Functional Characterization of Wild-type and 49 CYP2D6 Allelic Variants for N-Desmethyltamoxifen 4-Hydroxylation Activity

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Summary: Genetic variations in cytochrome P450 2D6 (CYP2D6) contribute to interindividual variability in the metabolism of clinically used drugs, e.g., tamoxifen. CYP2D6 is genetically polymorphic and is associated with large interindividual variations in therapeutic efficacy and drug toxicity. In this study, we performed an in vitro analysis of 50 allelic variants of CYP2D6 proteins. Wild-type CYP2D6.1 and 49 variants were transiently expressed in COS-7 cells, and the enzymatic activities of the CYP2D6 variants were characterized using N-desmethyltamoxifen as a substrate. The kinetic parameters $K_m$, $V_{max}$, and intrinsic clearance ($V_{max}/K_m$) of N-desmethyltamoxifen 4-hydroxylation were determined. Among the 50 CYP2D6 variants, the kinetic parameters for N-desmethyltamoxifen 4-hydroxylation were determined for 20 CYP2D6 variants. On the other hand, the kinetic parameters of 30 CYP2D6 variants could not be determined because the amount of metabolite produced was at or below the detection limit at the lower substrate concentrations. Among them, 8 variants, i.e., CYP2D6.2, .9, .26, .28, .32, .43, .45, and .70, showed decreased intrinsic clearance at <50% of CYP2D6.1. The comprehensive in vitro assessment of CYP2D6 variants provides novel insights into allele-specific activity towards tamoxifen and may be valuable when interpreting in vivo studies.

Keywords: cytochrome P450; CYP2D6; genetic polymorphisms; tamoxifen; drug metabolism; pharmacogenomics

Introduction

Cytochrome P450 2D6 (CYP2D6) is a major form of cytochrome P450 that metabolizes approximately 25% of clinically used drugs, including dextromethorphan, and tamoxifen. Interindividual variability in P450 activity is an important contributor to pharmacokinetics, efficacy, and adverse drug reactions. Genetic polymorphisms of CYP2D6 have been studied extensively with regard to alterations in enzymatic activity. Currently, there are over 100 variants and subvariants defined by the human CYP Allele Nomenclature Committee (http://www.cypalleles.ki.se/cyp2d6.htm). Consequently, such differences in CYP2D6 activity would lead not only to severe adverse effects in clinical therapy but also to lack of drug response, e.g., no observable analgesic effects of prodrugs such as codeine in poor metabolizers (PMs). CYP2D6 activity differs widely among ultrarapid metabolizer (UM), extensive metabolizer (EM), intermediate metabolizer (IM), and PM phenotypes. The PM phenotype is due to the presence of two nonfunctional (null) alleles, whereas the EM phenotype is due to one or two alleles with normal function, such as CYP2D6*1 and CYP2D6*2. An IM phenotype is usually observed in populations harboring a combination of one CYP2D6 null allele and another allele with impaired expression and/or function, such as CYP2D6*10.
Recently, the potential effect of CYP2D6 genetic variants on the clinical response to tamoxifen treatment in breast cancer patients has gained considerable attention. Tamoxifen is considered a prodrug with very little affinity for the estrogen receptor, and the pharmacological actions of tamoxifen are most likely due to its metabolites. The major metabolite of tamoxifen is N-desmethyltamoxifen, which is generated by CYP3A4/5 and accounts for approximately 90% of tamoxifen metabolites. The major metabolite of tamoxifen is 4-hydroxytamoxifen. A series of in vivo studies have shown that the CYP2D6 genotype alters the biotransformation of tamoxifen to its active metabolite and is associated with the clinical efficacy of tamoxifen. However, some studies have shown negative results for this association. There may be several reasons for these discrepancies among the studies showing positive and negative associations. As several reviews have pointed out, considerable heterogeneity in sample collection or analysis among the studies makes it difficult to compare them easily for the following reasons: (i) differences in the dosage and duration of tamoxifen treatment; (ii) incompleteness of genotyping; and (iii) selection of the study participants. In addition, no report has investigated an association between low-frequency CYP2D6 alleles and tamoxifen metabolism. The functional characterization of CYP2D6 variants may be substrate-dependent. For example, CYP2D6*17 is generally considered an allele with reduced function, but displays remarkable variability in activity toward its substrates, such as dextromethorphan, risperidone, codeine, and haloperidol. Thus, it is necessary to analyze CYP2D6 function using N-desmethyltamoxifen as a CYP2D6 substrate to investigate the association between CYP2D6 variant alleles and tamoxifen metabolism.

