POTENT INHIBITION OF CYTOCHROME P-450 2D6-MEDIATED DEXTROMETHORPHAN O-DEMETHYLATION BY TERBINAFINE

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ABSTRACT:
Cytochrome P-450 (CYP) 2D6 is responsible for the biotransformation of over 35 pharmacologic agents. In the process of studying CYP2D6 we identified phenotype-genotype discordance in two individuals receiving terbinafine. This prompted evaluation of the potential for terbinafine to inhibit CYP2D6 in vitro. Human hepatic microsomes and heterologously expressed CYP2D6 were incubated with terbinafine or quinidine and the formation of dextrorphan from dextromethorphan was determined by HPLC. Additionally, preliminary conformational analyses were conducted to determine the fit of terbinafine into a previously described pharmacophore model for CYP2D6 inhibitors. The apparent $K_m$ and $V_{max}$ of dextrorphan formation from four human hepatic microsome samples ranged from 5.8 to 6.8 μM and from 172 to 300 pmol/min/mg protein, respectively. Values of $K_m$ and $V_{max}$ in the heterologously expressed CYP2D6 system averaged 6.5 ± 2.1 μM and 1342 ± 147 pmol/min/mg protein, respectively. Terbinafine inhibited dextromethorphan O-demethylation with an apparent $K_i$ ranging from 28 to 44 nM in human hepatic microsomes and averaging 22.4 ± 0.6 nM for the heterologously expressed enzymes. Results of quinidine in these systems produced values for $K_i$ ranging from 18 to 43 nM. Such strong inhibition of CYP2D6 by terbinafine would not have been predicted by the previously proposed pharmacophore model of CYP2D6 inhibitors based on molecular structure. Terbinafine is a potent inhibitor of CYP2D6 with apparent $K_i$ values well below plasma and tissue concentrations typically achieved during a therapeutic course. This agent needs to be evaluated in vivo to determine the impact of CYP2D6 inhibition by terbinafine on the metabolism of concomitantly administered CYP2D6 substrates.

Characterization of cytochrome P-450 (CYP) $^1$ 2D6 activity in our laboratory led to the discovery of CYP2D6 phenotype-genotype discordance in two individuals who were receiving terbinafine for the treatment of onychomycosis (Leeder et al., 1998). Terbinafine is the most recent oral agent to become available for the treatment of superficial dermatophytosis and has, as a purported therapeutic advantage over currently existing agents, a lack of interaction with the CYPs (Hernandez, 1980; Schuster, 1985). Preliminary conformational analyses were conducted to determine the fit of terbinafine into a previously described pharmacophore model for CYP2D6 inhibitors. The apparent $K_m$ and $V_{max}$ of dextrorphan formation from four human hepatic microsome samples ranged from 5.8 to 6.8 μM and from 172 to 300 pmol/min/mg protein, respectively. Values of $K_m$ and $V_{max}$ in the heterologously expressed CYP2D6 system averaged 6.5 ± 2.1 μM and 1342 ± 147 pmol/min/mg protein, respectively. Terbinafine inhibited dextromethorphan O-demethylation with an apparent $K_i$ ranging from 28 to 44 nM in human hepatic microsomes and averaging 22.4 ± 0.6 nM for the heterologously expressed enzymes. Results of quinidine in these systems produced values for $K_i$ ranging from 18 to 43 nM. Such strong inhibition of CYP2D6 by terbinafine would not have been predicted by the previously proposed pharmacophore model of CYP2D6 inhibitors based on molecular structure. Terbinafine is a potent inhibitor of CYP2D6 with apparent $K_i$ values well below plasma and tissue concentrations typically achieved during a therapeutic course. This agent needs to be evaluated in vivo to determine the impact of CYP2D6 inhibition by terbinafine on the metabolism of concomitantly administered CYP2D6 substrates.

