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# Functional characterization of 40 CYP2B6 allelic variants by assessing efavirenz 8-hydroxylation

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**Abstract**

Genetic variations within cytochrome P450 2B6 (CYP2B6) contribute to inter-individual variation in the metabolism of clinically important drugs, including cyclophosphamide, bupropion, methadone and efavirenz (EFZ). In this study, we performed an *in vitro* analysis of 40 CYP2B6 allelic variant proteins including seven novel variants identified in 1070 Japanese individuals. Wild-type and 39 variant proteins were heterologously expressed in 293FT cells to estimate the kinetic parameters ($K_m$, $V_{max}$, and $CL_{int}$) of EFZ 8-hydroxylation and 7-ethoxy-4-trifluoromethylcoumarin (7-ETC) *O*-deethylation activities. The concentrations of CYP2B6 variant holo-enzymes were measured by using carbon monoxide (CO)-reduced difference spectroscopy, and the wild-type and 28 variants showed a peak at 450 nm. The kinetic parameters were measured for the wild-type and 24 variant proteins. The values for the remaining 15 variants could not be determined because the enzymatic activity was not detected at the highest substrate concentration used. Compared to wild-type, six variants showed significantly decreased EFZ 8-hydroxylation $CL_{int}$ values, while these values were significantly increased in another six variants, including CYP2B6.6. Although 7-ETC *O*-deethylation $CL_{int}$ values of CYP2B6 variants did not differ significantly from that of CYP2B6.1, the $CL_{int}$ ratios obtained for 7-ETC *O*-deethylation were highly correlated with EFZ 8-hydroxylation.

Furthermore, three-dimensional structural modeling analysis was performed to elucidate the mechanism of changes in the kinetics of CYP2B6 variants. Our findings could provide evidence of the specific metabolic activities of the CYP2B6 proteins encoded by these variant alleles.

***Keywords:*** Cytochrome P450; CYP2B6; Genetic polymorphisms; Efavirenz; Pharmacogenetics; Drug metabolism

**Chemical compounds cited in this article:**

Efavirenz (PubChem CID: 64139); 8-hydroxyefavirenz (PubChem CID: 487643); 7-ethoxy-4-trifluorometylcoumarin (PubChem CID: 24869840); 7-hydroxy-4-trifluoromethylcoumarin (PubChem CID: 24862915); Dimanganese decacarbonyl (PubChem CID: 517769); Sodium hydrosulfite (PubChem CID: 23665763)

## 1. Introduction

The Cytochrome P450 2B6 enzyme (CYP2B6) plays a major role in the biotransformation of several therapeutically important drugs, including cyclophosphamide, bupropion, methadone, and efavirenz (EFZ) [1]. The contribution of CYP2B6 to drug metabolism is relatively small, accounting for approximately 7% [2]. However, the inter-individual variability in CYP2B6 activity influences drug responsiveness, efficacy, and the occurrence of adverse effects. The observed impact of CYP2B6 on drug metabolism lies in the extensively polymorphic nature of the *CYP2B6* gene, with numerous variants in coding and non-coding regions, believed to be responsible for the observed inter-individual and inter-ethnic differences in drug response [3]. A large number of *CYP2B6* genetic polymorphisms have been identified to date, and the Pharmacogene Variation Consortium website (https://www.pharmvar.org/gene/CYP2B6) currently lists 38 distinct star-alleles (last accessed: July 1st, 2018). Thus far, many studies have reported the pharmacogenetic impacts of CYP2B6 *in vivo* and *in vitro*. However, the continual discovery of new variants means that in-depth studies of their effects on drug metabolism have become indispensable in the clinical setting.

*CYP2B6* genetic polymorphisms are associated with drug plasma concentrations

and with treatment efficacy, continuation, adverse reactions, relapse, and survival rates. *CYP2B6*6* (516G>T and 785A>G), which results in two amino acid changes, Gln172His and Lys262Arg, is the most common variant allele in various populations, including Asians and Caucasians [3]. The *CYP2B6*5* allele (1459C>T, Arg487Cys) occurs predominantly in 9–12% of Caucasians [4]. Homozygous *CYP2B6*6* correlates with high plasma EFZ levels in human immunodeficiency virus (HIV)-1 patients, an increased risk of central nervous system side effects, and with EFZ treatment discontinuation [5–7]. Additionally, homozygous *CYP2B6*6* allele patients with heroin addiction who also exhibit poor methadone metabolism have also shown disparities in the dose required for effective treatment of methadone [8]. Furthermore, patients with the *CYP2B6*1*/**5* genotype that undergo autologous hematopoietic cell transplantation, an important procedure for lymphoma treatment after cyclophosphamide-containing conditioning, have a higher two-year relapse rate and overall decreased survival rate than patients with the wild-type allele [9]. Thus, it has become necessary to consider *CYP2B6* genetic polymorphisms for successful clinical practice.

