Q172H Replacement Overcomes Effects on the Metabolism of Cyclophosphamide and Efavirenz Caused by CYP2B6 Variant with Arg262

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ABSTRACT:

There are a number of reports indicating that CYP2B6*6 (c.516G>T and c.785A>G) is responsible for decreased clearance of efavirenz (EFV), although increased disposition of cyclophosphamide (CPA) in individuals with this polymorphism was observed. Thus, we hypothesized that the effects of the two single nucleotide polymorphisms (SNPs) of CYP2B6*6 on the metabolism of drugs might be considerably different between these two agents. To clarify this possibility, we expressed two major variants of this enzyme, CYP2B6.6 (Q172H and K262R) and CYP2B6.4 (K262R), and investigated metabolic activities of these variants toward EFV and CPA. Kinetic analyses clearly indicated that CYP2B6.4 possessed enhanced metabolic activity toward EFV compared with that of the wild-type enzyme (CYP2B6.1), whereas CPA was metabolized less efficiently by CYP2B6.4 than by CYP2B6.1. On the other hand, CYP2B6.6 showed a completely opposite character, suggesting that Q172H gives inverse effects on metabolic activities of CYP2B6 affected by K262R. Although it is recognized that effects of amino acid change in cytochrome P450 on the metabolic activity depend on substrates, this study revealed SNPs giving an opposite effect on the metabolism of two clinically important drugs currently used. Furthermore, this study provides the first evidence that Q172H can reverse the direction of the effect caused by K262R in CYP2B6 on the metabolism of certain drugs.

Introduction

It is well documented that genetic polymorphisms of cytochrome P450 (P450) affect drug-metabolizing activities, although the degree of the effect depends on substrates. For example, a marked decrease in the metabolism of phenytoin by I359L in CYP2C9 occurs both in vitro (Takanashi et al., 2000) and in vivo (Odani et al., 1997), but the decrease in the metabolism of diclofenac is not remarkable either in vitro (Takanashi et al., 2000) or in vivo (Shimamoto et al., 2000). However, in most cases, a nonsynonymous SNP of the P450 genes gives a unidirectional change in catalytic activity toward different substrates. For example, replacement of Ile at codon 359 by Leu due to CYP2C9*3 decreases catalytic activities toward all seven different substrates (Takanashi et al., 2000).

CYP2B6 is known as the most important P450 isoform responsible for the metabolism of anticancer prodrug, cyclophosphamide (CPA) (Roy et al., 1999), and a non-nucleoside HIV-1 reverse transcriptase inhibitor, efavirenz (EFV) (Ward et al., 2003). We reported previously for the first time that CYP2B6 possessing a His instead of a Gln residue at codon 172 caused a drastic increase in CYP2B6 activity using 7-ethoxycoumarin as a substrate in vitro (Ariyoshi et al., 2001). Increased catalytic activity for the metabolism of another reagent, 7-ethoxy-4-trifluoromethyl-coumarin, has also been reported (Jinno et al., 2003). We have also observed enhanced metabolism of certain drugs (S. Afuso and N. Ariyoshi, unpublished observations, 2003). Supporting our observation, Xie et al. (2003) reported that CPA 4-hydroxylation was significantly enhanced in human liver microsomes from CYP2B6*6 carriers. They also demonstrated that the presence of the CYP2B6*6 allele might increase the rate of 4-hydroxy-CPA formation in vivo (Xie et al., 2006). Nakajima et al. (2007) also reported that the clearance of CPA in cancer patients who are homozygotes for CYP2B6*6 was significantly higher than that in those who are homozygotes for CYP2B6*1.

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ABBREVIATIONS: P450, cytochrome P450; SNP, single nucleotide polymorphism; CPA, cyclophosphamide; EFV, efavirenz; POR, NADPH-cytochrome P450 oxidoreductase; HPLC, high-performance liquid chromatography.
effects on catalytic activity of CYP2B6 between CPA and EFV. However, because there are some reports suggesting that CYP2B6 is related to decreased expression of CYP2B6 protein (Lang et al., 2001; Desta et al., 2007), the mechanism of the reduced clearance of EFV may simply be due to a low CYP2B6 expression level in vivo. If the latter hypothesis is correct, the metabolic activity of EFV may not be significantly altered by Q172H replacement. To clarify whether or not the metabolic activity of EFV by CYP2B6 is affected by several natural amino acid replacements, we performed kinetic analyses using recombinant enzymes without considering the effects of these SNPs on the enzyme expression.

