Short Communication

CYP2D6 CATALYZES 5-HYDROXYLATION OF 1-(2-PYRIMIDINYL)-PIPERAZINE, AN ACTIVE METABOLITE OF SEVERAL PSYCHOACTIVE DRUGS, IN HUMAN LIVER MICROSOMES

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ABSTRACT:
1-(2-Pyrimidinyl)-piperazine (1-PP) is a major active metabolite of several psychoactive drugs including buspirone. 1-PP is also the major metabolite in the human circulation and in rat brains following oral administration of buspirone. This study was conducted to identify the enzyme responsible for the metabolic conversion of 1-PP to 5-hydroxy-1-(2-pyrimidinyl)-piperazine (HO-1-PP) in human liver microsomes (HLMs). The product HO-1-PP was quantified by a validated liquid chromatography-tandem mass spectrometry method. In the presence of NADPH, 1-PP (100 μM) was incubated separately with human cDNA-expressed cytochrome P450 isoforms (including CYP2D6, 3A4, 1A2, 2A6, 2C9, 2C19, 2E1, and 2B6) at 37°C. CYP2D6 catalyzed the formation of HO-1-PP from 1-PP. This catalytic activity was >95% inhibited by quinidine, a CYP2D6 inhibitor. HO-1-PP formation rates correlated well with the bufuralol 1-hydroxylase (CYP2D6) activities of individual HLMs. The formation of HO-1-PP followed a Michaelis-Menten kinetics with a Km of 171 μM and Vmax of 313 pmol/min mg protein in HLMs. Collectively, these results indicate that polymorphic CYP2D6 is responsible for the conversion of 1-PP to HO-1-PP.

Materials and Methods
Materials. 1-PP, HO-1-PP, and [3H,15N,13C]1-PP, an internal standard, were synthesized at Bristol-Myers Squibb. Their chemical structures are shown in Fig. 1. Ammonium formate and NADPH were purchased from Aldrich Chemical Co. (Milwaukee, WI). Formic acid, methanol, and acetonitrile were purchased from EM Scientific (Gibbstown, NJ). All organic solvents were of HPLC grade. HLM preparations (pooled from 12 donors), individual HLMs with low, medium, and high CYP2D6 activities, and human cDNA-expressed P450 isozymes (CYP2D6, 3A4, 1A2, 2A6, 2C9, 2C19, 2E1, and 2B6) were purchased from BD Gentest (Woburn, MA). The HLM preparations had a protein concentration of 20 mg/ml (420 pmol P450/mg protein), and human cDNA-baculovirus-insect cell-expressed P450 isoforms (CYP2D6, 3A4, 1A2, 2A6, 2C9, 2C19, 2E1, and 2B6) were purchased from BD Gentest (Woburn, MA). The HLM preparations had a protein concentration of 20 mg/ml (420 pmol P450/mg protein), and human cDNA-expressed P450 isoforms had a concentration of 1000 pmol P450/ml and 5 mg/ml proteins. Microsomal preparations and the expressed P450 isoforms were stored in a −80°C freezer. A stock solution of NADPH (5 mM) was prepared fresh in water and used on the day of preparation. A sodium phosphate buffer (1 M, pH = 7.4) was prepared in deionized water. The buffer was prepared fresh and kept on ice. Ketoconazole and quinidine solutions (0.1 mM and 0.5 mM each) were made by dissolving the compounds in acetonitrile.

Incubation with Microsomal Preparations. The incubations were done in triplicates in a total volume of 0.25 ml. HLMs with different CYP2D6