Characterization of the functional effect of variant alleles on CYP2B6 activity has involved the use of various *in vitro* heterologous expression systems over the years. Using COS-1 mammalian cells, Jinno *et al.* have expressed six variants (CYP2B6.2–

CYP2B6.7) and examined their enzymatic properties using 7-ethoxy-4-trifluorometylcoumarin (7-ETC) *O*-deethylation [10]. Additionally, using bupropion and EFZ, Radloff *et al.* analyzed 10 variants (CYP2B6.5, CYP2B6.6, and eight amino acid-substituted enzymes expressed in COS-1 cells [11]. Furthermore, using COS-7 cells, we have previously performed a comprehensive analysis of 26 allelic variants by determining the kinetic parameters of 7-ETC *O*-deethylation, selegiline *N*-demethylation/*N*-depropagylation, and artemether demethylation [12, 13]. However, the effects of *CYP2B6* genetic polymorphisms on drug metabolism remains poorly understood, and has not been sufficiently applied in clinical practice. Recently, the Tohoku Medical Megabank Organization (ToMMo) has reported the whole-genome sequences (WGS) of 1070 healthy Japanese individuals and has constructed a Japanese population reference panel (1KJPN) [14]. Seven novel *CYP2B6* single nucleotide variants (SNVs), with unknown functional alterations, were identified in these individuals (Fig. 1).

To develop the most suitable pharmacotherapy for CYP2B6-metabolized drugs, further investigation is required to understand the differences in allele-specific CYP2B6 variant activity. In this study, we performed an *in vitro* analysis of 39 *CYP2B6* allelic variants with amino acid substitutions (*CYP2B6*2–CYP2B6*21*, *CYP2B6*23–CYP2B6*28*, *CYP2B6*31–CYP2B6*35*, *CYP2B6*37*, and seven novel *CYP2B6* variants)

(Table 1). The wild-type CYP2B6.1 protein and all 39 variants were heterologously expressed in 293FT cells under the same conditions, and their kinetic parameters—the Michaelis constant ($K_m$), maximum velocity ($V_{max}$), and intrinsic clearance ($CL_{int} = V_{max}/K_m$)—for EFZ 8-hydroxylation were determined. We further measured the kinetic parameters of 7-ETC *O*-deethylation in order to confirm the substrate specificity among CYP2B6 variants. EFZ, commonly used as an anti-HIV agent, is mainly metabolized by CYP2B6 [15, 16], and 7-ETC is a specific substrate for CYP2B6 [17]. We assessed the enzymatic activity of CYP2B6 holo-protein and performed a three-dimensional (3D) structural modeling analysis to elucidate the mechanism underlying the observed changes in the kinetics of CYP2B6 variants. The approach was then adopted to elucidate which CYP2B6 amino acid alterations affect the function of the protein. We believe this comprehensive *CYP2B6* genetic polymorphism research will further clarify the genotype-phenotype association intended for the clinical application of precision medicine.

## 2. Materials and methods

### *2.1. Chemicals*

The following reagents were purchased from the listed sources: EFZ, from Tokyo Kasei Industry (Tokyo, Japan); 8-hydroxyefavirenz (8-HEZ) and 8-hydroxyefavirenz-d4 (8-HEZ-d4), from Toronto Research Chemicals (Ontario, Canada); 7-ETC, 7-hydroxy-4-trifluoromethylcoumarin (7-HTC), and dimanganese decacarbonyl (DMDC), from Sigma-Aldrich (Steinheim, Germany); oxidized β-nicotinamide-adenine dinucleotide phosphate oxidized form ($NADP^+$), glucose-6-phosphate (G-6-P), and glucose-6-phosphate dehydrogenase (G-6-PDH), from Oriental Yeast (Tokyo, Japan); Sodium hydrosulfite ($Na_2S_2O_4$), from Nacalai Tesque, (Kyoto, Japan); polyclonal anti-human CYP2B6 antibody, from Abcam (Cambridge, MA, USA); polyclonal anti-calnexin antibody, from Enzo Life Sciences (Farmingdale, NY, USA); and horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG, from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other chemicals and reagents were of the highest commercially available quality.

### *2.2. CYP2B6 Sanger sequencing analysis*

To confirm the *CYP2B6* sequence alterations identified by WGS, we performed

Sanger sequencing according to previously described methods [18]. PCR amplification was conducted using peripheral blood leukocyte genomic DNA isolated from whole blood by using the Gentra Puregene Blood Kit (Qiagen, Hilden, Germany) according to previously described methods [14]. Whole blood samples were obtained from Japanese subjects participating in the community-based cohort study conducted by ToMMo. Primer pairs were used to amplify sequences containing each *CYP2B6* SNVs (Table 2).

This study was conducted in accordance with the Declaration of Helsinki and all participants provided written informed consent according to the protocols approved by the Tohoku University Graduate School of Pharmaceutical Sciences committee (permission number 14-08), and the Tohoku Medical Megabank Organization committee (permission number 2017-4-26, 2017-4-58, and 2017-4-090).