Materials and Methods

**Human Hepatic Microsome Assays.** Dextromethorphan (DM) was used as a surrogate marker of CYP2D6 activity as it is preferentially metabolized via O-demethylation by CYP2D6 to its primary metabolite dextrorphan (DX; Schmid et al., 1985). Control studies to determine the kinetics of DX formation from DM were conducted according to previously published methods (Pearce et al., 1996). All assays were performed in round bottom, 96-well microtiter plates (Fisher Scientific, Pittsburgh, PA). Briefly, 0.25 mg/ml of human liver microsomes, phenotyped for CYP2D6 activity (Gentest Corp., Woburn, MA), were placed in a shaking incubator at 37 ± 1°C with potassium phosphate buffer (50 mM, pH 7.4), MgCl$_2$ (3 mM), EDTA (1 mM), and varying

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concentrations of DM (0–100 μM) in a total volume of 100 μl. The reaction was initiated by the addition of an NADPH-generating system (5 mM glucose 6-phosphate, 1 U/ml glucose 6-phosphate dehydrogenase, 1 mM β-NADP) and subsequently terminated after 30 min by the addition of an equal volume of cold methanol containing 1.7 μg/ml of the internal standard levallophan tetrarotate (Hoffmann-La Roche, Nutley, NJ). The protein was sedimented by centrifugation at 3000 rpm and 4°C for 10 min (Beckman GS-6R, Palo Alto, CA) and the supernatant was analyzed for total DX formed by HPLC as described below.

Inhibition studies were conducted in five different human hepatic microsome samples using terbinafine (0, 0.05, 0.1, 0.2, 0.5, 0.75, 1, and 2 μM) and quinidine (0, 0.02, 0.03, 0.04, 0.0625, and 0.125, μM), a previously characterized CYP2D6 inhibitor (Otton et al., 1984). Stock solutions of DM were prepared in water. Stock solutions of terbinafine and quinidine were prepared in dimethyl sulfoxide and methanol, respectively, and subsequently diluted in the reaction wells to a final concentration of 1%. An equivalent volume of solvent was added to the control wells. Previous studies evaluating the effect of methanol and dimethyl sulfoxide demonstrate only slight to no diminution of CYP2D6 activity at solvent concentrations of 1% v/v (Chauret et al., 1998; Hickman et al., 1998). A separate set of studies was performed as described above except that microsomes, inhibitor, and NADPH-generating system were incubated for 10 min before the addition of DM to initiate the reaction. Three DM substrate concentrations were evaluated in duplicate with each assay. Substrate concentrations were selected to bracket the Km determined from the initial experiments. All experiments were performed in triplicate.

Heterologously Expressed CYP2D6 Activity. Assays using baculovirus-expressed CYP2D6 were performed according to the procedures described above for the human hepatic microsomes. Control studies to determine the kinetics of DX formation in the baculovirus system were conducted and subsequent inhibition assays were performed as above with 0.05 mg/ml of protein (204 pmol P-450/mg protein) (Genest). The remainder of experimental conditions were identical with those stated above. All assays were conducted in 100 μl total volume and were allowed to incubate for 30 min. Three DM substrate concentrations were evaluated with each assay and all experiments were performed in triplicate.

Analytical Procedure. DX concentrations were determined by a validated HPLC assay adapted from the previously published method of Lam and Rodriguez (1993). An aliquot of supernatant (25 μl) was injected onto a Novapak Phenyl column (3.9 × 150 mm). The mobile phase consisted of buffer (20 mM potassium phosphate, 20 mM hexane sulfonic acid, pH 4.0) and acetonitrile (65:35) at a flow rate of 1.2 ml/min. Chromatography was performed with fluorescence detection on a Hewlett-Packard 1046 Programmable Fluorescence detector (Hewlett-Packard, Palo Alto, CA) with excitation and emission wavelengths of 235 and 310 nm, respectively. All chromatography was performed at 50°C. Data were collected using Hewlett-Packard Chemstation V A.04.01 software. External and internal standard were prepared on the day of analysis from a stock solution in a potassium phosphate buffer. A five-point standard curve using the peak-height ratio of active compound to internal standard used to calculate all DX concentrations. The limit of detection for the assay was 0.1 μM. The analytical method demonstrated linearity over the range of standard concentrations evaluated, 0 to 3 μM (r² > 0.999). Intraday and interday assay variability for DX concentrations between 100 and 0.1 μM ranged from 1.9 to 4.8 and 2.8 to 10%, respectively.

