Gln172His Replacement Overcomes Effects on the Metabolism of 
Cyclophosphamide and Efavirenz Caused by CYP2B6 Variant with Arg262

Noritaka Ariyoshi, Miyuki Ohara, Mayumi Kaneko, Sakino Afuso, Takuya Kumamoto, 
Hiroyoshi Nakamura, Itsuko Ishii, Tsutomu Ishikawa, and Mitsukazu Kitada

Division of Pharmacy, University Hospital, Chiba University School of Medicine (N.A., H.N., M.K.),
Laboratory of Clinical Pharmacology (M.O., M.K., S.A., I.I.) and Department of Medicinal Organic 
Chemistry (T.K., T.I.), Graduate School of Pharmaceutical Sciences, Chiba University, Japan
Running title: Unique Effects of Q172H on Catalytic Activity of CYP2B6

Address correspondence to:

Noritaka Ariyoshi, PhD.

Division of Pharmacy, University Hospital, Chiba University School of Medicine, 1-8-1 Inohana, Chuo-ku, Chiba 260-8677, Japan

Tel/Fax: +81-43-226-2208; E-mail: ariyoshi@faculty.chiba-u.jp

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ABBREVIATIONS: bp, base pair; CPA, cyclophosphamide; CYP, cytochrome P450; EFV, efavirenz; HPLC, high-performance liquid chromatography, MOI, multiplicity of infection; PCR, polymerase chain reaction; POR, NADPH-CYP oxidoreductase; RT, reverse transcription; SNP, single nucleotide polymorphism
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 an increased disposition of cyclophosphamide (CPA) in individuals with this polymorphism was observed. Thus, we hypothesized that the effects of the two 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 (Gln172His and Lys262Arg) and CYP2B6.4 (Lys262Arg), 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 to 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 the completely opposite character, suggesting that Gln172His gives inverse effects on metabolic activities of CYP2B6 affected by Lys262Arg. Although it is recognized that effects of amino-acid change in CYP 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 Gln172His can inverse direction of the effect caused by Lys262Arg in CYP2B6 on the metabolism of certain drugs.
Introduction

It is well-documented that genetic polymorphisms of CYP affect drug metabolizing activities, although degree of the effect depends on substrates. For example, marked decrease occurs in the metabolism of phenytoin by Ile359Leu in CYP2C9 both in vitro (Takanashi et al., 2000) and in vivo (Odani et al., 1997), but the decrease is not remarkable on the metabolism of diclofenac both in vitro (Takanashi et al., 2000) and in vivo (Shimamoto et al., 2000). However, in most cases, non-synonymous SNP of the CYP gene gives unidirectional change in catalytic activity toward different substrates. For example, replacement of Ile at codon 359 to Leu due to CYP2C9*3 decreases catalytic activities toward all of seven different substrates (Takanashi et al., 2000).

CYP2B6 is known as the most important CYP isoform responsible for the metabolism of an anti-cancer prodrug, cyclophosphamide (CPA) (Roy et al., 1999) and a non-nucleoside HIV-1 reverse transcriptase inhibitor, efavirenz (EFV) (Ward et al., 2003). We previously reported, for the first time, that CYP2B6 possessing histidine (His) instead of glutamine (Gln) residue at codon 172 caused drastically 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 (4-hydroxylation) of CPA by CYP2B6.6 harboring double amino-acid replacement (Gln172His and Lys262Arg) but not by CYP2B6.4 with a single amino-acid replacement (Lys262Arg), suggesting that the change in Gln to His
residue in amino-acid 172 plays a key role in stimulation of the metabolism for certain drugs (unpublished observations, 2003). Supporting our observation, Xie and co-workers (2003) reported that CPA 4-hydroxylation was significantly enhanced in human liver microsomes from CYP2B6*6 carriers. They also demonstrated that the presence of 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 with homozygotes of CYP2B6*6 was significantly higher than that in homozygotes of CYP2B6*1.

In contrast, a number of studies have shown that the rate of metabolism in vivo of EFV is much lower in patients possessing homozygous CYP2B6*6 or a SNP, c.516G>T (Haas et al., 2004; Tsuchiya et al., 2004; Rodríguez-Nóvoa et al., 2005; Rotger et al., 2007). Thus, the presence of His at codon 172 may have opposite effects on catalytic activity of CYP2B6 between CPA and EFV. However, since there are some reports suggesting that CYP2B6*6 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 be simply due to low CYP2B6 expression level in vivo. If the latter hypothesis is correct, the metabolic activity of EFV may not be significantly altered by Gln172His replacement.