### *2.3. CYP2B6 cDNA cloning and construction of expression vectors*

*CYP2B6* complementary DNA (cDNA) fragments, obtained from a human liver cDNA library, (TaKaRa, Shiga, Japan) were amplified by PCR using a forward primer (5′-CACCATGGAACTCAGCGTCCTCCTC-3′) and reverse primer (5′-TCAGCGGGGCAGGAAGC-3′) with PfuUltra High-Fidelity DNA Polymerase (Agilent Technologies, Santa Clara, CA, USA). The underlined sequences in the forward primer

were introduced for directional TOPO cloning. The amplified fragments were subcloned into the pENTR/D-TOPO vector (Thermo Fisher Scientific, Waltham, MA, USA). Plasmids carrying *CYP2B6*1* (wild-type) cDNA were used as a template to generate 29 *CYP2B6* constructs (*CYP2B6*2*–**5*, **8*, **9*, **11*, **12*, **14*, **15*, **17*, **18*, **21*, **23*–**25*, **27*, **28*, **31*–**33*, **35*, and the seven novel variants) using a QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies) according to the manufacturer's instructions. Ten *CYP2B6* constructs were generated from plasmids carrying other cDNA templates: *CYP2B6*10* from *CYP2B6*2* cDNA; *CYP2B6*6* and *CYP2B6*16* from *CYP2B6*4* cDNA; and *CYP2B6*7*, **13*, **19*, **20*, **26*, **34*, and **37* from *CYP2B6*6* cDNA. All prepared wild-type and variant cDNAs were confirmed by Sanger sequencing. The wild-type and variant *CYP2B6* cDNA sequences were then subsequently subcloned into the pcDNA3.4 mammalian expression vector (Thermo Fisher Scientific).

### *2.4. Expression of CYP2B6 variants in 293FT cells*

293FT cells (Thermo Fisher Scientific) were cultured in Dulbecco's modified Eagle medium (Nacalai Tesque) containing 10% fetal bovine serum at 37ºC and 5% $CO_2$. Cells were plated at a density of $2.0 \times 10^6$ cells/100-mm dish. Twenty-four hours after plating, the cells were transfected with a plasmid (5 μg) encoding *CYP2B6* cDNA using

the TransFectin lipid reagent (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. After incubation for 24 h at 37°C, the cells were scraped off, and microsomal fractions were prepared as previously described [12]. The protein concentration was determined using a bicinchoninic acid protein assay Kit (Thermo Fisher Scientific).

*2.5. Western blotting analysis*

Western blotting was performed according to standard procedures by using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), with 5 μg of microsomal protein loaded per lane. CYP2B6 was detected using a polyclonal anti-human CYP2B6 antibody (1:1000) and HRP-conjugated goat anti-rabbit IgG (1:2500). The loading control used was calnexin, which was detected using a polyclonal anti-calnexin antibody (1:5000) and HRP-conjugated goat anti-rabbit IgG (1:10,000). The immunoblots were visualized using the SuperSignal™ West Dura extended duration substrate (Thermo Fisher Scientific). Chemiluminescence was quantified using a ChemiDoc XRS$^+$ with Image Lab software (Bio-Rad Laboratories).

*2.6. Determination of microsomal P450 contents*

CYP2B6 variant holo-enzymes were measured according to previously reported methods [19, 20], with several modifications. The microsomal fraction (0.80 ± 0.20 mg of microsomal proteins) was diluted to a final volume of 200 μL using 100 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol (v/v). Protein samples (100 μL) were added to sample and reference cuvettes. A baseline between 400 and 500 nm was recorded using a Cary 300 UV-Vis spectrophotometer (Agilent Technologies). The sample cuvette was illuminated with over 20,000 lux LED-light for 30 s following the addition of 0.2 mM DMDC [21]. Samples were reduced by freshly prepared 500 mM $Na_2S_2O_4$ solution in both cuvettes. The carbon monoxide (CO)-difference spectra of both cuvettes, normalized to the baseline spectrum, were then recorded between 400 and 500 nm using a spectrophotometer. The spectra for each CYP2B6 variant were recorded in triplicate. Data analysis was conducted using a Jasco Spectra Manager (JASCO Corporation, Sendai, Japan). The CYP concentration was calculated using the maximum absorbance of the three recorded values normalized to the baseline spectrum because the spectrum baseline showed a difference in absorbance between 400 and 500 nm. Cuvettes (Sub-Micro Cells, 16.50-Q-10/Z20) were purchased from Starna Scientific, Ltd. (London, UK).

