Comparative Metabolic Capabilities and Inhibitory Profiles of CYP2D6.1, CYP2D6.10, and CYP2D6.17

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ABSTRACT:

Polymorphisms in the cytochrome P450 2D6 (CYP2D6) gene are a major cause of pharmacokinetic variability in human. Although the poor metabolizer phenotype is known to be caused by two null alleles leading to absence of functional CYP2D6 protein, the large variability among individuals with functional alleles remains mostly unexplained. Thus, the goal of this study was to examine the intrinsic enzymatic differences that exist among the several active CYP2D6 allelic variants. The relative catalytic activities (enzyme kinetics) of three functionally active human CYP2D6 allelic variants, CYP2D6.1, CYP2D6.10, and CYP2D6.17, were systematically investigated for their ability to metabolize a structurally diverse set of clinically important CYP2D6-metabolized drugs [atomoxetine, bufuralol, codeine, debrisoquine, dextromethorphan, (S)-fluoxetine, nortriptyline, and tramadol] and the effects of various CYP2D6-inhibitors [cocaine, (S)-fluoxetine, (S)-norfluoxetine, imipramine, quindine, and thioridazine] on these three variants. The most significant difference observed was a consistent but substrate-dependent decrease in the catalytic efficiencies of cDNA-expressed CYP2D6.10 and CYP2D6.17 compared with CYP2D6.1, yielding 1.32 to 27.9 and 7.33 to 80.4% of the efficiency of CYP2D6.1, respectively. The most important finding from this study is that there are mixed effects on the functionally reduced allelic variants in enzyme-substrate affinity or enzyme-inhibitor affinity, which is lower, higher, or comparable to that for CYP2D6.1. Considering the rather high frequencies of CYP2D6*10 and CYP2D6*17 alleles for Asians and African Americans, respectively, these data provide further insight into ethnic differences in CYP2D6-mediated drug metabolism. However, as with all in vitro to in vivo extrapolations, caution should be applied to the clinical consequences.

Despite representing only approximately 2 to 4% of total human liver cytochrome P450 content, CYP2D6 plays a prominent role in the oxidation of xenobiotics. Estimates indicate that CYP2D6 is involved in the metabolism of approximately 30% of drugs presently on the market, in particular, the metabolism of many of drugs targeting the central nervous and cardiovascular systems (Gardiner and Begg, 2006). CYP2D6 is a polymorphic member of the cytochrome P450 superfamily (Zanger et al., 2004), for which individuals referred to as poor metabolizers carry a gene deletion or null variants of the gene in which one or more nucleotide substitutions encode for a faulty message or truncated protein and thus are unable to produce active enzyme. There are differences in the proportions of extensive metabolizers to poor metabolizers in various ethnic groups (Sachse et al., 1997; Ji et al., 2002; Gaedigk et al., 2002). For example, the in vivo activity of CYP2D6 is absent in 7.7% of Caucasians in contrast to only ∼1% of Chinese and Japanese (Shimizu et al., 2003).

People broadly classified as extensive metabolizers also demonstrate a pronounced variation in their metabolic capacity. Despite the very low frequency of poor metabolizers of CYP2D6 in Asian populations, these groups actually display lower mean CYP2D6 activity than Caucasians with the reference allele CYP2D6*1. This reduced activity is represented by a right shift in the metabolic ratio (parent/metabolite ratio) for several CYP2D6 substrates, including debrisoquine (Kalow et al., 1980), sparteine (Droll et al., 1998), metoprolol (Horai et al., 1989), and dextromethorphan (Tateishi et al., 1999). This lower overall CYP2D6 activity has been attributed to the high frequency in the Asian populations of a reduced activity variant of CYP2D6, i.e., CYP2D6*10. This variant possesses Pro34Ser and Arg296Cys amino acid substitutions compared with CYP2D6*1 (http://www.cypalleles.ki.se/cyp2d6.htm) and is present in approximately 56.2% of Chinese and 38.8% of Japanese (Ji et al., 2002; Shimizu et al., 2003). CYP2D6*17 is another important variant of CYP2D6 that is highly prevalent (∼21%) in African Americans (Gaedigk et al., 2002). The CYP2D6*17 allele carries three non synonymous coding region single nucleotide polymorphisms conferring Thr107Ile, Arg296Cys, and Ser386Thr amino acid substitutions relative to CYP2D6*1. Phenotyping studies and in vitro data suggest that the metabolism of CYP2D6 substrates may be differentially decreased by

ABBREVIATIONS: P450, cytochrome P450; LC, liquid chromatography; MS/MS, tandem mass spectrometry.
CYP2D6*17 (Masimirembwa et al., 1996; Oscarson et al., 1997; Bapiro et al., 2002). Other functional allelic variants of CYP2D6 include CYP2D6*2, CYP2D6*9, and CYP2D6*41. Based on genotyping, it is possible to identify individuals with the poor metabolizer phenotype (i.e., those carrying two null alleles) with very high certainty. However, it is much more difficult to predict the metabolic capacity of extensive metabolizers who carry one or more altered function alleles.

Expression systems for the P450 enzymes have been used to investigate the metabolic capabilities of these enzymes and to determine the metabolic profile of various compounds. A recent study by Fukuda et al. (2000) found reduced affinity of CYP2D6.10 for venlafaxine and bufuralol. Others also observed in vitro significantly reduced enzymatic activity of CYP2D6.10, CYP2D6.17, and CYP2D6.2 (Ramamoorthy et al., 2001, 2002; Bapiro et al., 2002; Marcucci et al., 2002; Nakamura et al., 2002; Yu et al., 2002; Bogni et al., 2005) compared with that of the reference form CYP2D6.1. These studies suggest that direct extrapolation with other substrates of CYP2D6*1 to individuals with the reduced activity alleles may not be possible. Therefore, detailed kinetic analyses with CYP2D6.10, CYP2D6.17, and CYP2D6.1 for other clinically important CYP2D6-metabolized drugs are still required to fully understand potential differences in the metabolic clearance of these agents. Investigation of allele-specific enzyme kinetics will not only have implications in ethnic-specific pharmacotherapy but also may be critical to rational design of in vitro metabolism studies in support of drug discovery and development.