The in vivo analysis of tamoxifen metabolism in patients with CYP2D6 variant alleles, e.g., CYP2D6*3, *4, *5, and *10, has been reported. However, the relevance of other CYP2D6 genotypes to tamoxifen metabolism has not yet been fully clarified.

For this association, it is necessary to analyze the catalytic properties of a large number of CYP2D6 variants that are expressed under the same conditions and compared comprehensively with the properties of the wild-type allele.

Although most of the CYP2D6 variant alleles identified carry amino-acid substitutions, CYP2D6*3, *6, *8, *11, *13, *15, *19–*21, *38, *41, *42, *44, *56, *60, and *69 are null alleles because of frame shift, whole gene deletion, splicing defect, or nonsense variations. In addition, CYP2D6*76–*105 variants have been recently identified. In the present study, we focused on the CYP2D6*1–*75 variant alleles with amino-acid substitutions. The purpose of this study was to investigate the in vitro functional characterization of the CYP2D6 variants on N-desmethyltamoxifen 4-hydroxylation. We generated 50 expression constructs (Supplemental Table 1), which were transfected into the COS-7 cell line. Their kinetic parameters, Vmax and intrinsic clearance (Cl_int; Vmax/Km), for N-desmethyltamoxifen 4-hydroxylation were determined. Furthermore, bufuralol hydroxylation and dextromethorphan demethylation were used as other markers for CYP2D6 activity, in addition to N-desmethyltamoxifen 4-hydroxylation. This approach was adopted to elucidate how alterations in the amino acid sequence of CYP2D6 affect its function.

Materials and Methods

Chemicals: N-Desmethyltamoxifen hydrochloride was purchased from Toronto Research Chemical, Inc. (Toronto, Canada). Endoxifen was synthesized using methods described by Detsi et al. and Yu and Forman. Propranolol hydrochloride was purchased from Wako Pure Chemical Industries (Osaka, Japan). NADP+, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were obtained from Oriental Yeast (Tokyo, Japan). The polyclonal anti-human CYP2D6 antibody was purchased from NovoNordisk (Kanagawa, Japan). Polyclonal goat anti-rabbit immunoglobulin/horseradish peroxidase was purchased from Dako Cytomation (Glostrup, Denmark). All other chemicals were of the highest available commercial grade or analytical grade.

Cloning and site-directed mutagenesis of CYP2D6 cDNA: CYP2D6 cDNA fragments obtained from a human liver cDNA library (TaKaRa, Shiga, Japan) were amplified by polymerase chain reaction with the forward primer 5’-CACCATGGGGGCTAGAAGCAC-3’ and the reverse primer 5’-CTAGGCCCCAGCACAAAG-3’ using the GeneAmp High Fidelity Polymerase Chain Reaction System (Applied Biosystems, Foster City, CA). The underlined sequence was introduced for direction cloning. The amplified fragments were subcloned into the pENTR/D-TOPO vector (Invitrogen, Carlsbad, CA). A plasmid carrying CYP2D6*1 cDNA was used as the template to generate CYP2D6 allelic variant constructs (CYP2D6*7, *10, *12, *14A, *14B, *17, *18, *20, *30, *31, *35, *36, *37, *40, *46, *47, *50, *51, *52, *54, *55, *57, *61, *62, *63, *64, *65, *71, *72, and *75) (Supplemental Table 1) using a Quick Change Site-directed...
Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. All of the constructs were sequenced to confirm that appropriate mutagenesis had occurred. The wild-type and variant CYP2D6 cDNAs were subsequently subcloned into the mammalian expression vector pcDNA-DEST40 (Invitrogen).