*2.7. EFZ 8-hydroxylation assay*

CYP2B6-mediated EFZ 8-hydroxylation was measured as previously described [15, 16], with several modifications. The incubation mixture consisted of the microsomal fraction (30 μg), EFZ (1, 2, 3, 5, 7.5, 10, 15, or 20 μM), 3.3 mM $MgCl_2$, and 100 mM potassium phosphate buffer (pH 7.4) in a total volume of 150 μL. Following pre-incubation at 37ºC for 5 min, reactions were initiated by the addition of NADPH-generating medium. The mixture was incubated at 37ºC for 30 min. Reactions were terminated by adding 150 μL of acetonitrile containing 2 μM of 8-HEZ-d4 (internal standard). EFZ 8-hydroxylation assessment, using 20 μM of EFZ and 30 μg of the microsomal fraction containing wild-type and variant CYP2B6, showed that 8-HEZ formation was linear for incubations of up to 30 min. Moreover, when the reaction containing 20 μM of EFZ was incubated for 30 min, 8-HEZ formation was linear in the presence of up to 30 μg of microsomal protein (data not shown).

After protein removal by centrifugation at 14,000 *g* for 5 min, 10 μL of the supernatant was injected into a liquid chromatography-tandem mass spectrometry (LC-MS/MS) system. 8-HEZ was measured using the LC-MS/MS system in the negative ion detection mode at the electrospray ionization interface (TSQ Quantum Ultra, Thermo Fisher Scientific). Separation by LC was conducted using NANOSPACE SI-2 (OSAKA SODA, Osaka, Japan). Chromatographic separation was performed using a Luna C18

100A column (2 × 150 mm, 5.0-μm particle size; Phenomenex, CA, USA) maintained at 40ºC. The flow rate was 300 μL/min, and the mobile phases were formed using water containing 0.1% formic acid as eluent A and acetonitrile containing 0.1% formic acid as eluent B. The gradient program was as follows: initial elution with 100% A, followed by a linear gradient to 50% B from 3.0 to 4.0 min, to 90% B from 4.0 to 6.0 min, and to 100% B from 6.0 to 8.0 min, held at 100% B for 12.0 min, and then immediately returned to initial conditions and maintained for 3.0 min until the end of the run. LC effluent was introduced into the mass spectrometer between 5.0 and 9.0 min after injection. Quantitative MS/MS analyses were performed in the selective reaction-monitoring mode. Each area under the peak of *m/z* 330 → 258 (collision energy, 21 V; S-Lens RF amplitude voltage, -91 V) for 8-HEZ and of *m/z* 334 → 258 (collision energy, 23 V; S-Lens RF amplitude voltage, -102 V) for 8-HEZ-d4 was measured. The optimized parameters for MS were as follows: spray voltage, 2.5 kV; sheath gas pressure, 15 psi; vaporizer temperature, 450ºC; capillary temperature, 300ºC; and collision pressure, 1.5 mTorr. The sheath gas was nitrogen, and the collision gas was argon. The LC-MS/MS system was controlled by Xcalibur software (Thermo Fisher Scientific), which was also used to analyze the attained data. The lower limit of 8-HEZ quantification was 5 nM. Standard curves for 8-HEZ were constructed in the 0.005–2.5 μM range using authentic metabolite

standards. Enzymatic activity was normalized to the amount of CYP2B6 holo-protein determined by CO-difference spectra.

*2.8. 7-ETC O-deethylation assay*

7-ETC *O*-deethylation by CYP2B6 was measured as previously reported [22], with several modifications. The incubation mixture consisted of the microsomal fraction (30 μg), 7-ETC (1.5, 3, 5, 7.5, 12, 20, 30, or 50 μM), 3.3 mM $MgCl_2$, and 100 mM potassium phosphate buffer (pH 7.4) in a total volume of 150 μL. Following pre-incubation at 37ºC for 5 min, reactions were initiated by the addition of NADPH-generating medium, consisting of 1.3 mM $NADP^+$, 3.3 mM G-6-P, and 0.4 U/mL G-6-PDH. The mixture was incubated at 37ºC for 20 min. Reactions were terminated by adding 150 μL of acetonitrile. 7-ETC *O*-deethylation determination using 50 μM of 7-ETC and 30 μg of the microsomal fraction containing wild-type and variant CYP2B6 showed that 7-HTC formation was linear for incubations of up to 20 min. Moreover, when the reaction containing 50 μM of 7-ETC was incubated for 20 min, 7-HTC formation was linear in the presence of up to 30 μg of microsomal protein (data not shown).

After protein removal by centrifugation at 14,000 *g* for 5 min, 50 μL of the supernatant was injected into a high-performance liquid chromatography (HPLC) system

consisting of a Nexera series system (SHIMADZU, Kyoto, Japan), a RF10AXL fluorescence detector (SHIMADZU), and a YMC-Pack ODS-AQ column (4.6 × 150 mm, 5-μm particle size; YMC, Kyoto, Japan) maintained at 40ºC. We eluted 7-HTC isocratically with 20 mM potassium phosphate buffer (pH 7.4) and methanol (40:60, v/v) at a flow rate of 1.0 mL/min. The quantity of 7-HTC was measured at an excitation wavelength of 410 nm and emission wavelength of 510 nm. The lower limit of 7-HTC quantification was 1 nM. Standard curves for 7-HTC were constructed in the 0.001–1 μM range using authentic metabolite. The enzymatic activity was normalized to the amount of CYP2B6 holo-protein determined by CO-difference spectra.