The aims of the present studies are to determine the relative catalytic activities (enzyme kinetics) of three functionally active human CYP2D6 allelic variants, CYP2D6.1, CYP2D6.10, and CYP2D6.17 (expressed in Supersomes with NADPH-cytochrome P450 reductase), for their ability to metabolize a structurally diverse set of clinically important drugs and to gain an understanding of the effects of various CYP2D6 inhibitors on these three variants.

Materials and Methods

Chemical and Materials. The CYP2D6 substrates, their metabolites, and the CYP2D6-inhibitors (Fig. 1) were obtained from Sigma-Aldrich (St. Louis, MO), Fluka (Buchs, Switzerland), Lilly Research Laboratories (Indianapolis, IN), or the National Institute for Control of Pharmaceutical and Biological Products (Beijing, China). 0-Demethyltramadol was provided by Dr. F.-J. Nan from the National Center for Drug Screening (Shanghai, China). Microsomes prepared from insect cells containing baculovirus-expressing human CYP2D6.1 with NADPH-cytochrome P450 reductase, and CYP2D6.10 and CYP2D6.17 (expressed in Supersomes with NADPH-cytochrome P450 reductase), for their ability to metabolize a structurally diverse set of clinically important drugs and to gain an understanding of the effects of various CYP2D6 inhibitors on these three variants.

**FIG. 1.** Structures of the substrates, their metabolites, and the inhibitors of CYP2D6 examined.
cells infected with the baculovirus vector was used as a control for native activity. β-NADPH, glucose 6-phosphate monosodium salt, and glucose-6-phosphate dehydrogenase were purchased from Sigma-Aldrich. High-performance liquid chromatography grade acetonitrile was obtained from Merck (Darmstadt, Germany). Ammonium formate (>99%) was obtained from Aldrich Chemical Co. (Milwaukee, WI). Other organic solvents and chemicals used were of analytical grade and were obtained from Sinopharm Chemical Reagent Co. (Shanghai, China). High-purity high-performance liquid chromatography water was made by distilling predeionized water twice in house.

**Incubation Conditions for Comparison of the Catalytic Activities of CYP2D6 Allelic Variants.** Pilot studies were performed with each biotransformation (listed in Table 1) to ensure that the comparison of metabolic capabilities of the three CYP2D6 allelic variants, i.e., CYP2D6.1, CYP2D6.10, and CYP2D6.17, was determined under linear metabolism conditions with respect to time and enzyme-protein concentration. Incubations were performed in duplicate in 96-well plates in a total assay volume of 100 μl with each well containing CYP2D6 Supersomes (at 5, 10, and 5 pmol P450/ml for CYP2D6.1, CYP2D6.10, and CYP2D6.17, respectively, except for debrisoquine 4-hydroxylation and (S)-fluoxetine N-demethylation, for which the concentrations of 20, 30, and 20 pmol P450/ml, respectively, were used), substrate (concentration ranges as indicated), 100 mM potassium phosphate buffer (pH 7.4), an NADPH-generating system comprising 3.3 mM magnesium chloride, 3.3 mM glucose 6-phosphate, 0.5 μM glucose-6-phosphate dehydrogenase, and 1.3 mM NADP. Before commencement of the reaction by adding the NADPH-generating system, the incubation mixture was preincubated for 3 min at 37°C. The optimal incubation time for the biotransformations was 30 min except for debrisoquine 4-hydroxylation (60 min). For each biotransformation, six to eight substrate concentrations were examined over the following ranges: 0.60 to 96 μM for atomoxetine 4-hydroxylation, 0.625 to 80 μM for bufuralol 1’-hydroxylation, 33 to 8000 μM for codeine O-demethylation, 2.74 to 1000 μM for debrisoquine 4-hydroxylation, 0.625 to 320 μM for dextromethorphan O-demethylation, 0.10 to 200 μM for fluoxetine N-demethylation, 0.625 to 320 μM for nortriptyline 10-hydroxylation, and 1.8 to 4000 μM for tramadol O-demethylation. The reactions were terminated by adding 100 μl of acetonitrile (4°C), and the resulting samples were centrifuged at 3000 rpm for 10 min to remove protein before 5 μl of the supernatant were applied to LC-MS/MS analysis.

**Inhibition Constant Determination for CYP2D6 Inhibitors.** The effects of the CYP2D6 inhibitors (Table 2) on the activities of the three CYP2D6 allelic variants mediating atomoxetine 4-hydroxylation and dextromethorphan O-demethylation were further measured in terms of inhibition constant (Kᵢ). Both the reactions were performed in 96-well plates and the incubation conditions were similar to those of enzyme kinetic assays as described above. A substrate concentration range from 0.2- to 5-fold of the Kᵢ values for the biotransformation was used for determining the Kᵢ values of the inhibitors affecting atomoxetine 4-hydroxylation or dextromethorphan O-demethylation mediated by CYP2D6.1, CYP2D6.10, and CYP2D6.17. The inhibitor concentration ranges for the atomoxetine 4-hydroxylation were 0.20 to 5, 0.19 to 15, 0.19 to 15, 2.5 to 62.5, 0.08 to 2, and 0.005 to 0.5 μM for cocaine, (S)-fluoxetine, (S)-norfluoxetine, imipramine, thioridazine, and quinidine, respectively, whereas those for the dextromethorphan O-demethylation were 0.8 to 20, 0.19 to 15, 0.19 to 15, 3.2 to 62.5, 0.025 to 0.5, and 0.01 to 0.5 μM, respectively.

**Analyses of Metabolites.** To determine metabolism rates, an LC-MS/MS system consisting of a TSQ Quantum triple stage quadrupole mass spectrometer (Thermo Fisher Scientific, San Jose, CA) interfaced via an electrospray ionization probe with an Agilent 1100 series liquid chromatography graph that included a vacuum degasser, a binary pump, a thermostatted autosampler and a thermostatted column compartment (Agilent, Waldbronn, Germany) was used. The Agilent Chemstation and Finnigan Xcalibur software packages were used for controlling the LC-MS/MS system, as well as for data acquisition and processing.