Expression of CYP2D6 variant proteins in COS-7 cells: COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) containing 10% fetal bovine serum (FBS) at 37°C in the presence of 5% CO₂. At 24 h before transfection, the cells were plated at a density of 0.9 × 10⁶ cells/100-mm dish. Subsequently, Opti-MEM medium (Invitrogen) was added to the culture medium, and plasmids (5.0 µg) carrying CYP2D6 cDNA were transfected using the TransFectin Lipid Reagent (Bio-Rad, Hercules, CA) according to the manufacturer’s instructions. The plasmids carrying CYP2D6 allelic variants were purified using the EndoFree Plasmid Maxi Kit (Qiagen, Gaithersburg, MD). After 6 h incubation at 37°C, the culture medium was replaced with 10% FBS-DMEM, and the cells were incubated for 18 h at 37°C. Cells of 10 dishes were scraped off and pooled. The cell suspension was centrifuged (1,500 × g for 5 min) and resuspended in a homogenization buffer containing 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 10% glycerol. Microsomal fractions were prepared by differential centrifugation at 9,000 × g for 20 min followed by centrifugation of the resultant supernatant at 105,000 × g for 60 min. Microsomal pellets were resuspended in 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 20% glycerol, 150 mM KCl, and a protease inhibitor and stored at –80°C. Protein concentration was determined using a BCA Protein Assay Kit (Pierce, Rockford, IL). Enzyme activity was determined from the microsomes of a single transfection experiment.

Determination of protein expression levels by immunoblotting: Western blotting was performed on microsomal proteins according to standard procedures with 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) with an ATTO mini-gel PAGE system (Tokyo, Japan), and 5 µg microsomal proteins were loaded into each lane. The recombinant CYP2D6 BACULOSOMES Reagent (BD Gentest, Woburn, MA) was co-analyzed as the standard (range, 0.008–0.063 pmol) on each gel and was used to quantify the content of the CYP2D6 apoprotein. CYP2D6 protein was detected using the anti-human CYP2D6 antibody and horseradish peroxidase-conjugated goat anti-rabbit IgG. The immunoblots were developed using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL). Chemiluminescence was quantified using a lumino-imaging analyzer (LAS-1000; Fujifilm, Tokyo, Japan) and ImageJ software (National Institutes of Health, Bethesda, MD).

Enzymatic properties of wild-type and CYP2D6 allelic variant N-desmethyltamoxifen 4-hydroxylation: The N-desmethyltamoxifen 4-hydroxylase activity of CYP2D6 was determined as reported previously with minor modifications. The incubation mixture consisted of microsomal fractions (50 µg, 6.5–21.4 pmol CYP2D6/mg microsomal protein), N-desmethyltamoxifen (1, 2, 4, 8, 20, 40, 80, 160, and 240 µM), and 0.1 M potassium phosphate buffer (pH 7.4). Following pre-warming at 37°C for 3 min, the reaction was initiated by the addition of the NADPH-generating system (1.3 mM NADP, 3.3 mM glucose 6-phosphate, 82.5 mM magnesium chloride, and 0.4 units/mL glucose 6-phosphate dehydrogenase) in a final volume of 125 µL. The samples were incubated at 37°C for 30 min, and the reaction was terminated by the addition of 125 µL methanol containing 2 µM propranolol hydrochloride, which was used as the internal standard.

To determine the optimal reaction conditions for the assay of N-desmethyltamoxifen 4-hydroxylation in microsomal fractions from COS-7 expressed CYP2D6 variants, the dependence of the enzyme activities on incubation time and microsomal protein amount was studied. For these studies, substrate concentrations of 80 µM were used. When the determination of N-desmethyltamoxifen 4-hydroxylation by the microsomal fraction (50 µg) containing CYP2D6.1 was performed, the endoxifen formation was linear from 0 to 60 min of incubation time. Similarly, when the reaction was performed for 30 min, the endoxifen formation was linear from 0 to 50 µg of microsomal proteins (data not shown).

After the removal of protein by centrifugation at 10,000 × g for 3 min, 50 µL supernatant was subjected to high-performance liquid chromatography (HPLC). The HPLC system consisted of a Waters 2965 Separations Module, Waters 2475 Multi λ Fluorescence Detector (Waters, Milford, MA), Photochemical Reactor (Aura Industries, New York, NY), and a TSK-GEL ODS-80Tm column (4.6 mm × 150 mm, 40°C; TOSOH, Tokyo, Japan). The mobile phase was composed of 65% 20 mM potassium phosphate buffer (pH 3.0) and 35% acetonitrile for the first 28 min, which was subsequently replaced with 35% 20 mM potassium phosphate buffer (pH 3.0) and 65% acetonitrile from 29 to 38 min, and the mobile phase was finally restored to 65% 20 mM potassium phosphate buffer (pH 3.0) and 35% acetonitrile from 39 to 48 min. Before detection, the samples were derivatized with UV irradiation (254 nm). Elution was performed at a flow rate of 1.0 mL/min. Endoxifen formation was monitored at excitation and emission wavelengths of 256 and 380 nm, respectively. Under these conditions, the retention times of propranolol hydrochloride and endoxifen were 4.7 and 23.5 min, respectively. The lower limit of endoxifen quantification was 0.8 nM. Enzymatic activity was normalized against the expression level of CYP2D6.