*2.9. 3D structural modeling of CYP2B6*

The 3D structural modeling of CYP2B6 was based on the CYP2B6 X-ray structure reported by Gay *et al.* (Protein Data Bank code 3IBD) [23]. Because 3IBD includes two mutations, Tyr226His and Lys262Arg, these mutants were replaced with wild-type residues to construct the CYP2B6.1 model structure. EFZ was docked with the CYP2B6.1 model structure according to the CDOCKER protocol of Discovery Studio 2.5 (BIOVIA, CA, USA). Docking iterations were conducted while considering the binding orientation and binding energies in a volume of space defined as nine and the

heme iron charge was set to $Fe^{3+}$.

*2.10. Data analysis*

Kinetic data, i.e., $K_m$, $V_{max}$, and $CL_{int}$ ($V_{max}/K_m$), were obtained using the Enzyme Kinetics Module of SigmaPlot 12.5 (Systat Software, Inc., Chicago, IL, USA), a curve-fitting program based on nonlinear regression analysis. All values are expressed as the mean ± SD of experiments performed in triplicate. Statistical analyses of expression levels and kinetic parameters were performed through analysis of variance (one-way ANOVA), followed by Dunnett's T3 test or the Kruskal-Wallis method (IBM SPSS Statistics Ver. 22; IBM, Armonk, NY, USA). Differences with $P < 0.05$ were considered statistically significant.

## 3. Results

### *3.1. Re-sequencing analysis of SNVs within CYP2B6 exons identified in 1070 Japanese*

To confirm the sequence alterations identified by WGS, we performed Sanger sequencing analysis of *CYP2B6*. For SNVs within exons, the WGS results were the same as those obtained from Sanger sequencing. Seven novel *CYP2B6* variants were identified in 1070 Japanese subjects (Fig. 1).

### *3.2. Wild-type and variant CYP2B6 expression levels in 293FT cells*

Protein levels of CYP2B6 variants expressed in 293FT cells were assessed by western blotting with a polyclonal CYP2B6 antibody that recognized all CYP2B6 variants tested (Fig. 2). Truncated variants harboring Arg378Ter substitution (*CYP2B6*28*) were stained as intense bands at a lower molecular weight than wild-type CYP2B6.1. All of the remaining CYP2B6 variant bands were detected at the correct sizes (~ 56 kDa). Expression levels of calnexin, an endoplasmic reticulum-resident protein, were virtually constant in microsomes of transfected cells. CYP2B6 protein was not detected in cells transfected with empty vector (mock transfection).

### *3.3. Determination of microsomal CYP2B6 holo-protein contents*

We measured the reduced CO-difference spectra of the microsomal fractions for each CYP2B6 variant. The spectra of all CYP2B6 variants are shown in Fig. 3. We detected an increase in the maximum absorption wavelength ($\lambda_{max}$) at 450 nm after CO treatment for CYP2B6.1 and 28 variants (CYP2B6.2–.10, .13, .14, .17, .19, .20, .23, .25–.27, .31–.35, Arg35Cys, Arg145Trp, Arg158Gly, Glu339Asp, and Tyr380His). The CYP2B6.1 expression level was 31.5 pmol of CYP2B6/mg microsomal protein. Only CYP2B6.19 was expressed at a significantly lower level than CYP2B6.1 (11.1 pmol CYP2B6/mg microsomal protein, $P < 0.05$). The expression levels of the remaining 27 variants did not differ significantly from that of CYP2B6.1. The expression level of the remaining 11 variants (CYP2B6.11, .12, .15, .16, .18, .21, .24, Arg378Ter, .37, Ile382Asn, and Arg443Cys) could not be determined because there was no significant increase in their absorption maxima at 450 nm (Fig. 4).

### *3.4. Kinetics of EFZ 8-hydroxylation by CYP2B6 variants*

The kinetic parameters of EFZ 8-hydroxylation were calculated for CYP2B6.1 and 39 CYP2B6 variants (Table 3). The kinetic parameters for 15 variants (CYP2B6.8, .11–.13, .15, .16, .18, .21, .24, Arg378Ter, .35, .37, Arg145Trp, Ile382Asn,

and Arg443Cys) could not be determined because the product amounts were below the quantification limit at the highest substrate concentration used. CYP2B6.1 $K_m$, $V_{max}$, and $CL_{int}$ values were 4.09 μM, 0.35 pmol·min$^{-1}$·pmol$^{-1}$ CYP2B6, and 0.086 μL·min$^{-1}$·pmol$^{-1}$ CYP2B6, respectively. Compared to CYP2B6.1, both CYP2B6.14 and CYP2B6.19 had significantly higher $K_m$ values ($P < 0.05$), while Tyr380His showed a significantly lower $V_{max}$ value ($P < 0.05$), both of which result in reduced $CL_{int}$ values ($P < 0.005$, $P < 0.05$, and $P < 0.005$, respectively). CYP2B6.6 had a significantly higher $V_{max}$ value ($P < 0.05$), resulting in an increased $CL_{int}$ value ($P < 0.005$). Additionally, the $CL_{int}$ values of CYP2B6.7, 26, .34 ($P < 0.05$) and .32, .33 ($P < 0.005$) were significantly higher than that of CYP2B6.1. In contrast, the $CL_{int}$ values for CYP2B6.10, Arg158Gly, and Glu339Asp ($P < 0.05$, $P < 0.005$, and $P < 0.01$, respectively) were significantly lower than that of CYP2B6.1. The kinetic parameters of the remaining CYP2B6 variants were not significantly different from that of CYP2B6.1.