Materials and Methods

Chemicals. CPA was purchased from Wako Pure Chemicals (Osaka, Japan). An authentic standard of 4-hydroxy-CPA was kindly donated by Shionogi and Co. (Osaka, Japan). EFV was obtained from Merck (Whitehouse Station, NJ), and ritonavir was generously provided by Abbott Laboratories (Abbott Park, IL). 8-Hydroxy-EFV was synthesized from 5-chloro-2-nitroanisole as a starting material essentially by the method of Markwalder et al. (2001). All other chemicals and solvents were of the highest analytical or molecular biological grade commercially available.

cDNA Cloning and Site-Directed Mutagenesis. CYP2B6 cDNA was amplified by reverse transcriptase-polymerase chain reaction from a human liver sample provided by the Human and Animal Bridging Research Organization (Chiba, Japan). The amplicon was subcloned into pBluescript II SK(+)- vector (Promega, Madison, WI). Site-directed mutagenesis was performed to produce a cDNA corresponding to the CYP2B6*4 by the primer-directed enzymatic amplification method (Saiki et al., 1988). Then cDNA corresponding to CYP2B6*6 was produced by introducing the second mutation (c.516G>T) at codon 172 of the cDNA corresponding to CYP2B6*4.

Heterologous Expression of CYP2B6 with Human NADPH-P450 Oxidoreductase Proteins. Sf9 insect cells were purchased from Invitrogen (Carlsbad, CA). CYP2B6 cDNA together with human NADPH-P450 oxidoreductase (POR) cDNA, which was previously cloned in our laboratory, were cotransfected into pFastBacDual vector (Invitrogen). CYP2B6 and POR cDNAs were inserted downstream of p10 and the polyhedrin promoter, respectively. The conditions for amplification of the viruses and determination of virus titer were inserted downstream of p10 and the polyhedrin promoter, respectively. The expression of each CYP2B6 protein. Anti-CYP2B6 antibody was kindly provided by Dr. Y. Funae (Osaka City University Medical School, Osaka, Japan). Band intensity was quantified by NIH Image (version 1.63; National Institutes of Health, Bethesda, MD).

Immunoblot Analysis. Western immunoblotting was performed to confirm the expression of each CYP2B6 protein. Anti-CYP2B6 antibody was kindly provided by Dr. Y. Funae (Osaka City University Medical School, Osaka, Japan). CYP2B6 proteins were detected using an ECL Western Blotting Detection System (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) and visualized with an LAS-1000plus image analyzer (Fuji Photo Film, Tokyo, Japan). Band intensity was quantified by NIH Image (version 1.63; National Institutes of Health, Bethesda, MD).

Enzyme Assay. In the present study, the activity of POR was judged to be high enough to support the activity of P450, because the molar ratios of POR to P450 were calculated to be approximately 10. Thus, expressed CYP2B6-mediated reactions were performed under saturating conditions. CPA 4-hydroxylase activity was determined in 500 μl of a reaction mixture comprising 50 mM potassium phosphate (pH 7.4), 4.0 pmol of expressed CYP2B6, various concentrations of CPA (ranging from 0.05 to 10 μM), and 1 mM NADPH. Incubation was performed at 37°C for 15 min, and the reaction was terminated by the addition of 500 μl of ice-cold acetonitrile and then vortexed vigorously and immediately chilled on ice. After centrifugation at 2000g for 10 min, 500 μl of the supernatant was incubated at 30°C for 5 min in the presence of 1.6 (w/v)% dansylhydrazine under an acidic condition to produce a fluorescent derivative of 4-hydroxy-CPA. Samples were injected into an HPLC system (Hitachi, Tokyo, Japan) consisting of a L-2100 pump, an L-2200 autosampler, an L-2300 column oven, an L-2485 FL detector (excitation, 350 nm; emission, 550 nm), and an Inertsil ODS-3 column (5 μm, 4.6×150 mm) (GL Sciences, Tokyo, Japan) at 35°C. The mobile phase was 0.1 mM potassium phosphate buffer (pH 3.5)-acetoniitrile (2:1 by volume), and the flow rate was 1.5 ml/min. EFV 8-hydroxylase activity was measured in 250 μl of a reaction mixture that was essentially the same as that described above except for the amount of P450 (2.5 pmol), the substrate (ranging from 2.5 to 80 μM), and the incubation time (20 min). After incubation, the reaction was stopped by the addition of 500 μl of ice-cold acetonitrile with vigorous shaking. As an internal standard, 50 μl of 5 μM ritonavir was added. The mixture was alkalized by adding 500 μl of 0.5 M NaOH (pH 10 with phosphoric acid), and the metabolite was extracted by adding 3 ml of ethyl acetate and shaking for 10 min. After centrifugation, the organic phase was evaporated in vacuo to dryness. The residue was dissolved in 80 μl of the mobile phase for HPLC described below, and the aliquot was injected into an HPLC system. The mobile phase was 10 mM KH3PO4 (pH 2.4 with phosphoric acid)-acetoniitrile (55:45, v/v). The HPLC system was the same as that described above except for an L-4200 UV detector (Hitachi) and an Inertsil ODS-2 column (5 μm, 4.6×150 mm) (GL Sciences) at 30°C. The flow rate was 0.9 ml/min; absorbance was monitored at 245 nm. HPLC conditions were essentially the same as reported by Griskevicius et al. (2002) except for the column, column temperature, and flow rate of the mobile phase.