Bufuralol 1’-hydroxylation: The bufuralol 1’-hydroxylation activity was measured by the method reported by Marcucci et al. with minor modifications. The incubation mixture contained 80 µM bufuralol (Sigma-Aldrich, St. Louis, MO), a microsomal fraction (25 µg) obtained from COS-7 cells, 1.3 mM NADP, 3.3 mM glucose-6-phosphate, 0.4 U/mL glucose-6-phosphate dehydrogenase, 3.3 mM MgCl₂, and 100 mM potassium phosphate buffer (pH 7.4) in a final volume of 75 µL. The reactions were carried out at 37°C for 15 min and terminated by the addition of 75 µL methanol. The samples were then centrifuged at 10,000 × g for 3 min to obtain a protein pellet, prior to HPLC analysis. The HPLC system was the same as described above with a 4.6 mm × 150 mm Novapak Phenyl column (3.9 mm × 15 cm; particle size, 4 µm; temperature, 40°C; Waters Corp). The mobile phase was composed of 82% buffer (20 mM potassium phosphate and 20 mM hexane sulfonic acid, pH 4.0) and 18% acetonitrile for the first 12 min, which was subsequently replaced by 60% buffer and 40% acetonitrile from 12 to 17 min, and the mobile phase was finally restored to 82% buffer and 18% acetonitrile from 17 min to 27 min. The flow rate was set at 1.0 mL/min with fluorescence detection at excitation and emission wavelengths of 252 nm and 302 nm, respectively. Under these conditions, the retention times of 1’-hydroxybufuralol and bufuralol were 9 min and 17 min, respectively.

Dextromethorphan O-demethylation: The dextromethorphan O-demethylation activity was measured by the method
reported by Marcucci et al.\textsuperscript{27)} with minor modifications. The reaction mixture containing 80 µM dextromethorphan (Wako Pure Chemical Industries, Osaka, Japan) was incubated for 10 min at 37°C. The reactions were terminated by adding 75 µL methanol containing 1 nM levallorphan tartrate (Sigma-Aldrich), which was used as the internal standard. Other conditions for incubation and deproteination were identical to those used in the assay for bufuralol 1’-hydroxylation activity. Dextrophan was detected using the same HPLC system used for the determination of 1’-hydroxybufuralol. The mobile phase consisted of 75% buffer (20 mM potassium phosphate and 20 mM hexane sulfonic acid, pH 4.0) and 25% acetonitrile at a flow rate of 1.2 mL/min with fluorescence detection at excitation and emission wavelengths of 280 nm and 310 nm, respectively. Under these conditions, the retention times of dextrorphan and dextromethorphan were 4 min and 16 min, respectively.

Data analysis: The kinetic data were applied to the Enzyme Kinetics Module in SigmaPlot 9.01 (Systat Software, Inc., Chicago, IL), which is a curve-fitting program based on nonlinear regression analysis, and the $K_m$, $V_{max}$, and $CL_{int}$ values were determined. All values are expressed in terms of the mean ± standard deviation (SD) values of catalytic assays performed in triplicate. Statistical analyses were performed by one-way analysis of the variance together with the post-hoc Dunnett method. p < 0.05 was defined as significant.

Results

Enzymatic properties for catalysis of N-desmethyltamoxifen 4-hydroxylation by wild-type and variant CYP2D6s: The CYP2D6 variants were expressed in COS-7 cells, and their protein levels were measured by immunoblot analysis. All expressed CYP2D6 variant proteins were recognized by a polyclonal antibody against CYP2D6 (Fig. 2). The recombinant CYP2D6 was co-analyzed as the standard in each gel and was used to quantify the content of the CYP2D6 apoprotein. The Michaelis-Menten kinetics for N-desmethyltamoxifen 4-hydroxylation were measured for the 50 CYP2D6 variant proteins. As shown in Table 1, the Michaelis-Menten kinetics for N-desmethyltamoxifen 4-hydroxylation could be determined for 20 CYP2D6 variants: CYP2D6.1, .2, .9, .22, .28, .32, .34, .39, .43, .45, .48, .49, .53, and .70. The estimated kinetic parameters $K_m$, $V_{max}$, and $CL_{int}$ for N-desmethyltamoxifen 4-hydroxylation by CYP2D6.1 were 56.47 µM, 4.18 µmol/min/pmol CYP2D6, and 0.0081 µL/min/mmol CYP2D6 (% of wild-type).

