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Cytochrome P450 2A6 (CYP2A6) catalyzes important metabolic reactions of many xenobiotic compounds, including coumarin, nicotine, cotinine, and clinical drugs. Genetic polymorphisms of CYP2A6 can influence its metabolic activities. This study analyzed the functional activities of the six CYP2A6 allelic variants (CYP2A6*5, *7, *8, *18, *19, and *35) containing nonsynonymous single-nucleotide polymorphisms. Recombinant variant enzymes of CYP2A6*7, *8, *18, *19, and *35 were successfully expressed in Escherichia coli and purified. However, a P450 holoenzyme spectrum was not detected for the CYP2A6*5 allelic variant (G479V). Structural analysis shows that the G479V mutation may alter the interaction between the A helix and the F—G helices. Enzyme kinetic analyses indicated that the effects of mutations in CYP2A6 allelic variants on drug metabolism are dependent on the substrates. In the case of coumarin 7-hydroxylation, CYP2A6*8 and *35 displayed increased $k_m$ values whereas CYP2A6*18 and *19 showed decreased $k_{cat}$ values, which resulted in lower catalytic efficiencies ($k_{cat}/k_m$). In the case of nicotine 5-oxidation, the CYP2A6*19 variant exhibited an increased $k_m$ value, whereas CYP2A6*18 and *35 showed much greater decreases in $k_{cat}$ values. These results suggest that individual carrying these allelic variants are likely to have different metabolisms for different CYP2A6 substrates. Functional characterization of these allelic variants of CYP2A6 can help determine the importance of CYP2A6 polymorphisms in the metabolism of many clinical drugs.

Key words P450; CYP 2A6; allelic variant; polymorphism; coumarin; nicotine

The cytochrome P450 (CYP, P450) family is composed of major phase I enzymes responsible for metabolizing xenobiotic chemicals including clinical drugs. CYP2A6 was first determined to catalyze coumarin 7-hydroxylation in the human liver. CYP2A6 also metabolizes many clinical drugs, including valproic acid, losigamone, chlorothiazole, letrozole, fadrozole, methoxyflurane, and tegafur. This enzyme is known to catalyze the metabolism of tobacco-specific compounds such as nicotine, cotinine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, and N-nitrosornicotine, and its role in the bioactivation of procarcinogens has also been intensively investigated.

Genetic variations in metabolic enzymes can cause dramatic differences in the response to specific drugs. Some drug-metabolizing P450 enzymes are very polymorphic, and the study of their polymorphisms is of particular interest because it can help develop a rational means to optimize drug therapy and ensure maximum efficacy with minimal adverse effects. Previous studies reported the genetic polymorphisms of CYP2A6 and proposed their relevance to cancer risk due to variations in nicotine and N-nitrosamine metabolism. Interestingly, the ethnic distribution of CYP2A6 polymorphisms is slightly different from that of other P450 enzymes. A very low frequency of poor metabolizers (PMs) has been reported in Caucasians and Africans, but the occurrence of PMs is much more common in Asians. To date, at least 36 allelic variants of CYP2A6 have been identified, 26 of which are located in the coding region of the CYP2A6 gene (http://www.cypalleles.ki.se/). Nonsynonymous single nucleotide polymorphisms (SNPs) can alter the protein sequences of CYP2A6. The substrate preferences of these mutant enzymes are different from those of the wild-type enzymes, and therefore, the mutant enzymes can produce dramatic effects in CYP2A6 metabolism.

In this study, we analyzed the functional activities of six allelic variants of CYP2A6 (CYP2A6*5, *7, *8, *18, *19, and *35) containing nonsynonymous SNPs. They contain substitutions of the following amino acids: CYP2A6*5 (G479V); CYP2A6*7 (I471T); CYP2A6*8 (R485L); *18 (Y392F, *19 (Y392F, I471T); and *35 (N438Y) (http://www.cypalleles.ki.se/). These variants are frequently found in Asian populations. The allelic frequencies of CYP2A6*5, *7, *8, *18, and *35 in Japanese and Koreans were 0.5%, 9.8%, 1.4—2.2%, 0.5%, and 0.5—0.8%, respectively. In view of the importance of CYP2A6 in the metabolism of many clinical drugs and several tobacco-specific compounds, we determined the functional changes in these allelic variants.