### *3.5. Correlation between the $CL_{int}$ ratios for EFZ 8-hydroxylation and 7-ETC O-deethylation*

We further calculated the kinetic parameters of 7-ETC *O*-deethylation for CYP2B6.1 and 39 variants. The kinetic parameters of fifteen inactive variants in EFZ

metabolism could also not be determined in 7-ETC metabolism because the enzymatic activity was not detected at the highest substrate concentration used. The kinetic parameters of 7-ETC *O*-deethylation for CYP2B6.1 and 24 variants were determined (Table 4). The $CL_{int}$ values of these variants did not differ significantly from that of CYP2B6.1. However, the $CL_{int}$ ratios obtained for 7-ETC *O*-deethylation were highly correlated with EFZ 8-hydroxylation ($R^2 = 0.837$, $P < 0.001$) (Fig. 5).

### *3.6. 3D structural modeling analysis*

3D structural modeling was applied to analyze the molecular interactions of CYP2B6 variants and EFZ. The Lys139Glu substitution in CYP2B6.8 and .13 was found to be disconnected from Pro261 and Thr267 (Fig. 6A). Seven amino acid substitutions (Gly99Glu, Ile391Asn, Pro428Thr, Gly476Asn, Ile114Thr, Val183Gly, and Arg443Cys) were located near the heme moiety of the CYP2B6 protein. Among them, the Ile114Thr substitution in CYP2B6.35 formed hydrophobic interactions with EFZ. Additionally, the Gly110Val substitution in CYP2B6.35 formed alkyl interactions with Arg112 and Ala116 (Fig. 6B). We further performed 3D structural analysis for the combination of Gln172His and Lys262Arg found in various CYP2B6 variants. The Gln172His substitution formed various interactions with eight amino acid residues surrounding

Thr302 (Fig. 7A). The Lys262Arg substitution formed attractive charge interactions and hydrogen bonds with Glu148 and Asp263, respectively (Fig. 7B).

## 4. Discussion

CYP2B6 is a key drug-metabolizing enzyme and plays an important role in the metabolism of clinically relevant drugs. The highly polymorphic nature of CYP2B6 affects both substrate-dependent and -independent mechanisms of drug metabolism [3]. *CYP2B6* genetic polymorphisms are associated with large inter-individual responses to drug metabolism. In this study, we evaluated the functional changes caused by CYP2B6 variant alleles by calculating the kinetic parameters of 40 CYP2B6 variant proteins expressed in 293FT cells. We determined the kinetic parameters of EFZ 8-hydroxylation for CYP2B6.1 and 24 variants. The kinetic parameters of 15 variants could not be determined because metabolite amounts were below the quantification limit at the highest substrate concentrations assayed. Our study revealed the effects of the allelic polymorphisms presented in each variant on enzymatic properties. Because of higher correlation between the $CL_{int}$ ratios for EFZ 8-hydroxylation and 7-ETC *O*-deethylation among CYP2B6 variants, functional alterations of *CYP2B6* allelic variants gained from this study could be applied to various pharmacotherapy for CYP2B6-metabolized drugs.

Among the 40 CYP2B6 variants, 15 variants were inactive despite confirmation of protein expression by western blotting. Eleven of these variants (CYP2B6.11, .12, .15, .16, .18, .21, .24, Arg378Ter, .37, Ile382Asn, and Arg443Cys)

showed no increase in absorbance at 450 nm after CO-treatment, indicating the absence of functional CYP enzymes. Among these, amino acid substitutions in six variants (CYP2B6.12, .15, .21, .24, .37, and Arg443Cys) are located near the heme moiety of the CYP2B6 protein. Conformational changes near the heme group may affect protein function. Met46 is located near a highly conserved proline-rich region, which functions as a hinge between the endoplasmic reticulum membrane anchor and the heme-binding site [24]. In CYP2B6.11, the conformational alterations caused by the Met46Val substitution near the heme-binding site may lead to complete loss of enzymatic activity. Ile328Thr, the common amino acid substitution found in CYP2B6.16 and CYP2B6.18, is located in the J helix. Although there were no remarkable structural changes regarding these substitutions in this study, Kobayashi *et al.* have suggested that the multiple helices present in the structure surrounding the heme group in CYP2B6.18, which differs widely from those of CYP2B6.1, promote activity loss [25]. The Arg378Ter substitution, which did not show 420 or 450 nm peaks, results in a marked structural change and functional inactivity.