<table>
<thead>
<tr>
<th>Variants</th>
<th>$K_m$ (µM)</th>
<th>$V_{max}$ (µmol/min/pmol CYP2D6)</th>
<th>$CL_{int}$ ($V_{max}/K_m$) (µL/min/mmol CYP2D6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2D6.1</td>
<td>56.47 ± 7.19</td>
<td>0.45 ± 0.08</td>
<td>0.0081 ± 0.0021</td>
</tr>
<tr>
<td>CYP2D6.2</td>
<td>97.17 ± 15.22</td>
<td>0.16 ± 0.01***</td>
<td>0.0017 ± 0.0003 (21%)</td>
</tr>
<tr>
<td>CYP2D6.9</td>
<td>42.16 ± 2.50</td>
<td>0.13 ± 0.01***</td>
<td>0.0030 ± 0.0004 (37%)</td>
</tr>
<tr>
<td>CYP2D6.22</td>
<td>70.00 ± 22.48</td>
<td>0.54 ± 0.07</td>
<td>0.0081 ± 0.0015 (100%)</td>
</tr>
<tr>
<td>CYP2D6.23</td>
<td>60.56 ± 31.13</td>
<td>0.42 ± 0.05</td>
<td>0.0078 ± 0.0026 (96%)</td>
</tr>
<tr>
<td>CYP2D6.24</td>
<td>59.35 ± 7.58</td>
<td>0.56 ± 0.03*</td>
<td>0.0096 ± 0.0009 (116%)</td>
</tr>
<tr>
<td>CYP2D6.25</td>
<td>60.46 ± 40.85</td>
<td>0.21 ± 0.05***</td>
<td>0.0043 ± 0.0018 (53%)</td>
</tr>
<tr>
<td>CYP2D6.26</td>
<td>68.70 ± 26.05</td>
<td>0.20 ± 0.02**</td>
<td>0.0030 ± 0.0007 (37%)</td>
</tr>
<tr>
<td>CYP2D6.27</td>
<td>46.29 ± 10.54</td>
<td>0.29 ± 0.03***</td>
<td>0.0066 ± 0.0021 (81%)</td>
</tr>
<tr>
<td>CYP2D6.28</td>
<td>57.74 ± 6.35</td>
<td>0.19 ± 0.01***</td>
<td>0.0033 ± 0.0004 (41%)</td>
</tr>
<tr>
<td>CYP2D6.32</td>
<td>98.28 ± 4.18</td>
<td>0.19 ± 0.01***</td>
<td>0.0019 ± 0.0001 (23%)</td>
</tr>
<tr>
<td>CYP2D6.33</td>
<td>74.07 ± 17.00</td>
<td>0.56 ± 0.06*</td>
<td>0.0077 ± 0.0011 (95%)</td>
</tr>
<tr>
<td>CYP2D6.34</td>
<td>36.74 ± 7.68</td>
<td>0.16 ± 0.01***</td>
<td>0.0043 ± 0.0006 (53%)</td>
</tr>
<tr>
<td>CYP2D6.39</td>
<td>70.60 ± 21.61</td>
<td>0.40 ± 0.08</td>
<td>0.0059 ± 0.0009 (73%)</td>
</tr>
<tr>
<td>CYP2D6.43</td>
<td>70.22 ± 34.01</td>
<td>0.26 ± 0.05***</td>
<td>0.0040 ± 0.0010 (49%)</td>
</tr>
<tr>
<td>CYP2D6.45</td>
<td>53.87 ± 32.95</td>
<td>0.11 ± 0.02**</td>
<td>0.0024 ± 0.0010 (30%)</td>
</tr>
<tr>
<td>CYP2D6.48</td>
<td>51.24 ± 12.15</td>
<td>0.28 ± 0.02***</td>
<td>0.0056 ± 0.0010 (69%)</td>
</tr>
<tr>
<td>CYP2D6.49</td>
<td>4.75 ± 0.43*</td>
<td>0.05 ± 0.003***</td>
<td>0.0108 ± 0.0007 (133%)</td>
</tr>
<tr>
<td>CYP2D6.53</td>
<td>7.38 ± 0.63*</td>
<td>1.66 ± 0.06**</td>
<td>0.2257 ± 0.0111***</td>
</tr>
<tr>
<td>CYP2D6.70</td>
<td>45.83 ± 12.53</td>
<td>0.11 ± 0.01***</td>
<td>0.0026 ± 0.0007 (32%)</td>
</tr>
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</table>