LC separations were achieved using a 4-μm Synergi C18 column (50 × 2.0 mm, i.d.; Phenomenex, Torrance, CA), with a precolumn 0.2-μm filter (Upchurch Scientific, Oak Harbor, WA). Prepared samples were separated under one of the following LC binary gradient programs (I and H), with mobile phases of CH₃CN:H₂O (10:90, v/v), containing a mass fraction of 0.02% HCOOH (H₂) for solvent A and CH₃CN:H₂O (450:50, v/v), containing a mass fraction of 0.02% HCOONH₄ (H₄) for solvent B delivered at 0.3 ml/min. Gradient program I was used for analysis of 4-hydroxyatomoxetine and dextrophan, which consisted of an initial 0.7-min increase of B from 25 to 95%, followed

**TABLE 1**

Comparison of enzyme kinetic parameters for CYP2D6.1, CYP2D6.10, and CYP2D6.17-mediated metabolic formations

All reactions were run in duplicate. Error estimates are based on the best fit of the average values obtained at each point to the Michaelis-Menten equation using nonlinear regression analysis.

<table>
<thead>
<tr>
<th>Biotransformation</th>
<th>Kᵢ (μM)</th>
<th>Vₘₐₓ (pmol/min/mmol P450)</th>
<th>CLₘₐₓ (μl/min/mmol P450)</th>
<th>CLₘₐₓ Ratio % CYP2D6.1</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CYP2D6.1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bufuralol 1’-hydroxylation</td>
<td>10.2 ± 0.6</td>
<td>6.29 ± 0.12</td>
<td>0.617</td>
<td></td>
</tr>
<tr>
<td>Dextromethorphan O-demethylation</td>
<td>2.97 ± 0.39</td>
<td>3.49 ± 0.10</td>
<td>1.18</td>
<td></td>
</tr>
<tr>
<td>Debrisoquine 4-hydroxylation</td>
<td>73.7 ± 7.0</td>
<td>1.32 ± 0.04</td>
<td>0.0179</td>
<td></td>
</tr>
<tr>
<td>Atomoxetine 4-hydroxylation</td>
<td>2.73 ± 0.23</td>
<td>19.1 ± 0.4</td>
<td>6.99</td>
<td></td>
</tr>
<tr>
<td>(S)-Fluoxetine N-demethylation</td>
<td>0.834 ± 0.111</td>
<td>0.414 ± 0.012</td>
<td>0.496</td>
<td></td>
</tr>
<tr>
<td>Nortriptyline 10-hydroxylation</td>
<td>4.07 ± 0.36</td>
<td>0.96 ± 0.02</td>
<td>0.236</td>
<td></td>
</tr>
<tr>
<td>Tramadol O-demethylation</td>
<td>286 ± 44</td>
<td>12.9 ± 0.5</td>
<td>0.0451</td>
<td></td>
</tr>
<tr>
<td>Codeine O-demethylation</td>
<td>1079 ± 307</td>
<td>3.68 ± 0.33</td>
<td>0.00341</td>
<td></td>
</tr>
<tr>
<td><strong>CYP2D6.10</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bufuralol 1’-hydroxylation</td>
<td>12.9 ± 1.1</td>
<td>0.29 ± 0.01</td>
<td>0.0225</td>
<td>3.65</td>
</tr>
<tr>
<td>Dextromethorphan O-demethylation</td>
<td>9.14 ± 0.59</td>
<td>0.57 ± 0.01</td>
<td>0.0624</td>
<td>5.31</td>
</tr>
<tr>
<td>Debrisoquine 4-hydroxylation</td>
<td>94.5 ± 7.8</td>
<td>0.20 ± 0.01</td>
<td>0.00212</td>
<td>11.8</td>
</tr>
<tr>
<td>Atomoxetine 4-hydroxylation</td>
<td>3.45 ± 0.45</td>
<td>2.07 ± 0.07</td>
<td>0.600</td>
<td>8.58</td>
</tr>
<tr>
<td>(S)-Fluoxetine N-demethylation</td>
<td>0.455 ± 0.076</td>
<td>0.017 ± 0.001</td>
<td>0.0374</td>
<td>7.54</td>
</tr>
<tr>
<td>Nortriptyline 10-hydroxylation</td>
<td>48.1 ± 8.0</td>
<td>0.15 ± 0.01</td>
<td>0.00312</td>
<td>1.32</td>
</tr>
<tr>
<td>Tramadol O-demethylation</td>
<td>331 ± 107</td>
<td>1.03 ± 0.09</td>
<td>0.00311</td>
<td>6.90</td>
</tr>
<tr>
<td>Codeine O-demethylation</td>
<td>1206 ± 239</td>
<td>1.14 ± 0.07</td>
<td>0.00095</td>
<td>27.9</td>
</tr>
<tr>
<td><strong>CYP2D6.17</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bufuralol 1’-hydroxylation</td>
<td>9.47 ± 1.54</td>
<td>1.33 ± 0.07</td>
<td>0.140</td>
<td>22.8</td>
</tr>
<tr>
<td>Dextromethorphan O-demethylation</td>
<td>17.3 ± 1.0</td>
<td>3.41 ± 0.05</td>
<td>0.197</td>
<td>16.8</td>
</tr>
<tr>
<td>Debrisoquine 4-hydroxylation</td>
<td>108 ± 8</td>
<td>1.24 ± 0.03</td>
<td>0.0115</td>
<td>64.2</td>
</tr>
<tr>
<td>Atomoxetine 4-hydroxylation</td>
<td>8.33 ± 0.40</td>
<td>12.8 ± 0.18</td>
<td>1.53</td>
<td>21.9</td>
</tr>
<tr>
<td>(S)-Fluoxetine N-demethylation</td>
<td>2.57 ± 0.50</td>
<td>0.104 ± 0.005</td>
<td>0.0405</td>
<td>8.17</td>
</tr>
<tr>
<td>Nortriptyline 10-hydroxylation</td>
<td>18.5 ± 4.0</td>
<td>0.32 ± 0.02</td>
<td>0.0173</td>
<td>7.33</td>
</tr>
<tr>
<td>Tramadol O-demethylation</td>
<td>209 ± 52</td>
<td>3.36 ± 0.21</td>
<td>0.0161</td>
<td>35.7</td>
</tr>
<tr>
<td>Codeine O-demethylation</td>
<td>1470 ± 253</td>
<td>4.03 ± 0.24</td>
<td>0.00274</td>
<td>80.4</td>
</tr>
</tbody>
</table>
In the studies presented here, eight CYP2D6-mediated biotransformation reactions were studied for their kinetic properties. When inhibition assays were performed to determine enzyme kinetic parameters, the data were plotted on an Eadie-Hofstee plot for a pilot visual analysis. The estimated kinetic parameters were then determined by nonlinear regression analysis using the Michaelis-Menten equation (rate of metabolite formation as a function of substrate concentration) using GraFit software (version 5; Erithacus Software Ltd., Surrey, UK). The mass spectrometer was operated in the positive electrospray ionization mode and selected reaction monitoring scan modes for all the analytes. The instrumental parameters were tuned to maximize generation of the protonated analyte molecules ([M + H]⁺) and characteristic fragment ions. The precursor-to-product ion transitions were monitored at m/z: 272 → 44, 278 → 180, 286 → 165, 192 → 132, 258 → 157, 296 → 134, 280 → 215, and 250 → 58 for 4-hydroxyatomoxetine, 1′-hydroxybufuralol, morphine, 4-hydroxydebrisoquine, (S)-norfluoxetine, 10-hydroxy-nortriptyline, and O-demethyltramadol, respectively. An online motorized six-port divert valve was used to introduce the LC eluent flow to the mass spectrometer over the period of 2.0 to 5.5 min for data acquisition, with the other eluent flow diverted to the waste. The instrumental parameters were tuned to optimize the signal-to-noise ratio greater than 30, with a peak area of a given metabolite versus the nominal concentration (nanomolar) being linear and showing correlation coefficients (r) greater than 0.999.

