IMPORTANCE OF AMINE pKₐ AND DISTRIBUTION COEFFICIENT IN THE METABOLISM OF FLUORINATED PROPRANOLOL ANALOGS: METABOLISM BY CYP1A2

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ABSTRACT:

A series of 1'-mono-, di-, and trifluorinated analogs of propranolol and related steric congeners was prepared, and their metabolism was examined with recombinant-expressed CYP1A2. The structural changes in this series of compounds, principally added fluorines and methyl groups in the 1'-position of the N-isopropyl group, provided compounds that varied in pKₐ by more than 5 log units, in log D by 3 log units, and in size of the added substituents. N-Desalkylation and aromatic hydroxylation (formation of the 4'- and 5'-regioisomers) were catalyzed by CYP1A2. Correlations of the log D by 3 log units, and in size of the added substituents. N-Desalkylation and aromatic hydroxylation (formation of the 4'- and 5'-regioisomers) were catalyzed by CYP1A2. Correlations of the metabolic kinetic parameters Kₘ and catalytic efficiency (k_cat/Kₘ) with physicochemical properties pKₐ and log D showed that increased lipophilicity (higher log D values) was associated with increased affinity (lower Kₘ) and increased catalytic efficiency for CYP1A2. Comparison of log Kₘ and log k_cat/Kₘ with pKₐ showed that the less basic analogs had higher affinities and increased catalytic efficiencies. The changes associated with pKₐ reflect increased lipid partitioning of substrate (increased log D) caused by an increase in the proportion of nonionized substrate. Increased steric bulk in the N-substituent alone did not decrease substrate affinity for CYP1A2 but did increase the amount of aromatic hydroxylation versus N-desalkylation. Removal of the hydroxyl group from the propanolamine side chain of propranolol resulted in a similar change in regioselectivity of metabolism.

The major metabolites of P¹ observed in vivo and in vitro result from initial cytochrome P450-catalyzed N-dealkylation to DIP, catalyzed primarily by CYP1A2, and aromatic hydroxylation to regioisomeric ring hydroxylation products, principally 4'-OHP, catalyzed primarily by CYP2D6 (Fig. 1). In humans, 3-(1-naphthoxy)lactic acid is the major urinary metabolite (Walle et al., 1985) from P. It arises from the P450-catalyzed formation of DIP via oxidative loss of the aldehyde to 3-(1-naphthoxy)lactic acid.

In human liver microsomes, the rate of N-dealkylation of P is highly correlated with the rate of phenacetin O-deethylation, a known CYP1A2-catalyzed process (Masubuchi et al., 1994; Yoshimoto et al., 1995), and the content of immunochromatically determined CYP1A2 (Masubuchi et al., 1994). α-Naphthoflavone, a potent inhibitor of CYP1A2, inhibited all of the P oxidation activities, and the IC₅₀ value for N-desisopropylationase activity was much smaller than values for ring-hydroxylase activities (Masubuchi et al., 1994).

Studies in human lymphoblastoid cell- (Yoshimoto et al., 1995) and yeast-expressed (Ching et al., 1996) recombinant human CYP1A2 have confirmed that CYP1A2 catalyzes formation of DIP and of the 4'- and 5'-hydroxylated metabolites, as well. In the presence of CYP1A2-expressed in human lymphoblastoid cells, N-dealkylation of P occurred about 2 to 3 times as fast as aromatic hydroxylation at a high-substrate concentration (Yoshimoto et al., 1995). In a yeast expression system, CYP1A2 catalyzed both aromatic hydroxylation and N-dealkylation, with the Vₘₐₓ being about 2 times higher for N-dealkylation than for 4'-hydroxylation and 8 to 40 times higher than for 5'-hydroxylation when 2R-P and 2S-P were studied separately (Ching et al., 1996).