MATERIALS AND METHODS

Chemicals and Enzymes Coumarin, 7-hydroxycoumarin, nicotine, imidazole, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate (CHAPS), dilauroyl-L-phosphatidylcholine (DPLC), NADP+, and NADPH were purchased from Sigma Chemical (St. Louis, MO, U.S.A.) or Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Ni2+-Nitrolotriacetate agarose was purchased from Qiagen (Valencia, CA, U.S.A.). Other chemicals were of the highest commercially available grade. Escherichia coli DH5α cells were purchased from Invitrogen (Carlsbad, CA, U.S.A.). Rat NADPH-P450 reductase was het-
erologically expressed in E. coli (TOPP3 strain) and purified as described elsewhere.20

**Construction of CYP 2A6 Variants** We used a pCW plasmid containing the coding sequence of CYP2A6 to construct expression vectors for the CYP2A6 variants. The general approach was described previously.21 Briefly, site-directed mutagenesis was performed using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.) with the following primers: 5'-CCAAAACACGTGTTCTTGGC-3', 5'-GGCAAAGACCACGTTTGTTGGG-3' (CYP2A6*5); 5'-CCTAAAGGACACTGACGTGTC-3', 5'-GACACGTACGTTGCTAAGG-3' (CYP2A6*7); 5'-GCCACGATCCACCATGGGACCTAACATACC-3', 5'-GGGGTACTGTTAGTTGGGATTGCTGG-3' (CYP2A6*8); 5'-CCGAAAGTGGTTCTATGCTGGG-3', 5'-CCACACATAGGGAAAGCTCTCGG-3' (CYP2A6*18); and 5'-GGGAAGGCGGTACTGTTTGAGGAG-3', 5'-CTCGAAAACAGTACGCTTCCC-3' (CYP2A6*35). The CYP2A6*19 variant was used as the primers for CYP2A6*7 and *18. The open reading frames for CYP2A6 variants were isolated and prepared from TB expression cultures following the method described previously.21 Purification of CYP2A6 were isolated and prepared from TB expression cultures following the method described previously.21 Puriﬁcation of CYP2A6 enzymes using a Ni2+-nitrilotriacetic acid column was also performed by using a previously described method.23 Briefly, the prepared membrane fractions were solubilized overnight at 4°C in potassium phosphate buffer 50 mM and incubated overnight at 37°C. The expression levels of CYP2A6*1, *7, *8, *18, *19, and *35 ranged from 0.05 to 1 μmol, rat NADPH-P450 reductase 100 pmol, and DLPC 45 μM in 0.50 mL of potassium phosphate buffer 100 mM (pH 7.4), along with a speciﬁed amount of the substrates. An NADPH-generating system was used to start reactions after 3-min preincubation at 37°C. Incubation was generally for 10 min at 37°C and terminated with 0.05 mL of HCl 2M in the case of the coumarin oxidation reaction. CHCl2 was added to extract the oxidized products, followed by vortex mixing and centrifugation at 2000 rpm for 10 min. The organic (lower) layer was transferred to a clean test tube, and 1 mL of sodium borate buffer 0.1 M (pH 9.6) was added. The production of 7-hydroxycoumarin in a microtiter plate was measured a ﬂuorescence plate reader (350 nm/453 nm). For nicotine oxidation reaction, the enzyme reactions were terminated by vortexing after the addition of 0.05 mL of HClO4 and then placing the reaction mixture on ice for 1 min. The reaction mixtures were centrifuged at 13000 rpm for 30 min at 4°C. To measure oxidized nicotine, the reaction products were analyzed with HPLC using a reversed-phase ODS column (5 μm, 150×4.6 mm, Alltech Associates, Inc., Deerfield, IL, U.S.A.) equipped with a YL9110 HPLC system (YoungLin, Korea). Isoﬁcric cation was used with a mobile phase of potassium phosphate buffer 20 mM containing heptane sulfate 3.3 mM, 0.5% triethylamine, and 10% acetonitrile (pH 5.07) at a ﬂow rate of 0.9 mL/min, while monitoring A340. The oxidized nicotine formation was quantiﬁed by comparing HPLC peaks using an authentic standard. Kinetic parameters were estimated from the ﬁtted curves using a computer program (GraphPad Prism 4) designed for nonlinear regression analysis. Statistical analyses of the kinetic parameters were performed using the two-tailed Student t-test.

**NADPH Oxidation Assays** The NADPH oxidation rates were determined using phospholipid reconstituted systems containing CYP2A6 and NADPH-P450 reductase. Reconstituted enzymes were preincubated for 5 min at 37°C in the presence or absence of coumarin (100 μM). The reactions were initiated by the addition of 10 μL of NADPH 10 mM, and the reduction of A340 was monitored. The rates were calculated using ΔA340 = 6.22 mmol−1 cm−1.