Molecular Modeling. Computer-simulated molecular modeling was performed to identify whether terbinafine (Fig. 2.1) fit a previously proposed pharmacophore model of competitive inhibitors for CYP2D6 (Strobl et al., 1993). Molecular modeling was performed using the Insight II/Discover molecular modeling suite (Molecular Simulations, Inc.) A systematic conformational search of terbinafine was performed according to the default torsion-forcing methodology within the Discover module. The four bonds designated χ1 (C$_{\text{β-naphth}}$-C$_{\text{naphth}}$-C$_{\text{methylene}}$-N), χ2 (C$_{\text{methylene}}$-N-C$_{\text{methylene}}$-C$_{\text{methylene}}$-N), χ3 (C$_{\text{methylene}}$-N-C$_{\text{methylene}}$-C$_{\text{alkene}}$), and χ4 (N-C$_{\text{methylene}}$-C$_{\text{alkene}}$-C$_{\text{alkene}}$) (Fig. 3) were selected for constraint. These bonds were rotated within 360° by 30° (χ1 and χ4) or 60° (χ2 and χ3) increments. Conformations within 5 kcal/mol of the lowest energy conformation were collected and the rest discarded. Removal of torsional constraints and molecular mechanics minimization (conjugate; cvff force field, electrostatics included) resulted in two global minima (E$_{\text{rel}}$ = 0.0 kcal/mol) and eight low-energy local minima (E$_{\text{rel}}$ = 0.8–2.9 kcal/mol). These conformations (minimized set) were considered a reasonable estimate of available (populated), local minima for the purpose of this preliminary investigation. Five conformations in which the nitrogen was inverted were also generated during the minimization process and were discarded. The minimized set could be further grouped into three conformational families based on χ1 and χ2. Differences within the families occur in χ3 and χ4. Family one (χ1 = 80–82°; χ2 = 63–70°) contains three local minima (E$_{\text{rel}}$ = 2.7–2.9 kcal/mol). Family two (χ1 = 79–85°; χ2 = 74°) is comprised of one of the global minima and two local minima (E$_{\text{rel}}$ = 1.5 and 2.9 kcal/mol). Family three (χ1 = 105–112°; χ2 = 160–174°) contained the second global minimum and three local minima (E$_{\text{rel}}$ = 0.8, 1.6 and 2.6 kcal/mol).

Data Analysis. The kinetic parameters of DX formation (i.e., V$_{\text{max}}$ and K$_{\text{m}}$) were estimated from the best fit line using least-squares linear regression of inverse substrate concentration versus inverse velocity (Lineweaver-Burk plots) and the mean values were used to calculate kinetic parameters V$_{\text{max}}$ and K$_{\text{m}}$. Inhibition data were graphically represented by Dixon plots and the apparent inhibition rate constants were calculated from the intersection of the best fit through the line determined by inhibitor concentration versus inverse velocity.

Results

In Vitro Inhibition Assays. The kinetics of DX formation in our microsome samples were similar to those reported previously for DM turnover by CYP2D6. Average K$_{\text{m}}$ and V$_{\text{max}}$ for DX formation in our microsome samples ranged from 5.8 to 6.8 μM and from 172 to 300 pmol/min/mg protein, respectively, and were similar to previously established values (Kerry et al., 1994). The V$_{\text{max}}$ was considerably higher in the heterologously expressed CYP2D6 system (1342.0 ± 146.6 pmol/min/mg protein); however, K$_{\text{m}}$ (6.5 ± 2.1 μM) was consistent with those values observed in the microsome systems.