The active site for CYP1A2 accommodates a variety of substrates with partition coefficients that vary more than 6 orders of magnitude (e.g., from benzo[a]pyrene, log P = 6.25, to caffeine, log P = 0.01). Most are neutral or weakly basic, although exceptions occur. Many of the substrates have significant planar molecular portions (e.g., aromatic hydrocarbons, nitrogenous heterocyclic systems related to MeIQ, acetanilide, caffeine, and steroids). Collating of physical chemical factors, such as log P, log D, pKₐ, molecular size and shape, dipole moment, and frontier orbital energies, has been done in attempts to differentiate between substrate selectivities of CYP isoforms (Lewis, 2000). According to this work, CYP1A2 substrates commonly include polar planar molecules with low log P values at physiological pH that are neutral or weakly basic and have rectangular surfaces, fairly high dipole moments, a hydrogen-bonding donor or acceptor
site near the site of metabolism, and solvent-accessible volume of 200 Å³ or less (De Rienzo et al., 2000). Many known substrates of CYP1A2 do not meet all of these criteria.

Although CYP1A2 has been less extensively studied than CYP2D6, protein homology models based on the crystal structures of CYP102 and CYP101 have been reported. Models based on potential substrate–protein interactions, using CYP 102 as a template, have been reported (Lewis et al., 1999b; Lozano et al., 2000; De Rienzo et al., 2000). Theses models have relatively large binding pockets composed of mostly hydrophobic and aromatic residues, with a number of polar, potentially hydrogen-bonding residues also present near the heme. In these models, substrates are placed approximately orthogonal to the heme plane. More than one mode of binding is proposed to accommodate formation of multiple regioisomeric oxidation products from substrates like caffeine. Roles have been assigned to Phe226 and Tyr495 in key bonding residues also present near the heme. In these models, substrates and aromatic residues, with a number of polar, potentially hydrogen-bonding residues also present near the heme. In these models, substrates are placed approximately orthogonal to the heme plane. More than one mode of binding is proposed to accommodate formation of multiple regioisomeric oxidation products from substrates like caffeine. Roles have been assigned to Phe226 and Tyr495 in key bonding residues also present near the heme. In these models, substrates and aromatic residues, with a number of polar, potentially hydrogen-bonding residues also present near the heme. In these models, substrates are placed approximately orthogonal to the heme plane.

A model for CYP1A2 based on the study of N-hydroxylation of several congeners of MeIQ, mutagens that are weakly basic planar heterocyclic nitrogen-containing ring systems, has been proposed and refined (Lozano et al., 1997, 2000). Regions that are assigned interactions include in SRS-1, Thr115, Asp119, Thr124, Phe125; in SRS-2, Thr223 and Phe226; in SRS-4, Thr321; in SRS-5, Leu382 and Pro383; and in SRS-6, Gly223. Based on catalytic efficiencies for metabolism of MeIQ, 7-ethoxyresorufin and phenacetin, a series of CYP1A2 mutants, have been studied (Parikh et al., 1999; Josephy et al., 2000). Mutants Phe226Ile, Phe226Thr, and Phe226Tyr had reduced catalytic efficiencies for metabolism of these substrates. Mutations at other sites produced varying activities depending on the substrate.

Earlier point mutation studies in SRS-3 of rat CYP1A2 showed that changes that increase the hydrophobic character of this region resulted in greater catalytic activity for methoxy- and ethoxyresorufin dealkylation (Krainev et al., 1992). Mutations of Thr319 (Thr321 in CYP1A2) in SRS-1, Thr115, Asp119, Thr124, Phe125; in SRS-2, Thr223 and Phe226; in SRS-4, Thr321; in SRS-5, Leu382 and Pro383; and in SRS-6, Gly223. Based on catalytic efficiencies for metabolism of MeIQ, 7-ethoxyresorufin and phenacetin, a series of CYP1A2 mutants, have been studied (Parikh et al., 1999; Josephy et al., 2000). Mutants Phe226Ile, Phe226Thr, and Phe226Tyr had reduced catalytic efficiencies for metabolism of these substrates. Mutations at other sites produced varying activities depending on the substrate.