Fig. 2. Detection of CYP2D6 proteins expressed in COS-7 cells by immunoblot analysis

Immunoblotting was performed according to standard procedures with 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. CYP2D6 proteins were recognized using a polyclonal antibody against CYP2D6. The numbers correspond to each CYP2D6 allele.

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using other CYP2D6 substrates, bufuralol 1

to compare functional changes when
and variant CYP2D6s: 

proteins at substrate concentrations of 80 µM. Among

below the detection limit at the lower substrate concentrations.

be determined because the amount of metabolite produced was at
or below the detection limit at the lower substrate concentrations.

Among them, 20 variants, i.e., CYP2D6.7, .12, .14A, .31, .36, .37, .40, .47, .51, .52, .54, .55, .57, .62–.65, .71, .72, and .75, showed no activity, and 10 variants, i.e., CYP2D6.10, .14B, .17, .18, .29, .30, .35, .46, .50, and .61, exhibited significantly decreased activity (<15% of CYP2D6.1) at the higher substrate concentration of 80 µM (Fig. 3).

Enzymatic properties for catalysis of bufuralol 1′-hydroxylation and dextromethorphan O-demethylation by wild-type and variant CYP2D6s: To compare functional changes when using other CYP2D6 substrates, bufuralol 1′-hydroxylation and dextromethorphan O-demethylation were measured in 50 CYP2D6 variant proteins at substrate concentrations of 80 µM. Among the 50 CYP2D6 variants tested, 7 variants, i.e., CYP2D6.12, .14A, .36, .47, .51, .57, and .62, showed no activity, and 8 variants, i.e., CYP2D6.7, .10, .37, .40, .54, .64, .71, and .75, exhibited significantly decreased activity with less than 15% activity of CYP2D6.1 for all three substrates (Supplemental Fig. 1).

Discussion

CYP2D6 is a clinically important enzyme because it metabolizes numerous therapeutic drugs. The present study provides comprehensive data regarding functional alterations in CYP2D6 variants. Among the 50 CYP2D6 variants studied, the kinetic parameters for N-desmethyltamoxifen 4-hydroxylation were determined for 20 CYP2D6 variants. On the other hand, the kinetic parameters of 30 CYP2D6 variants could not be determined because the amount of metabolite produced was at or below the detection limit at the lower substrate concentrations.

Among the 30 variants that showed no activity or significantly decreased activity with less than 15% activity of wild-type CYP2D6.1 for N-desmethyltamoxifen 4-hydroxylation, 10 variants, i.e., CYP2D6.10, .14A, .36, .37, .52, .54, .57, .64, .65, and .72, harbor P34S and S486T substitutions. Since the CLint value of CYP2D6.39 harboring only S486T was similar to that of wild-type CYP2D6.1, the critical residue causing decreased N-desmethyltamoxifen 4-hydroxylation activity is likely P34S. These functional alterations are consistent with those of our previous in vitro study performed using bufuralol and dextromethorphan as CYP2D6 substrates. The P34S substitution is located in a proline-rich region that is highly conserved among microsomal P450s and may function as a hinge between the hydrophobic membrane anchor and the heme-binding portion of the enzyme. The P34S substitution in CYP2D6.10 resulted in an unstable protein with significantly reduced enzyme activity towards tamoxifen, dextromethorphan, and bufuralol when expressed in yeast and COS cells.