RESULTS

**Expression and Purification of Recombinant CYP2A6 Variant Enzymes** All six CYP2A6 variants were successfully constructed in the pCW expression vectors, and recombinant CYP2A6 variant proteins were expressed in E. coli. Recombinant CYP2A6 expression levels in the whole-cell cultures were determined spectroscopically. The expression levels of CYP2A6*1, *7, *8, *18, *19, and *35 ranged from 100 to 200 μmol P450 holoenzyme per liter of culture medium (Fig. 1). However, no P450 holoenzyme spectrum was observed for the CYP2A6*5 allelic variant (G479V) (Fig. 1). Immunoblot analysis using anti-his-tag antibody in the membrane fraction showed that the CYP2A6*5 variant protein was not detectable (data not shown). This result suggests that the G479V substitution may interfere with the accurate expression of the enzyme.

Bacterial inner membrane fractions containing the CYP2A6 variants (CYP2A6*7, *8, *18, *19, and *35) were isolated and...
prepared. Purified wild type and variant CYP2A6 proteins were successfully obtained using the Ni\textsuperscript{2+}-affinity column. All the purified proteins showed single bands of 57 kDa on SDS-PAGE as expected from the molecular mass of the open reading frame (supplementary Fig. 1).

**Substrate Binding Affinities** Titration of purified wild-type and variant CYP2A6 enzymes showed a typical type I spectral change, suggesting the loss of coordination of H\textsubscript{2}O to the P450 heme. Binding affinities of purified wild type and variant CYP2A6 enzymes for coumarin were determined (Fig. 2, Table 1). The binding of coumarin to wild type CYP2A6 yielded a $K_d$ value of 3.5±0.1 μM (Table 1). CYP2A6 variants did not exhibit significantly altered binding affinities for coumarin, although a small increase in the $K_d$ values was seen (Table 1). These results suggest that the amino acid changes in the five variants did not affect the architecture of the active site of the enzymes.

**Enzymatic Activities of CYP2A6 Variants** Catalytic activities of the CYP2A6 variant enzymes were determined

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**Table 1. Coumarin Binding Affinities of Purified Wild-Type and Variants of CYP2A6**

<table>
<thead>
<tr>
<th>Variants</th>
<th>Mutations</th>
<th>$K_d$, μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2A6*1</td>
<td>WT</td>
<td>3.5±0.1</td>
</tr>
<tr>
<td>CYP2A6*7</td>
<td>I471T</td>
<td>4.7±0.4</td>
</tr>
<tr>
<td>CYP2A6*8</td>
<td>R485L</td>
<td>8.1±0.7</td>
</tr>
<tr>
<td>CYP2A6*18</td>
<td>Y392F</td>
<td>6.4±0.7</td>
</tr>
<tr>
<td>CYP2A6*19</td>
<td>Y392F/I471T</td>
<td>9.6±0.7</td>
</tr>
<tr>
<td>CYP2A6*35</td>
<td>N438Y</td>
<td>7.9±0.4</td>
</tr>
</tbody>
</table>

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**Fig. 1. Expression of Recombinant CYP2A6 Allelic Variant Enzymes in E. coli**

CO-binding spectra of P450 enzymes in *E. coli* whole cells were measured, and the contents of the P450 holoenzyme were calculated.

**Fig. 2. Coumarin Binding Affinities of CYP2A6 Allelic Variant Enzymes**

(A) Binding titration of CYP2A6 with coumarin. Increasing concentrations of coumarin were added to both the sample and reference cuvettes. (B) Plots of $\Delta A_{385-418}$ nm vs. the concentration of coumarin for the CYP2A6 allelic variants.
by measuring the rates of coumarin 7-hydroxylation and nicotine 5-oxidation. Steady-state kinetic analysis of coumarin 7-hydroxylation indicated that the catalytic efficiencies ($k_{cat}/K_m$) of all the variants were lower than that of the wild-type CYP2A6*1 (Fig. 3A, Table 2). CYP2A6*8 and *35 showed greater increases in $k_{cat}$ values (with some increases in $K_m$ values), whereas CYP2A6*18 and *19 showed lower catalytic efficiencies ($k_{cat}/K_m$), mainly because of the decreased $K_m$ values (Fig. 3A, Table 2). In addition, all variants exhibited reduced catalytic efficiencies ($k_{cat}/K_m$) for nicotine 5-oxidation. The CYP2A6*19 variant exhibited an increased $K_m$ value, which resulted in lower catalytic efficiencies ($k_{cat}/K_m$), whereas CYP2A6*18 and *35 showed much greater decreases in the $k_{cat}$ values than in the $K_m$ values. CYP2A6*7 and *8 showed a small decrease in the $K_m$ values, with no significant change in their $K_m$ values (Fig. 3B, Table 2). These results suggest that the effects of mutations in CYP2A6 allelic variants on drug metabolism are highly dependent on the substrates.