1-(2-Pyrimidinyl)-piperazine (1-PP) is a major active metabolite resulting from the N-dealkylation of several antidepressant/anxiolytic 5-HT1A agonists including tandospirone, gepirone, ipsapirone, and buspirone (Caccia et al., 1985, 1986; Bianchi et al., 1988; Koenig et al., 1995). 1-PP is up to 20% as active as buspirone in in vitro pharmacological models for buspirone and in vivo anxiolytic and α2-adrenoceptor antagonist activity (Garattini et al., 1982; Caccia et al., 1986; Bianchi and Garattini, 1988; Berlin et al., 1995). In addition, 1-PP has an affinity for α2-adrenergic receptor with Kd = 40 nM and for 5-HT1A with Kd = 1035 nM (Zuideveld et al., 2002). 1-PP was a partial agonist for 5-HT1A and an antagonist for the α2-adrenergic receptor and induced hyperthermic response in rats (Komissarev et al., 1990). The concentration of 1-PP in rat brain after oral administration of buspirone was 15 to 30 times higher than the concentration of buspirone (Gammans et al., 1983). The human plasma levels of 1-PP of buspirone was 15 to 30 times higher than the concentration of buspirone (Gammans et al., 1986). CYP3A4 plays a major role in vitro and in vivo metabolism of buspirone with 1-PP as one of the major metabolites (Kivisto et al., 1999; M. Zhu, W. Zhao, H. Jimenez, D. Zhang, S. Yeola, R. Dai, N. Vachharajani, and J. Mitroka, manuscript submitted for publication). Incubation of 5-hydroxybuspirone in human liver microsomes (HLMs) produced a very minimal amount of HO-1-PP. Therefore, oxidative metabolism of 1-PP might play an important role for the clearance of 1-PP. This study was conducted to identify the enzyme responsible for the conversion of 1-PP to HO-1-PP.
activities (low, medium, and high) and the human cDNA-expressed P450 isozymes (CYP2D6, 3A4, 1A2, 2A6, 2C9, 2C19, 2E1, and 2B6) were carefully thawed, mixed well, and placed on ice prior to the experiment. 1-PP at a concentration of 100 μM was incubated with HLM preparations at the final protein concentration of 0.5 mg/ml (HLM), 200 pmol of P450s (CYP3A4, 1A2, 2A6, 2C9, 2C19, 2E1, and 2B6), or various amounts of CYP2D6 in 0.1 M phosphate buffer (pH 7.4). The concentrations of 1-PP were 5, 7.5, 15, 30, 50, 100, 150, 225, 300, 400, 500, 750, and 1000 μM to determine enzyme kinetic parameters. Incubations were also performed in the presence of the inhibitor ketoconazole (1 and 5 μM) or quinidine (1 and 5 μM) at a 1-PP concentration of 100 μM in the inhibition studies. To determine the effect of heat-inactivated HLM proteins on the cDNA-expressed CYP2D6 activities, the pooled HLM in a glass tube was incubated for 5 min in boiled water before being added to the incubations with the expressed CYP2D6. NADPH was added to the incubation mixture at a final concentration of 1.2 mM. The incubation mixture was incubated in a water bath at 37°C for 15 min. At the end of the incubation period, acetonitrile (0.25 ml) was added to each sample. The mixtures were vortexed and allowed to sit in an ice-water bath for 2 h. The supernatant (0.175 ml) was removed and the internal standard (0.025 ml) was added to the solution to a final concentration of 12.5 ng/ml. Dilution quality control samples were prepared by diluting the supernatant (0.020 ml) with 5 mM ammonium formate/acetonitrile (50:50) to give a 1:10 dilution, and the internal standard (0.025 ml) was added to a final concentration of 12.5 ng/ml. The samples were vortexed and centrifuged for 10 min at 3000 rpm. A 10-μl portion of the sample was injected and analyzed by LC/MS/MS. Under these conditions, the HO-1-PP formation rates were linear with time and protein concentrations used.