Data Analysis for Pharmacokinetic Assay. Kinetic parameters for both CPA 4-hydroxylation and EFV 8-hydroxylation were determined on the basis of the Michaelis-Menten equation using a nonlinear least-squares algorithm. The differences in all pharmaco kinematic parameters among three CYP2B6 forms were analyzed by one-way analysis of variance followed by Tukey-Kramer multiple comparison tests. P < 0.05 was considered to be statistically significant. Data analyses were conducted by using Prism (version 5.04; GraphPad Software Inc., La Jolla, CA).

Results and Discussion

Effects of SNPs on Post-Transcriptional Expression of CYP2B6. To clarify whether the SNPs (c.785A>G with or without c.516G>T) affect post-transcriptional expression of the enzyme, the level of CYP2B6 protein in the microsomal fraction of S9 insect cells was examined by Western immunoblotting. Almost identical specific activity of POR in microsomes prepared from each insect cells expressing CYP2B6.1, CYP2B6.4, or CYP2B6.6 confirmed that infection efficiency by the baculovirus for the different CYP2B6 forms was comparable. Specific contents of P450 were 14, 18, and 14 pmol/mg microsomal protein for CYP2B6.1, CYP2B6.4, and CYP2B6.6, respectively. Judging from the ratio of the band intensity of P450/POR, a slight, but not significant, increase in the level of CYP2B6 protein was observed in both CYP2B6.4 and CYP2B6.6 compared with that of CYP2B6.1 (data not shown). Jinno et al. (2003) reported that the expression level of CYP2B6.6 in COS-1 cells was lower than that of CYP2B6.1, although it was not significantly different. In contrast, Rotger et al. (2007) found a slightly higher expression level of CYP2B6.6 than that of CYP2B6.1 by using the same host cell lines. Thus, the single nucleotide change (c.516G>T) appears to have a negligible effect on the translation process. Meanwhile, decreased expression of CYP2B6 in liver microsomes prepared from individuals who were carriers of CYP2B6*6 was demonstrated in several studies (Lang et al., 2001; Hesse et al., 2004; Desta et al., 2007). Regarding the reason for the lower expression level in vivo of CYP2B6 in CYP2B6*6 carriers, Lamba et al. (2003) and Hofmann et al. (2008) suggested that decreased expression of CYP2B6 in the liver of CYP2B6*6 carriers is caused by aberrant splicing. Taken together, the results suggest that the decreased expression in vivo of CYP2B6 in individuals possessing CYP2B6*6 is a transcriptional process.

Effects of SNPs on CPA 4-Hydroxylase Activity. The kinetic profiles of 4-hydroxy-CPA formation revealed that the CPA 4-hydroxylation reaction by CYP2B6 followed the Michaelis-Menten equation (data not shown). Unlike the change in kinetics of 7-ethoxy-coumarin O-deethylation activity occurring by Q172H of CYP2B6 (Ariyoshi et al., 2001), amino acid replacement did not alter the
kinetics either by K262R (CYP2B6.4) or K262R plus Q172H (CYP2B6.6) for CPA 4-hydroxylation. Although a single amino acid replacement, K262R, in CYP2B6.4 decreased CPA 4-hydroxylation activity, one additional amino acid replacement, Q172H, in CYP2B6.6 canceled the effect of K262R but increased the catalytic activity. The catalytic efficiency (kcat/Km) of CYP2B6.6 was 63% higher than that of CYP2B6.1 because of a reduction in the Km value (Table 1). However, if the expression level of hepatic CYP2B6 in a group with CYP2B6*6*/6 is very low, for example, only 25% of that in a group with CYP2B6*1*/1* as reported by Desta et al. (2007), CPA 4-hydroxylase activity in vivo may not be higher but rather is lower in a group with CYP2B6*6*/6*, because roughly estimated relative activities were 0.41 for CYP2B6*6*/6 and 1.0 for CYP2B6*1*/1*. On the other hand, if the decreased level of CPA is approximately 73%, for example, in a group with CYP2B6*1*/6 as reported by Lang et al. (2001), CPA 4-hydroxylase activity in vivo may not be so different between groups with CYP2B6*6*/6 and CYP2B6*1*/1*. In this case, relative activities calculated were 0.96 for CYP2B6*1*/6 and 1.0 for CYP2B6*1*/1*, respectively. In fact, similar clearence of CPA between groups with CYP2B6*1*/6 and CYP2B6*1*/1* in a study by Nakajima et al. (2007) may support our rough estimation. However, CYP2B6 expression and activity show large interindividual variability even in a group with the same CYP2B6 genotype and can be modified by several drugs used concomitantly. Thus, effects of CYP2B6*6 on the pharmacokinetics/pharmacodynamics of CPA are complicated and may be difficult to predict in clinical circumstances.