The remaining four inactive variants (CYP2B6.8, .13, .35, and Arg145Trp), have detected 450 nm peaks after CO-treatment and for which expression levels have been determined, indicating that the observed catalytic activity loss is due to marked

alterations of the crystal structure. Additionally, three of these variants (CYP2B6.13, .35, and Arg145Trp) were observed to have a bimodal peak at 420 nm and 450 nm, indicating the existence of apo- and holo-protein, respectively. Lys139Glu, the common amino acid substitution in both CYP2B6.8 and CYP2B6.13, is located in the C/D loop region and is involved in the binding of P450 redox partners [23]. Structurally, position 139, on the proximal side of the mammalian CYP2B6 structure, is highly conserved within the CYP2 family [26]. Zhang *et al.* have indicated that cytochrome P450 reductase electron transfer is severely impaired in the ferric Lys139Glu variant, resulting in an abolished catalytic capacity [27]. In this study, a Lys139 to Glu charge-reversal mutation separates from the residues in the G/H loop. This interaction, between the C/D and G/H loops, could provide the physical coordination mechanism underlying the observed ligand-induced conformational changes in the electron-delivery system of this protein [25]. Interruption of these interactions within the region may influence enzymatic activity. CYP2B6.35, which contains Gly110Val and Ile114Thr substitutions, showed a lack of enzymatic activity with both 7-ETC and EFZ. Similarly, Radloff *et al.* have reported that these substitutions result in an almost complete loss of protein function in COS-1 cells with respect to bupropion and EFZ [11]. Our results show that the Gly110Val substitution forms alkyl interactions with Arg112 and Ala116 located on the C helix,

while the Ile114Thr substitution forms hydrophobic interactions with EFZ. All of these residues are located in the substrate recognition site (SRS)-1. Therefore, substrate interactions caused by conformational changes within SRS-1 may affect the function of CYP2B6.

CYP2B6.1 and all 24 variants with determined kinetic parameters of EFZ 8-hydroxylation, showed increased absorbance values around 450 nm after CO-treatment. Among these, eight variants (CYP2B6.10, .17, .19, .20, .26, .27, .33, and Glu339Asp) were observed to have a bimodal peak at 420 nm and 450 nm, indicating the existence of apo- and holo-protein, respectively. CYP2B6.14 exhibited a 1.8-fold $K_m$ increase, resulting in a 22% $CL_{int}$ decrease compared with that of CYP2B6.1. Lang *et al.* have also reported that CYP2B6.14 showed similar activity for bupropion hydroxylation [26]. Arg140 is highly conserved within the CYP2 family [26], and the Arg140Gln substitution found in CYP2B6.14 is located in the C/D loop. Structural changes in this region might affect the affinity of CYP2B6 for EFZ. Additionally, CYP2B6.19 contains Gln172His, Lys262Arg, and Arg336Cys substitutions and demonstrated EFZ metabolism activity that was 37% of that reported for CYP2B6.1. Moreover, in this study, the expression level of the CYP2B6.19 variant was 65% lower than that of CYP2B6.1. The Arg336Cys substitution located near the J helix might reduce protein stability,

resulting in decreased activity.

The combination of Gln172His and Lys262Arg are the most common amino acid substitutions found amongst CYP2B6 variants. The frequency of the *CYP2B6*6* allele, with these amino acid changes, ranges from 15% to over 60% in various populations [3]. In our EFZ 8-hydroxylation analysis, the CYP2B6.6 allele showed a significantly increased $V_{max}$ (2.8-fold) and $CL_{int}$ value (2.7-fold) compared to that of CYP2B6.1. Furthermore, CYP2B6.7, .26, and .34 $CL_{int}$ values were significantly higher than those of CYP2B6.1. All of these variants contain Gln172His and Lys262Arg substitutions. Jinno *et al.* have reported that CYP2B6.6 exhibited a 1.9-fold increase than that of CYP2B6.1 in its $CL_{int}$ value for 7-ETC metabolism [10]. Radloff *et al.* have also reported that CYP2B6.6 showed a 1.8-fold increase in $CL_{int}$ value while using EFZ as a substrate [11]. This increased ratio was similar to that found in our results. Gay *et al.* have reported that Arg262 structurally constitutes part of a small hydrogen-bonding network that includes His252, Thr255, and Asp266 [23]. This network of hydrogen bonds could influence the orientation of active site residues on the G helix. In our 3D structural analysis, the Gln172His substitution forms various interactions with a number of residues surrounding Thr302. Thr302 is the covalent modification site of CYP2B6, and any structural change in this region may be responsible for enzymatic activity

alterations [28]. Additionally, the Lys262Arg substitution interacts with amino acid residues in the G/H loop, and conformational changes found between the C/D and G/H loops might affect CYP2B6 activity as mentioned above. Our study suggests that conformational changes caused by the interaction between the C/D and G/H loops leads to increased enzymatic activity and that the overlapping observed between Gln172His and Lys262Arg causes structural changes that might enhance metabolic activity.