Data Analysis. Michaelis constant (Km) and maximum velocity (Vmax) values were determined by nonlinear regression analysis using the Michaelis-Menten equation (rate of metabolite formation as a function of substrate concentration) using GraFit software (version 5; Erithacus Software Ltd., Surrey, UK). The ratio of Vmax and Km was used to calculate in vitro intrinsic clearance (Clint). When inhibition assays were performed to determine kinetic parameters, the data were plotted on an Eadie-Hofstee plot for a pilot visual inspection of the inhibition type, and the entire data set was fitted to the appropriate equations by nonlinear regression analysis with simple weighting using GraFit software.

Results

Enzyme Kinetics of CYP2D6.1, CYP2D6.10, and CYP2D6.17. In the studies presented here, eight CYP2D6-mediated biotransformation reactions, i.e., atomoxetine 4-hydroxylation, bufuralol 1′-hydroxylation, codeine O-demethylation, dextromethorphan O-demethylation, debrisoquine 4-hydroxylation, (S)-fluoxetine N-demethylation, nortriptyline 10-hydroxylation, and tramadol O-demethylation, were examined. The classic single-site Michaelis-Menten equation best fit these biotransformations catalyzed in vitro by the three recombinant CYP2D6 variants. Figure 2 contains representative plots of metabolite formation rate versus substrate concentration for CYP2D6-mediated turnover of atomoxetine and dextromethorphan. The parameters such as Km and Vmax for different CYP2D6 substrates, as well as the corresponding in vitro intrinsic clearance (Vmax/Km) values for each variant are presented in Table 1.

Bufuralol 1′-Hydroxylation, Dextromethorphan O-Demethylation, and Debrisoquine 4-Hydroxylation (Three Commonly Used CYP2D6 Probe Substrates). Bufuralol and dextromethorphan are two commonly used in vitro substrates for CYP2D6 probe substrates; debrisoquine is less commonly used but is of historical interest. The estimated kinetic parameters Km and Vmax for bufuralol 1′-hydroxylation, dextromethorphan O-demethylation, and debrisoquine 4-hydroxylation mediated by CYP2D6.1 were 10.2 μM and 6.29 pmol/min/pmol P450, 2.97 μM and 3.49 pmol/min/pmol P450, and 73.7 μM and 1.32 pmol/min/pmol P450, respectively, which resulted in intrinsic clearance (Clint) values of 0.62, 1.18, and 0.02 μl/min/pmol P450, respectively. Compared with the reference protein (CYP2D6.1), estimates of Km for bufuralol, dextromethorphan, and debrisoquine were 1.3-, 3.1-, and 1.3-fold for CYP2D6.10 and 0.92-, 5.8-, and 1.5-fold for CYP2D6.17. In addition, Vmax values for the reference protein were 21.6-, 6.1-, and 6.6-fold higher than those for CYP2D6.10 and 4.7-, 1.0-, and 1.1-fold higher than those for CYP2D6.17. The Clint values of these three substrates catalyzed by CYP2D6.10 were reduced to approximately 5.31 to 11.8% of that observed with the reference protein and reduced 16.8 to 64.2% with CYP2D6.17.