Preparation of Standards. A stock solution of 1-PP (15 mM) was prepared in water. Stock solutions (1 mg/ml) of HO-1-PP and the internal standard were prepared in methanol. A 100 ng/ml working internal standard solution was prepared in acetonitrile/5 mM ammonium formate, pH 3.5 (50:50 v/v) from the stock solution. Working solutions of HO-1-PP (10, 1, 0.1, and 0.01 μg/ml) from the stock solution were prepared in acetonitrile/5 mM ammonium formate, pH 3.5 (50:50 v/v). Calibration standards were prepared by spiking the working solutions to a mixture containing 0.5 mg/ml microsomes, 100 mM phosphate buffer, pH 7.4, and 50% acetonitrile in a 1-ml final volume to give 1000, 500, 200, 100, 50, 25, 10, 5, 1, and 0.5 ng/ml HO-1-PP concentrations. Quality control samples were prepared from 10, 1, and 0.01 μg/ml working solutions to give 800, 500, 80, and 1 ng/ml HO-1-PP concentrations. All quality control and calibration standard samples were used on the day of preparation. A dilution quality control sample (1000 ng/ml) was prepared in HLMs and diluted 40 times to give a concentration of 25 ng/ml. The samples were vortexed and centrifuged for 10 min at 3000 rpm. A 10-μl portion of the sample was injected and analyzed by LC/MS/MS.

LC/MS/MS Quantitation. A YMC ODS-AQ column (3 μm, 2 × 50 mm, 120 Å) (Waters, Milford, MA) was used to analyze samples for HO-1-PP. The HPLC system was equipped with two pumps (model PerkinElmer series 200; PerkinElmer Life and Analytical Sciences, Boston, MA) and an autoinjector (model PerkinElmer series 200). A two-solvent system was used for HPLC separations. Solvent A contained 100% 5 mM ammonium formate (pH 3.5), and solvent B contained 100% acetonitrile. The flow rate was 0.2 ml/min. The solvent B in a linear gradient was held at 10% for 1.5 min and then increased from 10% to 90% in 0.1 min. The gradient was held at 90% B up to 3.5 min and then brought to the initial condition. The column eluate was sent to a mass spectrometer using positive ion electrospray. The mass spectrometer was an API 3000 LC/MS/MS (Applied Biosystems/MD Sciex, Foster City, CA), interfaced with the HPLC. Nitrogen was used as the nebulizing and curtain gas at flow rates of 10 l/h and 11 l/h, respectively. The source temperature was 400°C. Data acquisition was via multiple reaction monitoring. Ions representing the (M + H)+ species for both the analyte and the internal standard were selected in MS1 and collisionally dissociated with argon at a pressure of 2.5 × 10−3 mbar to form specific product ions that were subsequently monitored by MS2. The transitions monitored were m/z 181→138 for HO-1-PP and m/z 204 RAGHAVAN ET AL.
172–127 for the stable labeled 1-PP as the internal standard. Declustering potential, focusing potential, collision energy, and collision exit potential were optimized at 25 V, 140 V, 25 V, and 7 V, respectively.

Data Analysis. LC/MS/MS data were acquired, and the chromatographic peaks were then integrated using Analyst software (Version 5.0; Agilent Technologies, Palo Alto, CA). Peak area ratios of the analyte to the internal standard were calculated. A weighted (reciprocal of calibration standard concentration, 1/X) linear regression model was fit to peak area ratio versus calibration standard concentration data. The equation of this regression line was then used to calculate the predicted concentrations in all samples. For kinetic parameters, the data points were analyzed in Microsoft Excel by XLFit using the Michaelis-Menten equation: \( V = \frac{V_{\text{max}} \cdot S}{K_m + S} \).

Results

For quantitation of HO-1-PP in microsomal incubations, calibration standard curves for HO-1-PP (ranging from 0.5 to 1000 ng/ml) were linear with \( r^2 \) values of ≥0.996. The mean observed concentrations of the analytical quality control samples showed less than 0.04% deviation from the nominal concentration. The overall coefficients of variation for between- and within-day variability of the analytical quality controls were <9%.