As a known substrate of CYP1A2, P meets most of the criteria proposed by Lewis (1999a) and can serve as a model on which to make systematic structural changes to test some of the requirements for metabolism by CYP1A2. The series of fluorinated and nonfluorinated analogs, which varied in physicochemical properties including basicity, lipophilicity, and substituent size (Tables 1 and 2) and showed significant changes in metabolism by baculovirus-insect cell expressed human recombinant CYP2D6 (Upthagrove and Nelson, 2001), were examined in CYP1A2. Changes in observed Michaelis-Menten kinetic parameters were correlated to changes in physicochemical properties, and the regioselectivity of oxidation was examined.

Materials and Methods

Propranolol-Related Substrates and Standards. Propranolol enantiomers (25-P and 2R-P) and, log P and log D values for these compounds were also obtained as described in the accompanying paper (Upthagrove and Nelson, 2001).

Basicity and Lipophilicity. The pKₐ, log P, and log D values (Table 1) for this series of amines (Upthagrove and Nelson, 2001) were determined at pH 7.4. (Cambridge, MA), using their validated potentiometric method (Avdeef et al., 1993). The log P and log D values for these compounds were also determined at pH 7.4, according to their validated potentiometric method (Avdeef, 1992; Slater et al., 1994).

Incubations in Insect Cell Expressed Human CYP1A2. Baculovirus-insect cell microsomes coexpressing human CYP2D6 and human P450 reductase were purchased from GENTEST (Woburn, MA). Duplicate incubation mixtures, having a final volume of 200 μl, contained 10 pmol of CYP1A2/ml, 100 mM phosphate buffer (pH 7.4), and 4 μl of a solution of substrate in a 1:1 mixture of CH₃OH and H₂O (final CH₃OH concentration, 1%). After preincubation at 37°C for 3 min, NADPH in phosphate buffer (final concentration, 1 mM) was added to initiate the reaction. After 2 min, the reaction was stopped by addition of 20 μl of 7% perchloric acid, and 5 mg of ascorbic acid was added to stabilize ring-hydroxylated metabolites. Substrate concentrations of 0.24, 0.49, 0.98, 1.95, 3.91, and 7.81 μM were used. Preliminary incubations of P and TFP were performed to establish the linearity of metabolite formation with respect to time and the amount of enzyme.

HPLC Separation. Following addition of the internal standard, incubation samples were vortexed and centrifuged to precipitate protein. One hundred microliters of the supernatant was analyzed by HPLC using a Hewlett-Packard 1100 LC system (Palo Alto, CA) with Chemstation instrument control and data analysis software. For all substrates, separation was achieved on a Microsorb-MV phenyl column (250 × 4.6 mm; Varian Corp., Walnut Creek, CA). Mobile phase gradients consisted of an aqueous phase containing 1% by volume triethylamine, 0.8% by volume phosphoric acid (to bring the pH to 2.2), and acetonitrile. The HPLC gradient used for metabolite separation from all substrates except FP and TFE was 25% (v/v) acetonitrile (75% aqueous

suggested a relatively large active site for this isoform (Tuck et al., 1993).

