP2C9 (sulconazole K_i, 0.01 μM), CYP2D6 (miconazole K_i, 0.70 μM; sulconazole K_i, 0.40 μM), CYP2E1 (tioconazole K_i, 0.40 μM), and CYP3A4 (clo-trimazole 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.

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testosterone hydroxylation with IC50 values ranging from 0.18 to 3.3 \( \mu \)M (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, chlorzoxazone, clotrimazole, cotinine, coumarin, dextromethorphan hydrobromide, dextrophan, diclofenac sodium, diethyldithiocarbamate, 7-ethoxy-4-trifluoromethylcoumarin (7-ETC), 7-hydroxycoumarin, 7-hydroxy-4-trifluoro-methylcoumarin, ketoconazole, miconazole, \( \alpha \)-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-Hydroxychlorzoxazone and 4′-hydroxyclofenac 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 lymphoblast 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 \( V_{\text{max}} \) 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 \( \mu \)M 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 \( \mu \)M) 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), trioxsalen 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 \( \mu \)l of mobile phase before high-performance liquid chromatography analysis [HP Spherisorb ODS2 column; UV, 280 nm; acetonitrile/H2O/acetic acid (40:60:0.25) 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 \( \mu \)M), 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 \( \mu \)l of 20% acetic acid before high-performance liquid chromatography analysis [HP Spherisorb ODS2 column; UV, 280 nm; acetonitrile/H2O/acetic 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 \( \mu \)M or 20 and 200 \( \mu \)M), and known selective P450 inhibitors were selected as controls according to previously published reports (Bourrie et al., 1996; Eagling et al., 1998; Hickman et al., 1998). The controls were \( \alpha \)-naphthoflavone (CYP1A2), pilocarpine (CYP2A6), orphenadrine (CYP2B6), sulfaphenazole (CYP2C9), S(+)-mephentoin (CYP2C19), budipine (CYP2D6), and ketoconazole (CYP3A4). Of note, orphenadrine has not shown to be CYP2B6-selective in previous studies.

**TABLE 1**

<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 O-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>0.15</td>
<td>30</td>
<td>Yamazaki et al. (1997)</td>
</tr>
<tr>
<td>2D6</td>
<td>Dextromethorphan O-demethylation</td>
<td>0.4</td>
<td>25</td>
<td>Rodrigues et al. (1996)</td>
</tr>
<tr>
<td>2E1</td>
<td>Chloroazoxane 6-hydroxylation</td>
<td>0.4</td>
<td>30</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-
pharmacological O-demethylation, and chlorozoxazone 6-hydroxylation exhibited biphasic kinetics. For these, the substrate concentration was carefully selected to equal the \( K_m \) values for the production of metabolite by the P450 enzyme of interest.

**P450 Inhibition Screening with Antifungals.** Figure 2 shows the inhibition profiles of the imidazole derivatives KET, CLO, MIC, SUL, and TIO with the major P450s in cDNA-expressing microsomes. CLO, MIC, SUL, and TIO to some extent inhibited all the P450s tested. CLO strongly inhibited (<15% remaining activity) CYP2A6, 2B6, 2C9, 2C19, and 3A4, with minimal effects on CYP1A2, 2D6, and 2E1 at 20 and 200 \( \mu M \). MIO showed high potency of inhibition with all P450s tested, except for CYP1A2 and 2E1. SUL seemed to strongly inhibit all P450s examined, whereas TIO potently inhibited all P450s except for CYP2E1. It seems that CYP2C9, 2C19, 2B6, and 3A4 were all uniformly potently inhibited by the four imidazoles investigated. In contrast, the interaction of KET, an imidazole with a larger N-substitution than the other imidazole studies, seemed to be a more selective CYP3A4 inhibitor in human liver microsomes. KET seemed to strongly inhibit CYP3A4 by almost 100% at 20 and 200 \( \mu M \), with CYP2C9 being inhibited by approximately 20% at 20 \( \mu M \).

**Potency of P450 Inhibition by Imidazole Derivatives.** As shown in Table 2, low micromolar and submicromolar \( K_i \) values were observed for the compounds examined across different P450s. Every P450 was associated with \( K_i \) values in this range by at least one of the compounds. MIO, SUL, and TIO inhibited CYP2C19, 2C9, 2B6, and 2D6, with \( K_i \) values in the nanomolar or low micromolar range. SUL and TIO also potently inhibited CYP1A2, both with a \( K_i \) value of 0.4 \( \mu M \). Furthermore, TIO inhibited CYP2E1, with a \( K_i \) value of 0.4 \( \mu M \). CYP2A6 inhibitory potencies were similar for CLO, MIC, SUL, and TIO, with \( K_i \) values ranging from 1.7 to 2.7 \( \mu M \). CLO also inhibited CYP2C19, with a \( K_i \) value of 1.4 \( \mu M \). All compounds examined inhibited CYP3A4 strongly, with \( K_i \) values in the nanomolar range (18–100 \( Nm \)). \( K_i \) values across P450s were not determined for KET because of the relative P450 selectivity demonstrated in the inhibition screening experiments (Fig. 2).

**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 Penman, 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.

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 CYP1A2, 2B6, 2C9, 2C19, 2D6, and 3A4, which all demonstrated submicromolar \( K_i \) 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, MIC, 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

<table>
<thead>
<tr>
<th>P450</th>
<th>Clotrimazole</th>
<th>Miconazole</th>
<th>Sulconazole</th>
<th>Tioconazole</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A2</td>
<td>7.9</td>
<td>3.2</td>
<td>0.40</td>
<td>0.40</td>
</tr>
<tr>
<td>2A6</td>
<td>2.7</td>
<td>2.2</td>
<td>2.0</td>
<td>1.7</td>
</tr>
<tr>
<td>2B6</td>
<td>12.0</td>
<td>0.050</td>
<td>0.040</td>
<td>0.10</td>
</tr>
<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>

**Table 2**

*P450 inhibition by clotrimazole and its analogs in microsomes from human lymphoblast cells.*
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_i values observed across several P450s indicate that care should be taken when administering these compounds with other P450-interacting compounds, particularly those with low therapeutic 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