The formation of DX from DM was inhibited by terbinafine in all microsome samples evaluated and in the heterologously expressed system. Apparent enzyme kinetic parameters from these experiments are reported in Table 1. Under competitive assay conditions (i.e., all components incubated simultaneously) terbinafine displayed potent inhibition with K$_{i}$ values ranging from 28.6 to 44.6 nM in human hepatic microsomes and averaging 22.4 ± 0.6 nM in the heterologously expressed system (Fig. 1). The apparent K$_{i}$ for quinidine run concurrently resulted in values between 17.8 to 42.9 nM, which are comparable to those reported by other investigators (Broly et al., 1989). Preincubation of microsome with terbinafine and the NADPH-

<table>
<thead>
<tr>
<th>Density</th>
<th>K$_{m}$</th>
<th>K$_{i}$ Terbinafine</th>
<th>K$_{i}$ Quinidine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human Microsome H023</td>
<td>225.7 ± 25.8</td>
<td>32.7 ± 19.2</td>
<td>17.8</td>
</tr>
<tr>
<td>Human Microsome H056</td>
<td>171.9 ± 15.7</td>
<td>33.2 ± 0.1</td>
<td>21.5</td>
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<tr>
<td>Human Microsome H066</td>
<td>300.9 ± 75.4</td>
<td>28.6 ± 4.8</td>
<td>39.4</td>
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<tr>
<td>Human Microsome H042</td>
<td>187.3 ± 9.7</td>
<td>44.6 ± 24.7</td>
<td>42.9</td>
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Values above represent the mean ± S.D. of three experiments.

Table 1: Kinetic parameters for the formation of DX from DM via O-demethylation and its inhibition by terbinafine and quinidine.
Fig. 1. Representative Dixon plots for terbinafine-induced inhibition of CYP2D6-mediated DX formation in H023 (a), H056 (b), H066 (c), H042 (d), and baculovirus (e) expressed CYP2D6.
generating system before addition of substrate generated essentially equivalent values for $K_i$ (data not shown).

**Molecular Modeling.** Computer-simulated molecular modeling was performed to identify whether terbinafine (Fig. 2.1) fit a previously proposed pharmacophore model of competitive inhibitors for CYP2D6. Strobl et al. (1993) developed a preliminary template for their model using ajmalicine (Fig. 2.2), a potent inhibitor of CYP2D6, the structure of which is relatively inflexible (i.e., few rotatable bonds). The model was subsequently refined using a number of structurally similar compounds of varying potency and conformational integrity. The authors put forth criteria required for CYP2D6 inhibitors which included: 1) a positively charged nitrogen atom at physiologic pH, 2) a flat hydrophobic moiety extending maximally 7.5 Å from the nitrogen, the plane of which is almost perpendicular to the N-H axis (region A), and 3) a less well defined hydrophobic moiety containing two electronegative sites located 4.8 to 5.5 and 6.6 to 7.5 Å from the protonated nitrogen (region B). The proposed model appears to favor heteroatom-containing functionalities of relatively low polarity (e.g., ether, ester) at this site, presumably improving inhibitory activity via hydrogen-bonding with CYP2D6. According to this model, inhibitors fulfilling these requirements are excellent CYP2D6 inhibitors. Molecules that partially fulfill these criteria exhibit diminished inhibition.

As expected, based on flexibility of the terbinafine molecule, a large number of conformations were generated. As a tertiary amine, terbinafine satisfies criterion 1 in the model of Strobl et al. (1993). We had initially anticipated the aromatic naphthyl moiety to occupy region A (criterion 2); however, our molecular modeling clearly showed that the naphthalene moiety could not adopt a position perpendicular to the N-H axis of protonated terbinafine. Placing the planar 2-ene-4-yne moiety in region A proved more satisfying. Although neither of the global minima possessed an appropriate conformation to fit the model, several of the local minima provided a reasonable overlay of the enyne moiety and the aromatic region of ajmalicine (see Fig. 4). Working from these local minima, only conformers of family three occupied region B. The best fit of the pharmacophore model was provided by the conformation shown in Fig. 3 ($E_{rel} = 2.6$ kcal/mol; $\chi_1 = 85^\circ$, $\chi_2 = -74^\circ$, $\chi_3 = 155^\circ$, $\chi_4 = 156^\circ$). Note that the terbinafine naphthalene group does not appear to adequately occupy the molecular volume that the pharmacophore model suggests for region B (and exhibited by ajmalicine). However, how this space is to be utilized in the absence of H-bonding functionality was not addressed by Strobl et al. (1993) and it is this lack of functionality that is the most glaring exception to the pharmacophore model. Thus, this terbinafine conformation cannot be dismissed as a CYP2D6 inhibitor. Nevertheless, the failure of a global minimum conformation to provide an appropriate enyne conformation (for region A) and the uncertainty surrounding the placement of the naphthyl moiety in region B would predict marginal inhibitory potency at best. Yet we have shown terbinafine to be a potent inhibitor of CYP2D6.