Figure 2 shows HO-1-PP formation activities in the expressed P450 isozymes. CYP2D6 showed the highest activity of approximately 42 pmol/min/pmol P450. Other major human cDNA-expressed P450s (CYP3A4, 1A2, 2A6, 2C9, 2C19, 2E1, and 2B6) showed no more than 1% of the activity of CYP2D6. These results suggest that CYP2D6 is the major isozyme responsible for the conversion of 1-PP to HO-1-PP. The CYP2D6 inhibitor (quinidine at 5 \( \mu \)M) decreased the HO-1-PP formation rate by >95% in the pooled HLMs (Fig. 3A). The similar inhibition was observed in the incubations with the expressed CYP2D6 (Fig. 3B). A 10 to 30% rate reduction in HLMs and the expressed CYP2D6 by the CYP3A4 inhibitor, ketoconazole, was due probably to nonspecific inhibition of CYP2D6 by ketoconazole at the concentrations used for inhibitor and substrate. Cross-inhibition of P450s by ketoconazole for drug metabolism is common (Newton et al., 1995). The result from these inhibition experiments further supports the con-
clclusion that CYP2D6 was the major HLM enzyme that catalyzed the conversion of 1-PP to HO-1-PP.

Figure 4 shows the correlation between HO-1-PP formation rates and the CYP2D6 activities of individual HLMs and the pooled HLM. One individual (M1) fell out of the range for an unknown reason. There is generally a good correlation ($r^2 = 0.82$) between the CYP2D6 rates (bufuralol 1-hydroxylation activities) and HO-1-PP formation activities ($r^2 = 0.97$ when M1 was excluded), which further supports CYP2D6 as the enzyme that catalyzes the conversion of 1-PP to HO-1-PP. Figure 5 shows that the HO-1-PP formation from 1-PP follows a Michaelis-Menten kinetics with a $K_m$ of 171 $\mu$M and $V_{max}$ of 313 pmol/min $\cdot$ mg protein in HLMs and with a $K_m$ of 17.5 $\mu$M and $V_{max}$ of 39.2 pmol/min $\cdot$ pmol P450 in the expressed CYP2D6.

Discussion

Through the use of human cDNA-expressed, isozyme-selective P450 chemical inhibitors, correlation studies with the known P450 activities in individual HLMs, and enzyme kinetic analyses, CYP2D6 was found to catalyze the conversion of 1-PP to HO-1-PP. A very minimal formation activity of HO-1-PP from 5-hydroxybuspirone, a major human metabolite, in HLMs suggests that N-dealkylation of 5-hydroxybuspirone might not be a significant pathway for the formation of HO-1-PP in vivo (M. Zhu, W. Zhao, H. Jimenez, D. Zhang, S. Yeola, R. Dai, N. Vachharajani, and J. Mitroka, manuscript submitted for publication). In addition, the conjugation metabolites of 1-PP are not known (Jajoo et al., 1989). Our finding suggests that CYP2D6 may play an important role for the clearance of 1-PP in vivo. Consistent with our finding, deramciclane, another anxiolytic drug and a CYP2D6 inhibitor, did not affect area under the curve of buspirone but led to a significant 84% increase in the area under the curve of 1-PP when coadministered with buspirone in 16 healthy subjects (Laine et al., 2003a,b). It is not surprising to find that CYP2D6 catalyzes the 5-hydroxylation of 1-PP. Pharmacophore modeling found that typical CYP2D6 substrates often contain a cationic nitrogen 5 or 7 Å from their major sites of oxidation, ideally situated to form ion-pair interactions with CYP2D6 active-site aspartic acid (D301) (De Groot et al., 1997). 1-PP would be a preferred CYP2D6 substrate for the ion-pairing orientation. CYP2D6 catalyzes 5-hydroxylation of gepirone, an analog of buspirone, which suggests that the N-substituted analog of 1-PP is also a preferred substrate for CYP2D6 (Greenblatt et al., 2003).