Atomoxetine 4-Hydroxylation. Atomoxetine, a potent and selective inhibitor of the presynaptic norepinephrine transporter, is used clinically for the treatment of attention-deficit hyperactivity disorder in children and adults (Newcorn et al., 2005). In humans, atomoxetine undergoes extensive metabolism of 4-hydroxylation by CYP2D6 to form 4-hydroxyatomoxetine and is also transformed into a minor metabolite, N-demethylatomoxetine, mainly by CYP2C19 (Ring et al., 2002; Sauer et al., 2003). The enzyme kinetics of CYP2D6.1, CYP2D6.10, and CYP2D6.17 variants toward atomoxetine 4-hydroxylation were characterized, and the results indicated that CYP2D6.10 and CYP2D6.17 exhibited enzyme efficiency (as Clint), which was 8.58 and 21.9% of CYP2D6.1. CYP2D6.10 exhibited a Km value for atomoxetine similar to that of the wild-type protein, but its maximum velocity was 9.2 times lower, which resulted in its decreased enzyme efficiency, whereas the decreased enzyme efficiency of CYP2D6.17 was attributed mainly to increased Km (by 3.1-fold).
and also to a 1.5-fold decreased $V_{\text{max}}$. For CYP2D6.1, the in vitro $CL_{\text{int}}$ of atomoxetine was approximately 11-, 6-, or 390-fold higher than that of bufuralol, dextromethorphan, or debrisoquine, respectively. Although exhibiting decreased catalytic efficiency relative to CYP2D6.1, as often observed for other CYP2D6-mediated biotransformations, the $CL_{\text{int}}$ value for atomoxetine 4-hydroxylation by CYP2D6.10 (0.600 µl/min/pmol P450) or CYP2D6.17 (1.53 µl/min/pmol P450) was still quite high and comparable with those for bufuralol 1'-hydroxylation and dextromethorphan $O$-demethylation catalyzed by CYP2D6.1.

**Fluoxetine, a racemic mixture of** $(R)$- and $(S)$-fluoxetine, is a selective serotonin reuptake inhibitor (Gram, 1994) and is indicated for the treatment of depression, associated anxiety, obsessive compulsive disorder, and bulimia, as well as premenstrual dysphoric disorder. The major metabolic route of fluoxetine is $N$-demethylation to form norfluoxetine. Identification of the enzyme involved in the formation of norfluoxetine demonstrated that $(S)$-fluoxetine $N$-demethylation correlated only with CYP2D6 catalytic activity, whereas both CYP2D6 and CYP2C9 significantly contributed to the formation of $(R)$-norfluoxetine (Margolis et al., 2000; Ring et al., 2001). Relative to the preceding CYP2D6 substrates in which the $K_m$ values were lower for the reference protein versus the variant, the kinetics of $(S)$-fluoxetine $N$-demethylation to $(S)$-norfluoxetine with CYP2D6.10 was different from that of CYP2D6.1, yielding $K_m$ values of 0.455 and 0.834 µM for CYP2D6.10 and CYP2D6.1, respectively. The corresponding $V_{\text{max}}$ value in CYP2D6.10 was only 4% of that for CYP2D6.1. Accordingly, the $CL_{\text{int}}$ value was reduced for CYP2D6.10, demonstrating approximately 7.54% of the efficiency of CYP2D6.1. In contrast, CYP2D6.17 demonstrated both increased $V_{\text{max}}$ and in vitro catalytic activity, whereas both CYP2D6 and CYP2C9 significantly contributed to the formation of $(S)$-norfluoxetine demonstrated that $(S)$-fluoxetine $N$-demethylation correlated only with CYP2D6 catalytic activity, whereas both CYP2D6 and CYP2C9 significantly contributed to the formation of $(R)$-norfluoxetine (Margolis et al., 2000; Ring et al., 2001). Relative to the preceding CYP2D6 substrates in which the $K_m$ values were lower for the reference protein versus the variant, the kinetics of $(S)$-fluoxetine $N$-demethylation to $(S)$-norfluoxetine with CYP2D6.10 was different from that of CYP2D6.1, yielding $K_m$ values of 0.455 and 0.834 µM for CYP2D6.10 and CYP2D6.1, respectively. The corresponding $V_{\text{max}}$ value in CYP2D6.10 was only 4% of that for CYP2D6.1. Accordingly, the $CL_{\text{int}}$ value was reduced for CYP2D6.10, demonstrating approximately 7.54% of the efficiency of CYP2D6.1. In contrast, CYP2D6.17 demonstrated both increased $K_m$ with $(S)$-fluoxetine and reduced maximal activity, resulting in a $CL_{\text{int}}$ that was 8.17% of the $CL_{\text{int}}$ for CYP2D6.1.

**Nortriptyline 10-Hydroxylation and Tramadol $O$-Demethylation.** Nortriptyline and tramadol were also used as substrates to compare the metabolic capabilities of CYP2D6.1, CYP2D6.10, and CYP2D6.17 allelic variants. Nortriptyline is a tricyclic antidepressant and in vitro studies demonstrated that CYP2D6 was the sole cytochrome P450 mediating the main metabolic pathway, hydroxylation to (E)-10-hydroxynortriptyline (Venkatakrishnan et al., 1999). The CYP2D6.1 enzyme was found to have the highest catalytic efficiency for nortriptyline 10-hydroxylation, whereas the intrinsic clearance values of CYP2D6.10 and CYP2D6.17 were only 1.32 and 7.33% of the CYP2D6.1. The reduced $CL_{\text{int}}$ with CYP2D6.10 or CYP2D6.17 was attributed to both an increased $K_m$ (11.8- or 4.5-fold) and a reduced $V_{\text{max}}$ (6.4- or 3-fold). Tramadol is an opioid analgesic of the aminocyclohexanol type, and its primary metabolites are $O$-demethyltramadol and $N$-demethyltramadol. Whereas the $N$-demethylation is catalyzed by CYP2B6 and CYP3A4, CYP2D6 is primary responsible for $O$-demethylation (Subrahmanyan et al., 2001). In contrast to nortriptyline, both CYP2D6.10 and CYP2D6.17 exhibited affinities similar to those of CYP2D6.1 for tramadol. A reduced maximum velocity resulted in the $CL_{\text{int}}$ values of tramadol catalyzed by CYP2D6.10 or CYP2D6.17, demonstrating 6.9 or 35.7% of the efficiency of CYP2D6.1, respectively.