To clarify whether or not the metabolic activity of EFV by CYP2B6 is affected by several natural amino-acid replacements, we carried out kinetic analyses using recombinant enzymes without considering effects of these SNPs on the enzyme expression.
Materials and Methods

Chemicals. CPA was purchased from Wako Pure Chemical Industries (Osaka, Japan). An authentic standard of 4-hydroxy-CPA was kindly donated by Shionogi & Co. (Osaka, Japan). EFV was obtained from MERCK (Rahway, 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 RT-PCR from a human liver sample provided by the Human & Animal Bridging Research Organization, Japan. The amplicon was subcloned into pBluescript II SK(+) vector (Promega, Madison, WI). Site-directed mutagenesis was carried out 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-CYP oxidoreductase proteins. Sf9 insect cells were purchased from Invitrogen (Carlsbad, CA). CYP2B6 cDNA together with human NADPH-CYP oxidoreductase (POR) cDNA, which was previously cloned in our laboratory, were co-transferred 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 conducted according to the manufacturer’s instruction. A recombinant CYP-enriched microsome fraction was prepared by a standard centrifugation method and was stored at -80°C until use in an enzyme assay described below. Protein concentration, CYP content, and POR activity was determined by the standard spectrophotometric method.

**Immunoblot analysis.** Western immunoblotting was performed to confirm the expression of each CYP2B6 protein. Anti-CYP2B6 antibody was kindly provided by Dr. Funae. CYP2B6 proteins were detected using an ECL Western Blotting Detection System (Amersham Biosciences, Piscataway, NJ) and visualized with an LAS-1000 plus image analyzer (Fuji Photo Film, Tokyo, Japan). Band intensity was quantified by NIH Image ver. 1.63.

**Enzyme assay.** In the present study, the activity of POR was judged to be high enough to support the activity of CYP, since the molar ratios of the POR to CYP were calculated to be approximately 10. Thus, expressed CYP2B6-mediated reactions were carried out under saturating conditions. CPA 4-hydroxylase activity was determined in 500 µL of a reaction mixture comprising 50 mM of potassium phosphate (pH 7.4), 4.0 pmol of expressed CYP2B6, various concentrations of CPA (ranging from 0.05 to 10 mM), and 1 mM NADPH. Incubation was carried out 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 2,000 x g for 10 min, 500 µL of the supernatant was incubated at 50°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 an 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 x 150 mm)(GL Sciences, Tokyo, Japan) at 35°C. The mobile phase was 0.1 mM potassium phosphate buffer (pH 3.5): acetonitrile (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 CYP (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 ethylacetate 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 KH₂PO₄ (pH 2.4 with phosphoric acid)/acetonitrile (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 x 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 one 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 pharmacokinetic parameters among three CYP2B6 forms were analyzed by one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison tests. \( P < 0.05 \) was considered to be statistically significant. Data analyses were conducted by using Prism ver. 5.04 (GraphPad Software, 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 Sf9 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 CYP 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 CYP/POR, a slight but not significant increase in the level of CYP2B6 protein was observed in both CYP2B6.4 and CYP2B6.6 compared to 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, though 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) appeared to have a negligible effect on 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 CPA 4-hydroxylation reaction by CYP2B6 followed the Michaelis-Menten equation (data not shown). Unlike the change in kinetics of 7-ethoxycoumarin O-deethylase activity occurred by Gln172His of CYP2B6 (Ariyoshi et al., 2001), amino-acid replacement did not alter the kinetics either by Lys262Arg (CYP2B6.4) or Lys262Arg plus Gln172His (CYP2B6.6) in the case of CPA 4-hydroxylation. Although a single amino-acid replacement, Lys262Arg, in CYP2B6.4 decreased CPA 4-hydroxylase activity, one additional amino-acid replacement, Gln172His, in CYP2B6.6 canceled the effect of Lys262Arg but increased the catalytic activity. The catalytic efficiency (kcat/Km) of CYP2B6.6 was 63% higher than that of CYP2B6.1 due to reduction of the Km value to 60% (Table 1). However, if the expression level of hepatic CYP2B6 in a group with CYP2B6*6/*6 is far 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 lower in a group with CYP2B6*6/*6, since 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 CYP2B6 is about 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*1/*6 and CYP2B6*1/*1. In this case, relative activities calculated were 0.96 for CYP2B6*1/*6 and 1.0 for
In fact, similar clearance 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/or activity show a large inter-individual 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 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 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-hydroxylase activity was clearly increased by a single amino-acid replacement, Lys262Arg, in CYP2B6.4. In contrast to CPA, the catalytic efficiency of EFV 8-hydroxylase 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 marked reduction of clearance in a group with CYP2B6*6/*6 may be caused not only by low CYP2B6 expression level but also decreased EFV 8-hydroxylase activity of CYP2B6.6. As shown in Table 2, our results support the data of Bumpus et al. (2006), who demonstrated that CYP2B6.4 shows higher EFV 8-hydroxylase activity than that of CYP2B6.1, mainly due to increase in turnover number of CYP2B6 for EFV 8-hydroxylation. Since the catalytic efficiency of EFV 8-hydroxylation by CYP2B6.6 (Lys262Arg and Gln172His) was lower than that of CYP2B6.4 (Lys262Arg), increased metabolism by
Lys262Arg appeared to be abolished by an additional amino-acid replacement, Gln172His, in CYP2B6.6.