The $V_{max}$ value for the 5-hydroxylation of 1-PP in the HLMs would be approximately 31.33 pmol/min/pmol CYP2D6 when a literature value of 10 pmol CYP2D6/mg HLM protein in the pooled HLM was used (Rodrigues, 1999). This value is very comparable with that (39.2 pmol/min/pmol P450) obtained from the incubation in the expressed CYP2D6, which further supports the finding that the 1-PP 5-hydroxylation activity in the HLMs was catalyzed by CYP2D6. The $K_m$ values in HLMs was approximately 10-fold higher than that in the cDNA-expressed CYP2D6. The protein concentration was 0.5 mg/ml in the incubations with HLMs, which was approximately 12 times that (0.04 mg/ml) used in the expressed CYP2D6. The solution-free fraction of 1-PP that is available to the enzyme would be lower in HLMs than in the expressed CYP2D6 due to nonspecific binding to HLM components. It has been demonstrated that microsomal protein concentrations could proportionally increase the apparent $K_m$ values of a drug molecule with a high nonspecific protein-binding property (Kalvass et al., 2001). However, bovine serum albumin (BSA) decreased the apparent $K_m$ values by 70% and 37% for phenytoin and tolbutamide metabolism, respectively, at a lower BSA concentration (0.25%) and then increased the $K_m$ values back to the level where no BSA was added, at a higher BSA concentration (2–4%) (Tang et al., 2002). Tang et al. (2002) concluded that using free fraction to calculate $K_m$ values might not be applicable to all reactions, and the impact of albumin on drug metabolism was more than that of protein binding. In addition, the binding of cationic drugs to phospholipids also had a profound effect on kinetic parameters of the CYP2D6 inhibition (Margolis and Obach, 2003). Therefore, the 10-fold difference in the $K_m$ values of 1-PP between HLMs and the expressed CYP2D6 might be caused by a combined nonspecific binding to microsomal proteins and phospholipids. To test this hypothesis, the heat-inactivated HLM was added to the incubations in the cDNA-expressed CYP2D6. The apparent $K_m$ values increased with the heat-inactivated HLM concentrations and up to approximately 10 times when the final concentrations of HLM were similar between the incubations with the HLM and with the expressed CYP2D6 added with heat-inactivated HLM (data not shown). For practical purposes, denatured HLM that contains natural proteins and phospholipids is the appropriate reagent for investigating the kinetic effects on hepatic enzymes. The present study
recommends addition of heat-inactivated HLMs to conduct enzyme kinetic studies with cationic drugs in the cDNA-expressed enzymes. The liver is generally the major organ involved in P450-mediated metabolism, but these enzymes are also present in extrahepatic tissues such as brain (Mikysk et al., 2002), and there is increasing evidence that they may contribute to the local in situ metabolism of drugs (Krishna and Klotz, 1994). CYP2D6 has a high interindividual variability, and much of this variability is attributed to the genetic polymorphism of CYP2D6. Approximately 10% of white people show a poor metabolizer phenotype for CYP2D6 due to inactive mutant alleles (Myer et al., 1990). CYP2D6 metabolizes a number of central-acting psychoactive drugs such as tricyclic antidepressants, neuroleptics, and anticonvulsants, and in situ metabolism by this enzyme in the brain may strongly influence the therapeutic efficacy and toxicity of these drugs. The severe adverse effects (including high fever, shivering, tremor, and tachycardia) in a 52-year-old woman prescribed with these drugs. The severe adverse effects (including high fever, shivering, tremor, and tachycardia) in a 52-year-old woman prescribed with these drugs. The severe adverse effects (including high fever, shivering, tremor, and tachycardia) in a 52-year-old woman prescribed with these drugs. The severe adverse effects (including high fever, shivering, tremor, and tachycardia) in a 52-year-old woman prescribed with these drugs.

In summary, through use of cDNA-expressed P450 isoforms, selected P450 inhibitors, individual HLMs for correlation studies, and enzyme kinetic analyses, we have identified that the polymorphic CYP2D6 metabolizes 1-PP, an active metabolite for several psychoactive drugs.

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References


Kalvass JC, Tess DA, Giragossian C, Linhares MC, and Maurer TS (2001) Influence of antiparkinsonian drugs, nonsteroidal antiinflammatory drugs, and anticonvulsants, and in situ metabolism by this enzyme in the brain may strongly influence the therapeutic efficacy and toxicity of these drugs.


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