Some work relating structural features of CYP1A2 inhibitors to their inhibitory potency has been reported. In the inhibition of phenacetin O-deethylation by a series of quinolone antibiotics, specific binding interactions similar to the suggested interactions of caffeine in the CYP1A2 active site were proposed, but no correlation with their lipophilicity was obtained (Fuhr et al., 1993). However, the range of inhibitory potency of these compounds was very limited. In a study of inhibitory potency of three structurally similar antiarrhythmics on 7-methoxyresorufin demethylation, a combination of factors, including a charge interaction of the amine nitrogen on each of these inhibitors with Asp313 of CYP1A2 and various possible hydrogen bonding and hydrophobic interactions, were suggested (Wei et al., 1999).
buffer described above) for 10 min, then a linear increase to 70% acetonitrile over the next 10 min, an additional 2 min at 70% acetonitrile, then returned to 25% acetonitrile over the next 5 min. For metabolites from FP, the gradient was 20% acetonitrile (80% aqueous buffer) for 10 min, then a linear increase to 60% acetonitrile over the next 10 min, held at 60% acetonitrile for 3 min, and then returned to 20% acetonitrile over the next 4 min. For metabolites from TFE, the gradient was 20% acetonitrile (80% aqueous buffer) for 10 min, then a linear increase to 70% acetonitrile over the next 10 min, held at 70% acetonitrile for 2 min, and then returned to 20% acetonitrile over the next 5 min. The internal standards used were propranolol O-methyl ether for metabolites of P, FP, DFP, TFP, and bBuMe, and P for metabolites of iPMe, TFE, desOHP, and desOHTFP.

Fluorescence Detection. For fluorescence detection, tables were programmed to excitation wavelength $\lambda_ex = 295 \text{ nm}$ and emission wavelength $\lambda_em = 370 \text{ nm}$ when N-desalkyl metabolite, internal standard, and substrate eluted and continued to the end of the HPLC run. At other times during the run, fluorescence excitation and emission wavelengths were set to $\lambda_ex = 320 \text{ nm}$ and $\lambda_em = 420 \text{ nm}$ to increase sensitivity for the 4'-hydroxylated metabolite.

Metabolite Quantitation. DIP, 4'-OH, 5'-OH, 4'-OH, deshydroxyisopropylpropranolol, and 4'-hydroxydeshydroxypropranolol were quantitated by comparison of peak area ratios of these compounds formed metabolically with standard curves of peak area ratios of known amounts of synthetic standards. Amounts of other metabolites were estimated by comparison of the area ratios with standard curve ratios of known amounts of synthetic standards. Amounts of other metabolites were estimated by comparison of the area ratios of unknown compounds to those of known standards.

Metabolite Identification. Fluorescence Excitation and Emission Spectra. Unknown metabolite peaks in the HPLC eluent were collected in silane-treated tubes. The fluorescence excitation and emission spectra of each fraction were obtained after transfer to a limited volume quartz cuvette, as described in the accompanying work (Upthagrove and Nelson, 2001).

Mass Spectra. Fraction collection was repeated, and combined fractions (total approximately 2 ml) were treated with 150 $\mu l$ of aqueous saturated Na$_2$CO$_3$, which was adequate to bring the eluent solution to approximately pH 8. The solutions were then extracted with 3 ml of EtOAc, vortexed for at least 15 s to mix, then centrifuged to separate the layers. The EtOAc layer was transferred to a silane-treated concentration tube, and the solvent evaporated using a stream of dry nitrogen and heat. The metabolite residues were dissolved in 50 $\mu l$ of CH$_3$OH and 50 $\mu l$ of H$_2$O. The metabolite residues dissolved in CH$_3$OH/H$_2$O were infused into a Finnigan LCQ mass spectrometer (Thermo Finnigan, San Jose, CA) equipped with a modified electrospray ionization source at a rate of 300 nI/min. Tandem mass spectroscopy spectra were obtained by collision-induced dissociation in the ion trap.

Calculation of Michaelis-Menten Kinetic Parameters. $K_m$ and $V_{max}$ for each of the enzyme-catalyzed reactions were determined by nonlinear fitting of initial velocity versus substrate concentration curves using the k.cat program (Biometallics, Inc., Princeton, NJ). The robust weighting option was used. All velocity values deviating from the calculated curve by less than 10% from the average residual were included in the fit. The rate constants ($k_{cat}$) for metabolism were calculated by dividing the $V_{max}$ values by the enzyme concentration. Substrate concentrations were corrected for substrate depletion by using the average of the initial substrate concentration and the final substrate concentration (Segel, 1973). Final substrate concentrations were calculated by subtracting the concentrations of all metabolites from the initial substrate concentrations.