In Asians, the most abundant variant allele is CYP2D6*10, which is generally considered to be an allele associated with the IM phenotype. Kiyotani et al. studied the impact of the CYP2D6*10 allele on recurrence-free survival in Japanese breast cancer patients receiving adjuvant tamoxifen therapy. In that study, in a comparison with women who had the CYP2D6*1/*1 genotype, the authors reported a 4-fold higher rate of recurrence in women with the CYP2D6*1/*10 genotype and a 16.63-fold higher rate of recurrence in women with the CYP2D6*10/*10 genotype. The plasma concentrations of endoxifen in patients with CYP2D6*10/*10 were reported to be as low as for the PM phenotype. In our present study, the kinetic parameters of CYP2D6.10 could not be determined because the amount of metabolite produced was below the detection limit at the lower substrate concentrations, and the N-desmethyltamoxifen 4-hydroxylation activity of CYP2D6.10, when using the higher substrate concentration (80 µM), was only 1% of that of wild-type CYP2D6.1 (Fig. 3). These lines of evidence imply that CYP2D6*10 genotype patients would have limited enzyme activity, similar to a PM allele, which would remarkably reduce the plasma levels of the active metabolite endoxifen and influence the clinical outcome of tamoxifen therapy.

CYP2D6.2 exhibited a 64% decrease of Vmax, resulting in 21% of the CLint value for N-desmethyltamoxifen 4-hydroxylation and 28% of enzyme activity when using the higher substrate concentration (80 µM), compared with those of CYP2D6.1 (Table 1,

0.45 pmol-min⁻¹·µmol⁻¹ CYP2D6, and 0.0081 µL-min⁻¹·µmol⁻¹ CYP2D6, respectively. Among them, 8 variants, i.e., CYP2D6.2, .9, .26, .28, .32, .43, .45, and .70, showed decreased CLint values that were <50% of CYP2D6.1. Among the 50 types of CYP2D6 variants tested, the kinetic parameters of 30 variants, i.e., CYP2D6.7, .10, .12, .14A, .14B, .17, .18, .29–.31, .35–.37, .40, .46, .47, .50–.52, .54, .55, .57, .61–.65, .71, .72, and .75, could not be determined because the amount of metabolite produced was at or below the detection limit at the lower substrate concentrations.

Among them, 8 variants, i.e., CYP2D6.10, .14A, .36, .37, .52, .54, .57, .64, .65, and .72, harbor P34S and S486T substitutions. Since the CLint value of CYP2D6.39 harboring only S486T was similar to that of wild-type CYP2D6.1, the critical residue causing decreased N-desmethyltamoxifen 4-hydroxylation activity is likely P34S. These functional alterations are consistent with those of our previous in vitro study performed using bufuralol and dextromethorphan as CYP2D6 substrates. The P34S substitution is located in a proline-rich region that is highly conserved among microsomal P450s and may function as a hinge between the hydrophobic membrane anchor and the heme-binding portion of the enzyme. The P34S substitution in CYP2D6.10 resulted in an unstable protein with significantly reduced enzyme activity towards tamoxifen, dextromethorphan, and bufuralol when expressed in yeast and COS cells.

In Asians, the most abundant variant allele is CYP2D6*10, which is generally considered to be an allele associated with the IM phenotype. Kiyotani et al. studied the impact of the CYP2D6*10 allele on recurrence-free survival in Japanese breast cancer patients receiving adjuvant tamoxifen therapy. In that study, in a comparison with women who had the CYP2D6*1/*1 genotype, the authors reported a 4-fold higher rate of recurrence in women with the CYP2D6*1/*10 genotype and a 16.63-fold higher rate of recurrence in women with the CYP2D6*10/*10 genotype. The plasma concentrations of endoxifen in patients with CYP2D6*10/*10 were reported to be as low as for the PM phenotype. In our present study, the kinetic parameters of CYP2D6.10 could not be determined because the amount of metabolite produced was below the detection limit at the lower substrate concentrations, and the N-desmethyltamoxifen 4-hydroxylation activity of CYP2D6.10, when using the higher substrate concentration (80 µM), was only 1% of that of wild-type CYP2D6.1 (Fig. 3). These lines of evidence imply that CYP2D6*10 genotype patients would have limited enzyme activity, similar to a PM allele, which would remarkably reduce the plasma levels of the active metabolite endoxifen and influence the clinical outcome of tamoxifen therapy.