Considering that the previous pharmacophore model emphasized the importance of heteroatoms in region B for strong inhibition, we took the opportunity to investigate whether a metabolite of terbinafine might be a better candidate for the inhibition of CYP2D6. Several catechol metabolites of terbinafine have been identified (Jensen, 1989), specifically 3,4- and 5,6-naphthalene diol metabolites (Figs. 2.5 and 2.6) whose functional groups would provide inhibition-enhancing H-bonding groups in region B based on our modeling results. Accordingly, our results would suggest the 3,4-diol metabolite as a likely candidate. Terbinafine metabolite 2.5 was constructed from the family three conformation shown in Fig. 3. Nitrogen-oxygen distances of 5.8 and 6.6 Å were obtained for 3-OH and 4-OH, respectively (criterion 3). These distances are consistent with the oxygen atoms of the methoxycarbonyl group of ajmalicine. A 20° clockwise rotation about the N-H axis of the terbinafine molecule shown in Fig. 4 nearly superimposes the 3,4-diol and methoxycarbonyl oxygens of the terbinafine metabolite and ajmalicine, respectively, while maintaining the overlap of the enyne and aromatic moieties in region A. For this putative inhibitor, it appears that more confidence can be placed in criterion 3.

**Discussion**

The overall significance of CYP2D6 in the biotransformation of a given substrate is influenced by the quantitative importance of alternative metabolic routes. For agents that are preferentially metabolized by CYP2D6, pharmacologic inhibitors can modify enzyme activity such that the magnitude of change in substrate metabolism may mimic that of genetically determined poor metabolizers (i.e., an apparent change in phenotype from an extensive metabolizer to a poor metabolizer). With inhibitors of CYP2D6, the metabolism of coadministered CYP2D6 substrates may be significantly altered in close to 93% of the population classified as extensive metabolizers (Brosen and Gram, 1989). Such interactions may decrease the efficacy of a prodrug requiring metabolic conversion to its active moiety or, alternately, may result in toxicity for CYP2D6 substrates that have a narrow therapeutic index.

Our results demonstrate that terbinafine inhibits CYP2D6 in vitro on the same order of magnitude as quinidine, a well characterized potent CYP2D6 inhibitor. Presumably, this inhibition results from the binding of terbinafine and/or one of its metabolites to CYP2D6 in a

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**Fig. 2.** Two-dimensional molecular structure of select CYP2D6 inhibitors including terbinafine (1), ajmalicine (2), quinidine (3), 2-methoxy-4,5-methyleneoxyamphetamine (4), 3,4-naphthalene diol metabolite (5), and 5,6-naphthalene diol metabolite (6).

**Fig. 3.** Three-dimensional structure for terbinafine conformation which best fit a previously proposed pharmacophore model of competitive CYP2D6 inhibitors.
manner sufficient to reduce catalytic activity. Inhibition of $DX$ formation occurred at nanomolar concentrations, with only a 2-fold difference observed in apparent $K_i$ between the lowest and highest values, despite the relatively wide range of CYP2D6 activities between microsome samples. An apparent $K_i$ could not be accurately determined in the microsome sample with minimal CYP2D6 activity (H112) a finding consistent with terbinafine inhibition occurring at the level of this isoform. Similar apparent $K_i$ values were observed for terbinafine within the heterologously expressed CYP2D6 system further confirming the results from the microsome experiments.