Results

Fluorinated and Steric Analogs of propranolol, $pK_a$ Values, log D, and Steric Parameters. The propranolol-related compounds for the metabolic studies are listed in Table 1. These included the 1'-mono, di-, and trifluorinated derivatives (FP, DFP, TFP) and steric congeners with two and three additional 1'-methyl groups (iPrMe, bBuMe). Additional analogs include the trifluoroethanol analog TFE and deshydroxy analogs desOHP and desOHTFP. Their $pK_a$ values and log D (pH 7.4) values (Upthagrove and Nelson, 2001) are included in Table 1. A measure of the relative sizes of the substituents is given by the Taft $E_v$ and Charton van der Waals radius $r_v$ parameters also in Table 1.

Identification and Quantitation of Metabolites. Incubations of all of the P analogs in CYP1A2 resulted in formation of the product of N-dealkylation and, in most cases, the 4'- and 5'-hydroxylated metabolites also. No other metabolites were observed. Representative chromatograms showing the retention times of the ring-hydroxylated metabolites from two of the substrates appear in the accompanying report (Upthagrove and Nelson, 2001). Ring hydroxylation regioisomers were identified as described therein.

Kinetic Parameters. The $K_m$ and $k_{cat}$ values for formation of each of the metabolites in CYP1A2 appear in Table 1. Total $k_{cat}$ values, which appear in Table 3, are the sums of the individual metabolite $k_{cat}$ values. For each substrate, there is some variability in the $K_m$ values determined for each metabolite; the $K_m$ values used in correlations with physicochemical parameters are the averages of the $K_m$ values for the individual metabolites. A small difference in the $K_m$ values and catalytic efficiencies of 2R-P and 2S-P were observed. 2S-P has a 2-fold higher $K_m$ and a 2-fold lower $k_{cat}/K_m$ than 2R-P. Among the TFP stereoisomers studied, 3-fold differences in $K_m$ and $k_{cat}/K_m$ were observed.

In this CYP1A2 system, as the number of fluorines on the carbon-beta to the amine nitrogen increases, $K_m$ decreases and total $k_{cat}/K_m$ increases. The two substrates with larger nonfluorinated alkyl groups, iPMe and bBuMe, also have $K_m$ values slightly lower than that of P, whereas TFE, the smaller trifluorinated compound, has a $K_m$ comparable to that of TFP. Removal of the side chain hydroxyl group also results in a decrease in $K_m$ relative to P (Table 3). 2R-P and 2S-P have the highest $K_m$ values, with the other substrates having higher affinities. The $K_m$ values differ about 9-fold across the series (Table 3). Larger differences in $K_m$ values for formation of the individual metabolites (Table 2) are observed.

Each of the structural changes made increases the total catalytic efficiency ($k_{cat}/K_m$) for metabolism by CYP1A2 over P itself. The $k_{cat}/K_m$ values show greater variance than the $K_m$ data, varying about 25-fold from 2S-P to 2S-TFP. More variance is observed in the $k_{cat}/K_m$ data for formation of individual metabolites (Table 2).

### Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>$pK_a$</th>
<th>log P</th>
<th>log D (pH 7.4)</th>
<th>$E_v/Tat^a$</th>
<th>Charton van der Waals Radius $r_v^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>P</td>
<td>-CH$_3$</td>
<td>9.53</td>
<td>3.48</td>
<td>1.35</td>
<td>-1.24</td>
<td>1.72 Å</td>
</tr>
<tr>
<td>F</td>
<td>-CH$_2$F</td>
<td>7.69</td>
<td>2.82</td>
<td>2.26</td>
<td>-1.48</td>
<td>1.82 Å</td>
</tr>
<tr>
<td>DFP</td>
<td>-CH$_2$F</td>
<td>6.13</td>
<td>4.06</td>
<td>4.03</td>
<td>-1.91</td>
<td>1.88 Å</td>
</tr>
<tr>
<td>TFP</td>
<td>-CF$_3$</td>
<td>4.37</td>
<td>3.66</td>
<td>3.66</td>
<td>-2.40</td>
<td>2.01 Å</td>
</tr>
<tr>
<td>iPMe</td>
<td>-CH$_2$(CH$_3$)$_2$</td>
<td>9.26</td>
<td>4.16</td>
<td>2.31</td>
<td>-1.71</td>
<td>1.96 Å</td>
</tr>
<tr>
<td>bBuMe</td>
<td>-C(CH$_3$)$_3$</td>
<td>9.02</td>
<td>4.62</td>
<td>3.00</td>
<td>-2.78</td>
<td>2.44 Å</td>
</tr>
<tr>
<td>desOHP</td>
<td>-CH$_3$(desOH)</td>
<td>10.25</td>
<td>4.14</td>
<td>1.54</td>
<td></td>
<td></td>
</tr>
<tr>
<td>desOHTFP</td>
<td>-CF$_3$(desOH)</td>
<td>5.15</td>
<td>4.27</td>
<td>4.27</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Hansch et al., 1995.

$^b$ $E_v$ = Charton van der Waals radius of H atom (Charton, 1975).
Correlation of log \( k_a \) and log \( k_{cat} \) versus \( pK_a \) and log \( k_{cat}/K_m \) versus log D with equations for the best fit lines of the data appear in Figs. 2 and 3, respectively. For this series of compounds, a significant positive correlation of log \( K_m \) with \( pK_a \) (\( r^2 = 0.47 \)) and a significant negative correlation of log \( k_{cat}/K_m \) with \( pK_a \) (\( r^2 = 0.63 \)) are observed. The more basic compounds have higher \( K_m \) values (lower affinities) and lower catalytic efficiencies. The opposite trends appear in the correlations with log D. A significant negative correlation of log \( K_m \) with log D (\( r^2 = 0.50 \)) and a significant positive correlation of log \( k_{cat}/K_m \) with log D (\( r^2 = 0.69 \)) are observed. The more lipid soluble compounds have lower \( K_m \) values (higher affinity for CYP1A2) and higher catalytic efficiencies.

**Discussion**

All of the compounds in this series are oxidized at sites analogous to the sites of metabolism in P. As would be expected for an enzyme with a relatively large active site that accommodates a variety of substrates, none of the small structural changes in the analogs prevented CYP1A2-catalyzed N-dealkylation and aromatic 4'- and 5'-hydroxylation.

The small difference in the \( K_m \) values and catalytic efficiencies of 2R-P and 2S-P (2S-P has a 2-fold higher \( K_m \) and a 2-fold lower \( k_{cat}/K_m \) than 2R-P) is in agreement with previously reported results in recombinant human 1A2 systems (Yoshimoto et al., 1995; Ching et al., 1996). The small (3-fold) difference in \( K_m \) and \( k_{cat}/K_m \) among the TFP stereoisomers indicates that regioselectivity of metabolism is only slightly dependent on the stereochemistry at C-2 in the side chain of TFP, like in P.

As shown in Tables 3 and 4, the sequential addition of fluorines leads to lower \( K_m \) values, by up to slightly more than 10-fold, indicating increased enzyme affinity. Rate constant values (\( k_{cat} \)) decrease only slightly with addition of fluorines, resulting in higher catalytic efficiencies. The catalytic efficiency (\( k_{cat}/K_m \)) varies across the series to a greater extent than the \( K_m \) value (about 25-fold versus 9-fold), providing a useful comparison of the relative rates of metabolism of these agents with each other under nonsaturating conditions.