**Codeine $O$-Demethylation.** Although glucuronidation of codeine to codeine-6-glucuronide and subsequent renal excretion is the major route of elimination, the minor biotransformation $O$-demethylation to morphine is mediated by CYP2D6 (only accounting for approximately 10% of codeine metabolism) which produces its analgesic, antitussive, and antiarrheal effects (Mortimer et al., 1990). All of the three forms exhibited low but comparable $K_m$ values for codeine, i.e., 1079, 1206, and 1470 µM for CYP2D6.1, CYP2D6.10, and CYP2D6.17, respectively. The decreased enzyme efficiency of CYP2D6.10 relative to CYP2D6.1 (27.9%) was attributed mainly to a 3.2-fold reduced $V_{\text{max}}$. Only a slight decrease in enzyme efficiency was observed for CYP2D6.17, which was approximately 80.4% of CYP2D6.1.

**Differences between CYP2D6-Substrates.** All of the substrates exhibited a reduced in vitro intrinsic clearance ($CL_{\text{int}}$) with CYP2D6.10, demonstrating only 1.32 to 27.9% of the efficiency
CYP2D6.1. The result indicates that the estimated CLint values of these substrates were also decreased to 7.33 to 80.4% of those of CYP2D6.1. The decreases in CLint observed with CYP2D6.10 or CYP2D6.17 relative to the CYP2D6.1 were the results of nonuniform increases in the Michaelis constant (Km) as well as in the maximum reaction velocity (Vmax) across the test CYP2D6-substrates. CYP2D6.10 exhibited only slight Km changes for atomoxetine, bufuralol, codeine, debrisoquine, (S)-fluoxetine, and tramadol compared with CYP2D6.1; thus the reduced CLint values were mainly attributed to the decreased Vmax. Meanwhile, both higher Km values and reduced Vmax values for dextromethorphan O-demethylation and nortriptyline 10-hydroxylation by CYP2D6.10 yielded a lower CLint than CYP2D6.1. The most dramatic difference in CLint between CYP2D6.10 and CYP2D6.1 was observed with nortriptyline as the substrate, whereas the difference was the least with codeine. Compared with CYP2D6.1, CYP2D6.10 appeared to display a greater substrate-specific change in Km than CYP2D6.10. Relative to CYP2D6.1, modest differences in Km were observed only with the substrates bufuralol, codeine, and tramadol. Meanwhile, atomoxetine, codeine, debrisoquine, and dextromethorphan exhibited comparable or slightly reduced Vmax values. The enzyme efficiencies of CYP2D6.17 toward codeine and debrisoquine were only slightly decreased by approximately 20 and 36%, respectively, compared with that for CYP2D6.1. Similar to the situation with comparison between CYP2D6.10 and CYP2D6.1, nortriptyline demonstrated the greatest difference in CLint between CYP2D6.17 and CYP2D6.1 of the substrates investigated.

**Interactions of CYP2D6 Inhibitors with CYP2D6.1, CYP2D6.10, and CYP2D6.17.** In addition to various CYP2D6 substrates, the interactions between six known CYP2D6 inhibitors of varying drug classes with CYP2D6.1, CYP2D6.10, and CYP2D6.17 were also studied. The inhibitors tested were cocaine, (S)-fluoxetine, (S)-norfluoxetine, imipramine, quinidine, and thioridazine. Cocaine has been reported to be a competitive inhibitor of CYP2D6-mediated sparteine 2-dehydrogenation (Tyndale et al., 1991). The CYP2D6 substrate fluoxetine and its N-demethylated metabolite norfluoxetine are about equal competitive CYP2D6 inhibitors (Stevens and Wrighton, 1993). Although fluoxetine is marketed as a racemic mixture, (S)-fluoxetine and (S)-norfluoxetine were found to be more potent inhibitors of CYP2D6 as evidenced by Kᵢ values that were approximately 5 times lower than the corresponding values for the (R)-enantiomers. As for other tricyclic antidepressants, imipramine competitively inhibits CYP2D6-catalyzed dextromethorphan O-demethylation, with the estimated Kᵢ value of 28.6 μM (Shin et al., 2002). The antiarrhythmic quinidine is a well documented potent competitive inhibitor of, but not substrate for, CYP2D6. Quinidine is often used as a positive control for in vitro and in vivo CYP2D6 inhibition studies. Thioridazine has been found to competitively inhibit dextromethorphan O-demethylation with an estimated Kᵢ value of 1.4 μM (Shin et al., 1999). To determine the Kᵢ values for these CYP2D6 inhibitors, both dextromethorphan O-demethylation and atomoxetine 4-hydroxylation were chosen because of their high selectivity and specificity for CYP2D6, and the results are reported in Table 2. In addition, representative Eadie-Hofstee plots for quinidine inhibiting dextromethorphan O-demethylation and atomoxetine 4-hydroxylation mediated by CYP2D6.1, CYP2D6.10, or CYP2D6.17 are shown in Fig. 3.
visual inspection of the Eadie-Hofstee plots. The ratios of $K_i$ values obtained in CYP2D6.10 compared with those in CYP2D6.1 were 0.32 for (S)-fluoxetine and 0.41 for (S)-norfluoxetine, showing higher affinity of CYP2D6.10 than that of CYP2D6.1: 1.51 for cocaine and 1.11 for imipramine, demonstrating little difference between the two alleles; and 4.64 and 11.8 for quinidine and thioridazine, respectively, exhibiting significantly reduced affinity for CYP2D6.10. CYP2D6.17 displayed greater $K_i$ values (lower affinity) for all of the tested inhibitors than CYP2D6.1 with the $K_i$ ratio (2D6.17/2D6.1) varying from 1.4 to 9.2. Similar to the preceding mixed $K_m$ changes for the tested CYP2D6-substrates, CYP2D6.10 exhibited mixed $K_m$ changes for the tested inhibitors compared with CYP2D6.1, whereas CYP2D6.17 displayed greater $K_m$ values for all the test inhibitors than CYP2D6.1 and greater $K_m$ values for most of the test substrates as well.