NADPH Consumption by CYP2A6 Variants When the rates of NADPH consumption by the CYP2A6 variants were analyzed, CYP2A6*7 and wild-type CYP2A6 showed similar NADPH oxidation rates (Table 3). CYP2A6*18, *19, and *35 showed slow rates of NADPH oxidation (ca. 40%) in the presence of the substrate (Table 3). These results suggest that the lower enzymatic activities of the allelic variants CYP2A6*18 and *19 in coumarin oxidation may be caused partly by a decrease in the utilization of electrons. However, the slow rate of NADPH oxidation did not alter its catalytic efficiency ($k_{cat}/K_m$) value in the variant CYP2A6*35 because both $k_{cat}$ and $K_m$ values were increased. In addition, the faster rate of NADPH consumption (ca. 37%) observed in the case of CYP2A6*8 may indicate the inefficient coupling of NADPH in the enzymatic reaction.

Positions of the Mutated Amino Acids in CYP2A6 Variants The X-ray crystal structure of CYP2A6 in complex with coumarin (PDB-entry code, 1Z10) was used to position the mutated residues in the CYP2A6 enzyme (Fig. 4).25 Mutated residues in CYP2A6*5, *7, and *8 (G479V, I471T, R485L, respectively) are located in the β3—β4 hairpin loop region near the C-terminus of the enzyme, and the Y392 residue in CYP2A6*18 is located in the β2-sheet. These residues are all located outside the formal active site of P450 (Fig. 4). Among these mutations, the G479V mutation in CYP2A6*5 is located spatially between the C-terminus of the A helix and the F—G helices, thereby resulting in the overall structural stability of the P450 enzyme. We also found that the N438Y mutation in CYP2A6*35 is located next to the proximal Cys

Table 2. Kinetic Parameters of Coumarin 7-Hydroxylation and Nicotine 5-Hydroxylation by Purified Wild-Type and Variants of CYP2A6

<table>
<thead>
<tr>
<th>Variants</th>
<th>Mutations</th>
<th>Coumarin 7-hydroxylation</th>
<th>Nicotine 5-oxidation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$k_{cat}$, min$^{-1}$</td>
<td>$K_m$, μM</td>
</tr>
<tr>
<td>CYP2A6*1</td>
<td>WT</td>
<td>11.3±0.4</td>
<td>7.6±1.0</td>
</tr>
<tr>
<td>CYP2A6*7</td>
<td>I471T</td>
<td>10.0±0.4*</td>
<td>8.1±1.1</td>
</tr>
<tr>
<td>CYP2A6*8</td>
<td>R485L</td>
<td>15.5±0.8**</td>
<td>20.6±2.8*</td>
</tr>
<tr>
<td>CYP2A6*18</td>
<td>Y392F</td>
<td>6.9±0.2**</td>
<td>6.6±0.6</td>
</tr>
<tr>
<td>CYP2A6*19</td>
<td>Y392F/I471T</td>
<td>8.8±0.3**</td>
<td>8.9±1.2</td>
</tr>
<tr>
<td>CYP2A6*35</td>
<td>N438Y</td>
<td>26.5±1.0**</td>
<td>20.7±2.2**</td>
</tr>
</tbody>
</table>

Results are presented as means±S.D.; *p<0.05, compared with WT (CYP2A6*1); **p<0.005, compared with WT (CYP2A6*1) by Student’s t-test.

Table 3. NADPH Oxidation with Purified Wild-Type and Variants of CYP2A6

<table>
<thead>
<tr>
<th>Variants</th>
<th>Mutations</th>
<th>Coumarin (1000μM)</th>
<th>NADPH Oxidationa</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP2A6*1</td>
<td>WT</td>
<td>+</td>
<td>178±4</td>
</tr>
<tr>
<td>CYP2A6*7</td>
<td>I471T</td>
<td>+</td>
<td>42±3</td>
</tr>
<tr>
<td>CYP2A6*8</td>
<td>R485L</td>
<td>+</td>
<td>151±3</td>
</tr>
<tr>
<td>CYP2A6*18</td>
<td>Y392F</td>
<td>+</td>
<td>61±2</td>
</tr>
<tr>
<td>CYP2A6*19</td>
<td>Y392F/I471T</td>
<td>+</td>
<td>50±10</td>
</tr>
<tr>
<td>CYP2A6*35</td>
<td>N438Y</td>
<td>+</td>
<td>35±0</td>
</tr>
</tbody>
</table>

a) nmol of reduced NADPH/min/nmol of purified P450 protein.