Higher binding affinity (decreasing log \( K_m \)) and higher log catalytic efficiency values are associated with increasing log D values (Figure 3), suggesting that hydrophobic interactions play an important role in binding to and turnover by CYP1A2. Lower affinity and lower catalytic efficiency associated with increasing \( pK_a \) values (Figure 2) mirror this trend almost exactly, reflecting the effect of protonation state of the amine nitrogen on partitioning of the substrates between lipid and aqueous phases. Compounds with lower \( pK_a \) values (increased proportion of nonionized species at pH 7.4) have lower \( K_m \) values indicating that increased lipophilicity increases enzyme affinity. The correlations of log D and \( pK_a \) show slightly less scatter in the plots against log \( k_{cat}/K_m \) (\( r^2 \) values, 0.63 and 0.69, respectively) than against log \( K_m \) (\( r^2 \) values, 0.43 and 0.50, respectively), accounting for a slightly greater proportion of the metabolic data.
The selectivity of metabolism is changed as well. The aromatic hydroxylation (desOHP and desOHTFP) or that is changed due to steric hindrance with a site in the enzyme that is absent in the deshydroxy analogs could be a specific interaction of the side chain hydroxyl group of P molecule to a hydrophobic region of the enzyme. Alternatively, there could be a specific interaction of the side chain hydroxyl group of P with a site in the enzyme that is absent in the deshydroxy analogs (desOHP and desOHTFP) or that is changed due to steric hindrance by substitution of a larger N-alkyl group (e.g., in tBuMe or TFP). Both of these possibilities are consistent with the current models for the CYP1A2 active site and with the view that individual residues of 1A2 control catalytic selectivity by altering kinetics of specific steps in the multistep P450 reaction cycle (Yun et al., 2000).

The possibility that the rate of electron/proton or hydrogen atom transfer is limiting the overall rate of N-dealkylation in this series of compounds seems unlikely. Across the series, the increased ratio of ring hydroxylation to N-dealkylation is due to an increase in $k_{cat}/K_m$ for 4'- and 5'-hydroxylated metabolites rather than a decrease in catalytic efficiency for N-dealkylation (Table 2). Our observations do not distinguish between hydrogen atom transfer or proton/electron transfer mechanisms, but the presence of fluorines on the carbon adjacent to the site of abstraction would disfavor either process (i.e., an increase in the energy of activation for either electron or hydrogen atom removal would be expected with fluorine addition). The presence of fluorines on the carbon-β to the amine nitrogen increases the one electron ionization potential, as reported for the analogous β-mono-, di-, and trifluoroethyl amines (Staley et al., 1977), and is supported by electron impact-mass spectrometry data on P, FP, DFP, and TFP (Upthagrove et al., 1999). β-Fluorination can also significantly affect radical abstraction reactions, and the effect depends on the nature of the abstracting radical. Fluorine inductively deactivates hydrogen atom abstraction by electrophilic radicals due both to increased carbon-hydrogen bond strength and to unfavorable polarization in the transition state. Fluorine can be activating for abstraction by nucleophilic radicals, however, due to a favorable polarization of the transition state (Smart, 1995).

At higher substrate concentrations, biphasic kinetics were observed for all the substrates with CYP1A2. Further work on this observation is beyond the scope of this study. For the kinetic parameters reported here, substrate concentrations were kept low enough to estimate only the higher affinity $K_m$ and $k_{cat}$ values. Biphasic binding curves have been reported for the binding of R- and S-1-cyclohexylethylamine in rat CYP1A2 (Krainev et al., 1993), and non-linear Michaelis-Menten kinetics have been reported for yeast recombinant rat CYP1A1-catalyzed metabolism of 7-ethoxyresorufin and aminopyrine (Inouye, et al., 2000). The biphasic behavior may be due to the simultaneous presence of two substrate molecules in the active site of CYP1A2, as has been suggested for other cytochrome P450 isoforms and other substrates. However, ratios of metabolites from P and TFP do not change significantly at higher substrate concentrations, as has been observed for other systems in which biphasic kinetics has been reported (Korzekwa et al., 1998). Differential source- and substrate-dependent autoactivation of CYP1A2 has also been reported (Ekins et al., 1998). Further studies would be required to explore this observation.