**Effects of SNPs on EFV 8-Hydroxylase Activity.** Substrate velocity plots of the EFV 8-hydroxylation reaction by CYP2B6 showed a typical hyperbolic curve explained by the Michaelis-Menten equation (data not shown). Consistent with the results of a study by Bumpus et al. (2006), EFV 8-hydroxylation activity was clearly increased by a single amino acid replacement, K262R, in CYP2B6.4. In contrast to that of CYP2B6.4, the catalytic efficiency of EFV 8-hydroxylation activity of CYP2B6.6 was reduced by 50% compared with that of CYP2B6.1 (Table 2). Thus, a significantly high plasma concentration of EFV resulting from a marked reduction of clearance in a group with CYP2B6*6*/6*, is responsible for decreased expression and activity of CYP2B6 in liver.

CYP2B6.4 overcomes the effects of K262R on the catalytic activity of two tyypical substrates of CYP2B6 investigated in this study. Further studies are necessary to clarify substrate dependence of the difference in effects of these natural amino acid replacements on catalytic activity of CYP2B6.

**TABLE 1**

**Differences in kinetic parameters of CPA 4-hydroxylation among CYP2B6 variants expressed in Sf9 cells**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>kcat (mM/min)</th>
<th>kcat/Km (mM)</th>
<th>km (mM)</th>
<th>Data are represented by mean ± S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2B6.1</td>
<td>2.68 ± 0.26</td>
<td>301 ± 10.3</td>
<td>112 ± 11.5 (100%)</td>
<td></td>
</tr>
<tr>
<td>CYP2B6.4</td>
<td>2.75 ± 0.55</td>
<td>223 ± 16.2*</td>
<td>81.3 ± 17.3 (72.6%)</td>
<td></td>
</tr>
<tr>
<td>CYP2B6.6</td>
<td>1.62 ± 0.18</td>
<td>297 ± 10.4††</td>
<td>184 ± 21.2 (164%)</td>
<td></td>
</tr>
</tbody>
</table>

* P < 0.05 versus CYP2B6.1 (Tukey-Kramer multiple comparison test).
† P < 0.05 versus CYP2B6.4 (Tukey-Kramer multiple comparison test).
‡ P < 0.05 versus CYP2B6.6 (Tukey-Kramer multiple comparison test).

The variant to wild-type ratios are indicated in parentheses.

**Unique Effects of Q172H on Catalytic Activity of CYP2B6**

Differences in kinetic parameters of CPA 4-hydroxylation among CYP2B6 variants expressed in Sf9 cells

**TABLE 2**

**Difference in kinetic parameters of EFV 8-hydroxylation among CYP2B6 variants expressed in Sf9 cells**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>kcat (mM/min)</th>
<th>kcat/Km (mM)</th>
<th>km (mM)</th>
<th>Data are represented by mean ± S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2B6.1</td>
<td>7.70 ± 1.55</td>
<td>9.06 ± 0.47</td>
<td>1.18 ± 0.25 (100%)</td>
<td></td>
</tr>
<tr>
<td>CYP2B6.4</td>
<td>9.16 ± 1.62</td>
<td>15.3 ± 0.74***</td>
<td>1.67 ± 0.31 (142%)</td>
<td></td>
</tr>
<tr>
<td>CYP2B6.6</td>
<td>12.4 ± 1.51</td>
<td>7.33 ± 0.27†††</td>
<td>0.59 ± 0.081 (50.0%)</td>
<td></td>
</tr>
</tbody>
</table>

**P < 0.001 versus CYP2B6.1 (Tukey-Kramer multiple comparison test).**
**P < 0.05 versus CYP2B6.4 (Tukey-Kramer multiple comparison test).**

The variant to wild-type ratios are indicated in parentheses.


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