In conclusion, two major genetic polymorphisms of the CYP2B6 gene, CYP2B6*4 and CYP2B6*6, have apparently opposite effects on the metabolism of CPA and EFV. The addition of Gln172His to CYP2B6.4 overcomes the effects of Lys262Arg on the catalytic activity of two typical substrates of CYP2B6 investigated in this study. Further studies are necessary to clarify substrate dependency of the difference in effects of these natural amino-acid replacements on catalytic activity of CYP2B6.
**Authorship Contributions.**

*Participated in research design:* Ariyoshi N, and Kitada M.

*Conducted experiments:* Ohara M, Afuso S, and Ariyoshi N.

*Contributed to synthesize authentic standard of metabolite:* Keneko M, Kumamoto T, and Ishikawa T.

*Performed data analysis:* Ohana M, Nakamura H, Ishii I, and Ariyoshi N.

*Wrote or contributed to the writing of the manuscript:* Ariyoshi N, and Kitada M.
References


Aberrant splicing caused by single nucleotide polymorphism c.516G>T [Q172H], a marker of CYP2B6*6, is responsible for decreased expression and activity of CYP2B6 in liver. *J Pharmacol Exp Ther* **325**: 284-292.


Footnotes

This study was supported by a grant from The Nakatomi Foundation.
## TABLE 1

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

<table>
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<tr>
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<th>$K_m$ [mM]</th>
<th>$k_{cat}$ [nmol/min/nmol CYP]</th>
<th>$k_{cat}/K_m$ [mL/min/nmol CYP]</th>
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<tbody>
<tr>
<td>CYP2B6.1</td>
<td>2.68 ± 0.26</td>
<td>301 ± 10.3</td>
<td>112 ± 11.5 (100%)</td>
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<tr>
<td>CYP2B6.4</td>
<td>2.75 ± 0.55</td>
<td>223 ± 16.2*</td>
<td>81.3 ± 17.3 (72.6%)</td>
</tr>
<tr>
<td>CYP2B6.6</td>
<td>1.62 ± 0.18</td>
<td>297 ± 10.4†</td>
<td>184 ± 21.2† (164%)</td>
</tr>
</tbody>
</table>

Data are represented by mean ± standard error.

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

† $P < 0.05$ versus CYP2B6.4 (Tukey-Kramer multiple comparison test).
### TABLE 2

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

<table>
<thead>
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<th>$K_m$</th>
<th>$k_{cat}$</th>
<th>$k_{cat}/K_m$</th>
</tr>
</thead>
<tbody>
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<td></td>
<td>[μM]</td>
<td>[nmol/min/nmol CYP]</td>
<td>[μL/min/nmol CYP]</td>
</tr>
<tr>
<td>CYP2B6.1</td>
<td>7.70 ± 1.55</td>
<td>9.06 ± 0.47</td>
<td>1.18 ± 0.25 (100%)</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>
</tr>
<tr>
<td>CYP2B6.6</td>
<td>12.4 ± 1.51</td>
<td>7.33 ± 0.27†††</td>
<td>0.59 ± 0.08† (50.0%)</td>
</tr>
</tbody>
</table>

Data are represented by mean ± standard error.

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

† $P < 0.05;$ ††† $P < 0.001$ versus CYP2B6.4 (Tukey-Kramer multiple comparison test).