Comparison of Results in CYP1A2 with CYP2D6. Correlations of $\log K_m$ and $pK_a$ values of these related substrates with low $K_m$ values (and higher catalytic efficiency) are consistent with lipophilicity being a major contributor to substrate affinity for CYP1A2. These results are in contrast with results of the studies with CYP2D6 in which higher enzyme affinity (low $K_m$ values) and higher catalytic efficiency are positively correlated with the increased degree of substrate protonation at pH 7.4 (higher $pK_a$) and smaller log D values (Upthagrove and Nelson, 2001).

A wider range of differences in affinities of the substrates (about 15-fold) was observed for CYP2D6 (ca. 370-fold change) than for CYP1A2 (ca. 25-fold change), indicating that the former isozyme is more sensitive to molecular changes in this series of congeners. This may represent greater specificity in the structural interactions of CYP2D6 with these substrates. The suggested specific function of Asp301 in CYP2D6 in ion pairing as part of substrate recognition and...
the absence of an equivalent specific interaction requirement in CYP1A2 is consistent with this observation. A combination of other differences in size, shape, lipophilicity, and charge distribution characteristics of the substrate binding sites between these two isozymes is also a likely contributor to these observed differences.

Only a small range of values of $k_{cat}$ about 3- to 5-fold, is observed for these compounds in results from CYP1A2 (Table 3) and in CYP2D6 (Uptaghove and Nelson, 2001). Small changes in $k_{cat}$ are consistent with the high degree of structural similarity in the substrate analogs and the analogous metabolic products formed. Under these conditions, only small changes in the contributions of substrate-dependent uncoupling to form reduced oxygen products would be expected. Significant contribution of one or more the specific steps that contribute to the P450 reaction cycle could also limit the range of observed $k_{cat}$ values. These steps include substrate binding, reduction of the porphyrin ferric iron, binding of oxygen to the reduced CYP1A2 ferrous iron, second electron addition, and formation of the activated oxygen species, all steps that occur before oxidative steps in the P450 reaction cycle.

$K_m$ values that principally reflects changes in the intrinsic affinity for the substrates. A combination of other differences for this isozyme than for CYP2D6, with increasing aromatic hydroxylation to $N$-desisopropylation by cloned human cytochrome P4501A1 and P4501A2. Drug Metab Dispos 22:294–299.

Decreased steric bulk in the N-substituent in this series is not detrimental to enzyme affinity nor to catalytic turn over by either CYP1A2 or CYP2D6, and changes in regioselectivity of metabolism (increased aromatic hydroxylation versus $N$-dealkylation with increasing substituent size) is a feature of CYP1A2 but not in CYP2D6. As noted previously, CYP1A2 seems to present a sterically less demanding active site than does CYP2D6, accommodating a wider range of different-sized substrates with less stringent structural requirements for the substrates. The change in regioselectivity of oxidation of these substrates by CYP1A2, with increasing aromatic hydroxylation to $N$-dealkylation product ratios is consistent with a less restrictive site for this isozyme than for CYP2D6. This result does not agree with the calculated volumes of representative substrates for CYP2D6 and CYP1A2 (De Rienzo, et al., 2000). However, propranolol itself, with a flexible aliphatic side chain, is larger than the more compact aromatic substrates for CYP1A2 upon which these comparisons are made. For CYP2D6, steric interaction with the bulkier N-alkyl group may prevent binding in an orientation that would allow $N$-dealkylation.

In summary, sequential $\beta$-fluorination to the amine nitrogen that results in changes in $pK_a$ values of these structurally related amines produces changes in partitioning characteristics at a physiological pH that alter significantly their affinity for CYP1A2 and CYP2D6 in an opposite fashion. The changes in metabolism of members of the series by these P450 isozymes are readily correlated to the physicochemical characteristics associated with substituent changes and are consistent with known characteristics of these isozymes.

References


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