**Inhibition of Atomoxetine 4-Hydroxylase.** To gain a better understanding of the effects of the inhibitors on CYP2D6.1, CYP2D6.10, and CYP2D6.17, the inhibition study was also performed using the probe reaction, atomoxetine 4-hydroxylation. All of the test CYP2D6 inhibitors demonstrated competitive inhibition of the three recombinant variants. The test inhibitors displayed similar spectra for the effects on the three allelic variants with atomoxetine 4-hydroxylation compared with those with dextromethorphan O-demethylation. In terms of 4-hydroxyatomoxetine formation, quinidine and thioridazine also exhibited the most potent inhibition of CYP2D6.1 with $K_i$ values of 0.01 and 0.026 μM, respectively, followed by cocaine, (S)-fluoxetine, and (S)-norfluoxetine with higher $K_i$ values of 0.35 to 1.04 μM. Imipramine was still the weakest inhibitor with a $K_i$ value of approximately 3.04 μM. The $K_i$ values for CYP2D6.10/CYP2D6.1 also demonstrated mixed effects on $K_i$ changes for the inhibitors, which could be divided into three groups with 1) (S)-norfluoxetine and (S)-fluoxetine exhibiting the ratios of 0.57 and 0.73, respectively, 2) cocaine, imipramine, and quinidine showing ratios between 1.23 and 2.3, and 3) thioridazine displaying the greatest ratio of 19. Similar to the results with dextromethorphan, with atomoxetine as the substrate CYP2D6.17 displayed greater $K_i$ values for all the inhibitors compared with CYP2D6.1 with the $K_i$ ratios (CYP2D6.17/CYP2D6.1) varying from 1.12 to 5.6. Except for the two most potent inhibitors quinidine and thioridazine, the differences in $K_i$ values between CYP2D6.10 and CYP2D6.17 appeared to be smaller with atomoxetine 4-hydroxylation than those with dextromethorphan O-demethylation. An unexpected observation in the inhibition studies with CYP2D6.17 is that the $K_i$ value for cocaine was 9.29 μM for competitive inhibition of dextromethorphan metabolism, but only 1.60 μM for that of atomoxetine metabolism, the variation in which was almost 6 fold. The reason behind this repeatable observation (data not shown) remains to be understood.

**Discussion**

In the study presented here, the experimental conditions were optimized to focus on measuring initial rates of CYP2D6-substrate biotransformations. Because of lower enzyme efficiencies for CYP2D6.10 and CYP2D6.17 relative to that of CYP2D6.1, sensitive LC-MS/MS methods were developed and used for measuring metabolite formation in incubations with the lowest amount of enzyme protein possible and less than 5% of the substrate consumed for all the compounds with all the variants. In addition, efforts were also made to minimize the matrix effects on the LC-MS/MS-based quantification to meet validation requirements on precision and accuracy, leading to the generation of reliable experimental data for this study.

Previous communications (Fukuda et al., 2000; Ramamooorthy et al., 2001, 2002; Bapiro et al., 2002; Marcucci et al., 2002; Yu et al., 2002; Nakamura et al., 2002) reporting the catalytic efficiencies of cDNA-expressed CYP2D6.10 or CYP2D6.17 compared with that of CYP2D6.1 examined the kinetics of common CYP2D6 probe substrate reactions, such as bufuralol 1’-hydroxylation, dextromethorphan O-demethylation, and debrisoquine 4-hydroxylation. Both CYP2D6.10 and CYP2D6.17 exhibited decreased catalytic efficiencies for the substrates compared with CYP2D6.1, but the results reported by the different authors were highly variable. For example, in these previous studies bufuralol 1’-hydroxylation mediated by CYP2D6.1, CYP2D6.10, or CYP2D6.17 exhibited $K_m$ values 1.7 to 25, 12 to 50, or 17 to 33 μM, respectively, and the calculated CLint value ranged from 0.04 to 7.88, 0.05 to 0.24, or 0.02 to 0.27 μl/min/ pmol P450, respectively. The reason for the discrepancy between the laboratories might be associated in part with different sources of enzyme used, as well as the different incubation conditions used in the studies.

Nakamura et al. (2002) reported the thermal instability of CYP2D6.10 at 39°C and higher temperature. These authors presumed that this instability may be important when abnormal body temperature, e.g., fever, in an individual is caused by a disease. As to the thermolability of the tested CYP2D6 variants in the present studies, we did not find CYP2D6.10 protein to possess any unusual instability at 37°C compared with CYP2D6.1 and CYP2D6.17. The relative enzyme activity of CYP2D6.1, CYP2D6.10, or CYP2D6.17 mediating dextromethorphan O-demethylation after preincubation for 30 min was 75.9 ± 3.7, 78.9 ± 1.7, or 76.6 ± 6.0%, respectively, whereas the relative enzyme activity for a 60-min preincubation was 56.1 ± 1.7, 58.5 ± 0.5, or 55.2 ± 1.8%, respectively. Similar results were obtained using the CYP2D6-mediated reaction, atomoxetine 4-hydroxylation. Because the effects of temperature were similar, no correction for enzyme thermolability was made for the comparison of the three CYP2D6 allelic variants with respect to metabolic capabilities and inhibitory profiles.

The cytochrome P450s require P450 reductase as an electron donor to function as monooxygenases. Although the ratio of P450 to the reductase may influence the catalytic activities of CYP2D6, Yamazaki et al. (2002) found that bufuralol 1’-hydroxylation activity by CYP2D6 was not enhanced in coexpression systems by the addition of exogenous P450 reductase. The level of P450 reductase in human liver microsomes was found to be subject to considerable variation (14-fold), but this was not reflected in variability in reductase activity (Forrester et al., 1992). In this study, no exogenous NADPH-cytochrome P450 reductase was supplemented to achieve a constant molar P450/reductase ratio for the three expressed CYP2D6 proteins with coexpressed P450/reductase ratios of 1:0.8, 1:0.4, and 1:1.5 for CYP2D6.1, CYP2D6.10, and CYP2D6.17, respectively. Literature values for the kinetics of dextromethorphan O-demethylation resulting from using purified CYP2D6 allelic variants (Yu et al., 2002) provides a valuable comparison for the systems used in the current study. Using purified CYP2D6.1, CYP2D6.10, and CYP2D6.17 with P450 and the reductase in a consistent 1:2 M ratio, Yu et al. (2002) measured $V_{max}$ of 9.0, 2.6, and 8.6 pmol/min/pmol P450 for this reaction, whereas Table 1 shows a similar order of magnitude in $V_{max}$, i.e., 3.49, 0.57, and 3.41 pmol/min/pmol P450, respectively.