CYP2D6.2 exhibited a 64% decrease of Vmax, resulting in 21% of the CLint value for N-desmethyltamoxifen 4-hydroxylation and 28% of enzyme activity when using the higher substrate concentration (80 µM), compared with those of CYP2D6.1 (Table 1,
in vivo expression system under the same conditions. If CYP2D6 has between the CYP2D6 protein and substrate concentrations used in this study, the amino acids intro-

metabolite endoxifen was below the detection limit at the lower values, resulting in a 50% decrease in the $K_n$ value compared with the $K_n$ value of CYP2D6.1 in 7-methoxy-4-(aminomethyl)-coumarin metabolism. Our present and previous results, using N-desmethyltamoxifen and bufuralol as substrates, respectively, revealed that CYP2D6.53 exhibited lower $K_n$ and higher $V_{max}$ values than CYP2D6.1, suggesting that the increased affinity of CYP2D6.53 should be attributed to the natural F120I variant. The increase in the $CL_{int}$ value of CYP2D6.53 is considered to be the main alteration affecting substrate binding, and individuals with CYP2D6*53 may exhibit the UM phenotype. In contrast, the $CL_{int}$ value of CYP2D6.49 harboring P34S, F120I, and S486T was similar to that of CYP2D6.1. With regard to CYP2D6.49, although F120I would cause lower $K_n$ values, P34S would cause lower $V_{max}$ values, resulting in comparable $CL_{int}$ values for CYP2D6.1.

CYP2D6.36, .57, .61, and .63 share 7 amino acid changes (P469A, T470A, H478S, G479A, F481V, A482S, and S486T) coded in exon 9, and their N-desmethyltamoxifen 4-hydroxylation activity was below the detection limit when using the higher substrate concentration (80 µM). Fukuda et al. reported that the $K_n$ value of recombinant CYP2D6.36 (P34S and the 7 codon changes in exon 9) expressed in yeast for bufuralol 1-hydroxylation was higher than that of recombinant CYP2D6.10 (P34S and S486T). Gaedigk et al. also reported that CYP2D6*36 was associated with a PM phenotype in vivo, with dextromethorphan as a sub-

strate. Although we were unable to calculate the $K_n$ values of CYP2D6.36, .57, .61, and .63 because the N-desmethyltamoxifen metabolite endoxifen was below the detection limit at the lower substrate concentrations used in this study, the amino acids introduced by the exon 9 conversion may induce an alteration in affinity between the CYP2D6 protein and N-desmethyltamoxifen, resulting in the loss of CYP2D6 activity.

This is the first study to functionally analyze a large number of recombinant CYP2D6 variant proteins with respect to N-desmethyltamoxifen 4-hydroxylation activity using a heterologous expression system under the same conditions. If CYP2D6 has a significant role in the metabolism of N-desmethyltamoxifen in vivo as well as in vitro, individuals with compromised CYP2D6 metabolism would have lower plasma concentrations of the active metabolite endoxifen than those with more active variants of this enzyme. However, because tamoxifen is metabolized by several phase I and II enzymes, e.g., CYP3A4/5, sulfotransferases (SULTs), and UDP-glucuronosyltransferases (UGTs), it would be difficult to assess the clinical outcome in subjects who express CYP2D6 polymorphically without in vivo data. To understand the mechanistic basis of our findings more fully, it would be of great value to examine the clinical relationships among CYP2D6, CYP3A4/5, SULT, and UGT genotypes and the plasma concentrations of tamoxifen and its metabolites.

There are some limitations to predicting in vivo activity or phenotype from in vitro data. For example, expression levels and protein stability may be considerably different in vivo compared to the amounts of protein expressed and their stability in vitro. It would be important to carefully determine the stability of the variant proteins using denaturation experiments at higher temperatures and to determine structure-function relationships. We have been analyzing the relationship by using three-dimensional docking simulation software. Moreover, some CYP2D6 variant proteins may decrease heme incorporation into the CYP2D6 apoprotein, but this possibility could not be assessed in the present study. The levels of CYP2D6 protein expressed in COS-7 cells were too low to be determined by the difference in the carbon monoxide-reduced spectra analysis. An analysis using the variant proteins produced in bacterial and baculovirus-mediated systems may allow the determination of the actual P450 concentration. Further studies will be required to determine the importance of the CYP2D6 allelic variants in clinical settings. Furthermore, the relationship between in vivo and in vitro studies using other substrates as well as the measurement of holoprotein levels is required to confirm these results.

In conclusion, we transiently expressed 50 CYP2D6 variants in COS-7 cells. Of these, most of the CYP2D6 variants tested in vitro exhibited functional alterations in CYP2D6 activity. The comprehensive in vitro assessment of CYP2D6 allelic variants can provide novel insights into allele-specific activity towards tamoxifen and may be valuable when interpreting in vivo studies.

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References


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