Fig. 3. Steady-State Kinetic Analysis of Coumarin 7-Hydroxylation and Nicotine 5-Oxidation by CYP2A6 Allelic Variant Enzymes

Each point presented is mean±S.D. of triplicate assays.
DISCUSSION

A previous study in a yeast expression system showed that the CYP2A6*5 allelic variant is an unstable enzyme with a G479L substitution. Similarly, our study also showed that the mutant recombinant enzyme containing the G479V substitution did not express the holoenzyme in E. coli (Fig. 1). Analysis of the X-ray crystal structure showed that the G479 residue is located between the A helix and F—G helices and plays a role in accurate folding of the enzyme structure (Fig. 4).

The I471T substitution in CYP2A6*7 and *19 was also found in several other alleles of CYP2A6 including CYP2A6*10 (I471T; R485L), *36 (N438Y; I471T), and *37 (N438Y; I471T; R485L) (http://www.cypalleles.ki.se/). Interestingly, the allelic frequencies of CYP2A6*7, *10, and *19 with the I471T substitution were relatively high in Korean and Japanese populations, whereas they were not detected in Caucasian and African populations. Previous studies on the bicistronic membrane fractions showed that the enzymes with the I471T substitution had reduced activities in metabolizing coumarin and nicotine. In our study using purified protein, the catalytic efficiencies showed an approximately 30% reduction (Table 2). Fukami et al. reported that the $k_{cat}$ and $K_m$ values of CYP2A6*19 (Y392F; I471T) were lower than those of CYP2A6*1 for nicotine oxidation, whereas they were higher than those of CYP2A6*1 in this study. These inconsistent results may be attributed to the use of different in vitro methods (bacterial membrane fractions vs. reconstitution with purified enzymes) for analyzing enzymatic activities. Further elaborate studies are required to understand the significance of the I471T substitution in humans.

A previous in vivo pilot study found that the catalytic activity of CYP2A6*8 was not affected by the R485L substitution; however, in our study, the purified enzyme showed a significant reduction in enzyme activities for the oxidation of coumarin (because of increased $K_m$) and nicotine (because of decreased $k_{cat}$) (Table 2). This substitution appears to have different effects in different substrates.

Fukami et al. reported that the allelic variation in CYP2A6*18 affected the enzymatic activity and induced decreased metabolism of coumarin, nicotine, and tegafur in vivo and in vitro. They reported that the $K_m$ value of CYP2A6*18 for coumarin 7-hydroxylation was higher than that of CYP2A6*1 and that the $K_m$ and $k_{cat}$ values of CYP2A6*18 for nicotine oxidation was higher than those of CYP2A6*1. However, the Y392F substitution was observed to reduce the $k_{cat}$ values for coumarin and nicotine oxidation significantly in our study (Table 2). Further studies on CYP2A6*18 are also required to understand the different enzymatic results from the bacterial membrane fractions and the reconstitution with
purified enzymes. Koudsi et al. reported that the N438Y substitution appeared to be associated with lower nicotine metabolism in vivo and decreased nicotine oxidation activity and thermal stability in vitro. They reported that the $K_m$ value of CYP2A6*35 for nicotine oxidation was higher than that of CYP2A6*1, resulting in lower catalytic efficiency. In our study, the enzyme containing this substitution showed a significantly decreased $k_{cat}$ value for nicotine 5-oxidation, and this resulted in lower catalytic efficiency (Table 2). However, the catalytic efficiency for coumarin 7-hydroxylation was not altered to a great extent because both the $k_{cat}$ and $K_m$ values were increased.

In conclusion, we expressed six allelic variants of CYP2A6 and analyzed their catalytic activities. CYP2A6*5 containing the G479V substitution was observed to be inactive in the recombinant expression system used, and the remaining variants showed changes in their catalytic activities toward the CYP2A6 substrates coumarin and nicotine. Because CYP2A6 is an important enzyme involved in the metabolism of many clinical drugs, the prevalence of these polymorphisms in CYP2A6 and their possible effects on the safety and efficacy of these drugs should be carefully examined.

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REFERENCES

23) Yun CH, Miller GP, Guengerich FP. Rate-determining steps in phenacetin oxidations by human cytochrome P450 1A2 and selected mutants. Biochemistry, 39, 11319—11329 (2000).