In the current study kinetic parameters for eight CYP2D6 biotransformations were determined with cDNA-expressed CYP2D6.10 and CYP2D6.17, which consistently exhibited lower catalytic efficiencies compared with CYP2D6.1. Moreover, the relationships between different CYP2D6 substrates also appear quite variable, suggesting that substrate-dependent intrinsic enzymatic differences exist among the three important active CYP2D6 variants. Therefore, substrate-specific and allelic-dependent metabolism may affect the dominating meta-
bolic pathway and the relative contributions of the various metabolizing enzymes involved in the clearance of a drug. These findings have important implications for genotype-phenotype prediction for therapeutically useful CYP2D6 substrates. Most in vitro studies have focused on the common CYP2D6 probe substrates such as dextromethorphan or bufuralol to simply clarify in vitro drug biotransformation data with in vivo phenotype data. However, the predicted in vivo consequence of a CYP2D6*17-containing genotype might be quite different if the practitioner is using the genotype information to predict fluoxetine clearance (Clint 8% of that observed with CYP2D6.1) versus that of codeine and by Nakamura et al. (2002) also showed that CYP2D6.10 exhibited a ratio of 0.5 for (S)-fluoxetine, and nortriptyline. The decreased intrinsic clearance of CYP2D6.10 toward the tested CYP2D6-substrates was mainly the result of a substantial decrease in Vmax by the less efficient variant rather than a different Km value. Studies on bufuralol 1-hydroxylation by Zanger et al. (2001) and by Nakamura et al. (2002) also showed that CYP2D6.10 exhibited small Km changes compared with CYP2D6.1, with Km(CYP2D6.10)/Km(CYP2D6.1) ratios of 1.8 and 1.7, respectively. In addition, the current results from the enzyme inhibition studies using dextromethorphan O-demethylase or atomoxetine 4-hydroxylase activity as the CYP2D6 probe (Table 2) indicated mixed differences of enzyme inhibitor affinity between CYP2D6.10 and CYP2D6.1, i.e., the ratios of Kc(CYP2D6.17)/Kc(CYP2D6.1) for cocaine, (S)-fluoxetine, imipramine, and (S)-norfluoxetine were 0.32 to 1.51, whereas the ratios for quinidine and thioridazine were 2.3 and 19, respectively.

CYP2D6.17 also exhibited mixed effects on Km compared with CYP2D6.1, demonstrating a ratio of Km(CYP2D6.17)/Km(CYP2D6.1) of 0.7 to 1.5 for bufuralol, codeine, debrisoquin, and tramadol and >3 for atomoxetine, dextromethorphan, (S)-fluoxetine, and nortriptyline. The results from the current enzyme inhibition studies indicated that both (S)-fluoxetine and (S)-norfluoxetine exhibited comparable enzyme inhibitor affinity for CYP2D6.17 and CYP2D6.1, but the other tested CYP2D6 inhibitors exhibited reduced enzyme inhibitor affinity for CYP2D6.17 compared with that for CYP2D6.1. In total, these results indicate that the effects of the variants, CYP2D6.10 and CYP2D6.17, on Clint relative to those of CYP2D6.1 are substrate-dependent and affect Km, Vmax, or both. Related is the observation that the affinity of inhibitors also varies between the variants and reference allele.

CYP2D6.10 possesses Pro134Ser and Ser188Thr amino acid substitutions, and the effects of these substitutions toward bufuralol, venlafaxine, and p-tyramine were elucidated (Fukuda et al., 2000; Niwa et al., 2004). The studies using site-directed mutagenesis suggested that Ser188Thr alone had no significant effects on Km, whereas Pro134Ser substitution appears to be related to the increased Km for these test substrates. In CYP2D6.17, site-directed mutation studies (Oscarson et al., 1997; Bapiro et al., 2002) indicated that both Thr107Ile and Arg296Cys substitutions were required instead of either single amino acid substitution to cause the change in Km. The results obtained here with the mixed difference in enzyme substrate affinity, as well as enzyme inhibitor affinity, between the less functional CYP2D6 allelic variants and CYP2D6.1 may contribute to improvement of in silico modeling of CYP2D6.

In summary, although CYP2D6.1 and its allelic variants CYP2D6.10 and CYP2D6.17 only differ by two or three amino acids, the results reported here demonstrated that the allelic variants are associated with substrate-dependent decreases in catalytic properties and enzyme inhibitor affinities. The notable finding from this study is that the changes with the functionally reduced allelic variants in enzyme substrate affinity, as well as enzyme inhibitor affinity, compared with those for CYP2D6.1 are mixed, depending on substrates or inhibitors. CYP2D6*10 and CYP2D6*17 alleles have been described for Asians and African Americans, respectively, with relatively high frequencies. The results reported here have implications for drug discovery and development; it is advised that CYP2D6-metabolized candidates or investigational drugs be tested for allele-specific metabolism/interactions. Meanwhile, different metabolism and inhibition by the multiple test compounds with CYP2D6.10 and CYP2D6.17 will have implications for drug dosing, toxicity, and the likelihood of drug-drug interactions in those with one or two of the variant enzymes. Ethnic variability in these kinetic effects may also be predicted by these studies, potentially allowing for ethnic-specific pharmacotherapy. As with all in vitro to in vivo extrapolation, the in vitro results should be interpreted cautiously in the clinical context, in which other drug metabolism pathways may also play a role. It would be interesting to perform further testing in human liver microsomes from individual donors who are homozygous for the observed differences in the cDNA-expressed CYP2D6.1, CYP2D6.10, and CYP2D6.17.

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