Support of the argument that terbinafine may well be a CYP2D6 inhibitor was provided by our molecular modeling. Preliminary modeling was performed to see if we could find a reasonable low-energy conformation that fit a proposed pharmacophore model described by Strobl et al. (1993). Terbinafine proved to meet criteria 1 and marginally satisfy criteria 2 and 3 for a CYP2D6 inhibitor as described above; however, the best fit for criterion 3 (i.e., those proposed to be suggestive of enhanced inhibitory activity) resulted from our modeling of terbinafine metabolites rather than the parent compound.

The inexact fit of criterion 2, despite the potent inhibitory activity that we observed in vitro, may suggest occupation of region A is a minor contributor to inhibitor binding at the active site of the enzyme. Precedence for this can be found within the cinchona alkaloids and certain amphetamine analogs. The molecular modeling of quinidine (Fig. 2.3) places the small ethylene moiety of quinidine in this region (Strobl et al., 1993). Similarly, certain amphetamine analogs reported to demonstrate activity as moderately potent inhibitors of CYP2D6 (Wu et al., 1997) have no substituent in region A. For example, protonated 2-methoxy-4,5-methylenedioxyamphetamine (Fig. 2.4) exhibits a reported $K_i$ of approximately 170 nM, yet preliminary modeling suggests this compound, like terbinafine, satisfies criterion 3 by placing the phenyl moiety in region B (data not shown). In summary, criteria 1 through 3 of the pharmacophore model appear to be confirmed by this preliminary modeling study. However, the strong inhibition exhibited by terbinafine observed in this study would probably not have been predicted by the model.

Although evaluation of in vitro activity is not necessarily predictive of in vivo response, the phenotype-genotype discordance reported previously by our group along with the case report of van der Kuy et al. (1998) suggest that the magnitude of CYP2D6 inhibition by terbinafine in vivo can markedly impair the metabolism of select CYP2D6 substrates. Using the following equation, one can predict the extent of enzyme inhibition that may be observed in the presence of a given concentration of inhibitor:

$$i = \frac{[I]}{[I] + K_i(1 + [S]/K_m)}$$

where $i$ refers to the fraction inhibited, $[I]$ the concentration of inhibitor, $K_i$ the apparent inhibitor rate constant (i.e., approximately 30 nM for terbinafine), $[S]$ the concentration of substrate, and $K_m$ the concentration of substrate at half-maximal velocity (Waley, 1985). In the example of nortriptyline, the reported $K_m$ for hydroxylation of nortriptyline to (E)-10-OH-nortriptyline, a CYP2D6 mediated process, ranges from 0.48 to 0.74 μM (Olesen and Linnet, 1997). At therapeutic nortriptyline concentrations (e.g., 190–570 nM) and an average steady-state concentration for terbinafine of 1.7 μM, one would expect approximately 97% inhibition of CYP2D6-mediated nortriptyline hydroxylation. For other substrates where $[S] \ll K_m$, the fraction inhibited approaches $[I]/([I] + K_i)$, thus, one may expect up to 98% inhibition of CYP2D6-mediated metabolic processes with a therapeutic course of terbinafine.

Our data clearly suggest that CYP2D6 inhibition occurs in the presence of terbinafine. Terbinafine has become the first line agent for the treatment of the fungal nail infection, onychomycosis, in a number of countries (Finlay, 1994; Goulden and Goodfield, 1995) and its use is expanding into other dermatophyte infections. Given the prevalence of superficial dermatophytoses in all age groups of the general population, it would not be surprising to find that a significant percentage of these patients may be receiving CYP2D6 substrates and thus, be at risk for terbinafine-induced drug-drug interactions. Although the in vivo significance of CYP2D6 inhibition by terbinafine remains to be...
characterized, our data suggest that sufficient caution should be warranted in the coadministration of CYP2D6 substrates with a low therapeutic index while patients are receiving therapy with terbinafine.

References