However, critical differences have been observed between *in vitro* and *in vivo* studies, particularly in *CYP2B6*6*. The mean plasma EFZ concentration in homozygous *CYP2B6*6* patients infected with HIV was significantly higher than that of patients with the heterozygote *CYP2B6*6* genotype or those lacking the *CYP2B6*6* allele [5, 7]. The discrepancy between our results and those identified in clinical research could be caused by reduced gene expression and function in the human liver. An investigation using the *CYP2B6* minigene expression system including intronic regions, the c.516G>T exon 4 mutation, a nonsynonymous mutation in the *CYP2B6*6* allele, was found to be responsible for aberrant splicing resulting in a major splice variant that lacks the region comprising of exons 4 to 6 [29]. This splicing error causes severely reduced levels of mRNA transcript, protein expression, and enzymatic activity, resulting in inter-individual differences in phenotypic *in vivo* activity. Given that we use the *CYP2B6*

cDNA expression system in this study, there is a limit as to what CYP2B6 properties we are able to evaluate compared with those assessed using human liver samples.

We investigated the function of seven novel CYP2B6 variants found in Japanese individuals. In our EFZ 8-hydroxylation analysis, Arg35Cys kinetic parameters were not significantly different from those of CYP2B6.1. On the other hand, three variants (Arg158Gly, Glu339Asp, and Tyr380His located in the D helix, J' helix, and β2 strand, respectively) showed significantly lower $CL_{int}$ values than CYP2B6.1. Structural changes in these regions might decrease the activity of CYP2B6 for EFZ. Furthermore, a different set of three variants (Arg145Trp, Ile382Asn, and Arg443Cys) was inactive. Ile382Asn and Arg443Cys did not exhibit peaks at 450 nm, indicating a lack of functional proteins. The Ile382 residue interacts with the β1 sheet while Arg443 belongs to the L helix. These segments are located near the heme of the CYP2B6 protein, and structural changes in these sites result in loss of function. Arg145Trp another inactive variant which causes the hydrogen bond between Lys262 and the G/H loop to be severed, showed an increase in its absorption maxima at 450 nm. The dissociation of these important residues may affect enzymatic activity. As a result, Japanese individuals carrying variants, which cause a significant decrease or loss of CYP2B6 activity, are expected to be poor metabolizers of their respective substrates.

In conclusion, wild-type and 39 CYP2B6 variants were expressed in 293FT cells and their enzymatic activities were characterized *in vitro*. These data revealed the functional effects caused by the genetic polymorphisms found in the CYP2B6 allelic variants. Comprehensive *in vitro* assessment of CYP2B6 allelic variants can provide novel insight into the allele-specific activity of CYP2B6-metabolized drugs such as EFZ. In the future, this approach could lead to the discovery of genotypic-phenotypic associations, which can be later used in the development of personalized medicine with respect to *CYP2B6* genetic polymorphisms.

## Conflict of interest

The authors declare no conflict of interest.

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## Figure Legends

**Fig. 1.** Seven novel *CYP2B6* SNVs identified in 1070 Japanese subjects. The shaded box numbers correspond to each *CYP2B6* exon. The frequencies of the CYP2B6 variants in the diagram were observed in 1070 Japanese subjects.

**Fig. 2.** Western blots showing immunoreactive CYP2B6 proteins (upper panel) and calnexin (lower panel). Western blotting was performed according to standard procedures using SDS-PAGE. Five micrograms of microsomal fractions, containing CYP2B6 variant proteins, were loaded onto each lane. CYP2B6 variants and calnexin were detected using polyclonal antibodies against the respective proteins. The numbers correspond to each CYP2B6 variant.

**Fig. 3.** Representative CO difference spectra of CYP2B6 variant proteins expressed in 293FT cells.

**Fig. 4.** Expression levels of CYP2B6 proteins expressed in 293FT cells. Each bar is presented as the mean ± S.D. of three independently performed CO-difference spectra readings. The numbers correspond to each CYP2B6 variant.

*$P < 0.05$ compared to CYP2B6.1.

**Fig. 5.** Correlation between the $CL_{int}$ ratios (relative to CYP2B6.1) for EFZ 8-hydroxylation and 7-ETC *O*-deethylation among CYP2B6 variants. EFZ 8-hydroxylation $CL_{int}$ ratios are plotted on the horizontal axis and 7-ETC *O*-deethylation $CL_{int}$ ratios are plotted on the vertical axis. Numbers correspond to CYP2B6 variants. Each bar is presented as the mean ± S.D. of three independently performed catalytic assays. The correlation was analyzed using a weighted linear regression analysis using IBM SPSS Statistics.

**Fig. 6.** Diagram showing part of the crystal structures of CYP2B6.1 (left panel) and CYP2B6 variants (right panel). The Lys139 and Glu139 residues in CYP2B6.1 are shown in yellow (A). The Gln110 and Ile114 residues in CYP2B6.1, as well as the Val110 and Thr114 residues in CYP2B6.35, are shown in yellow (B). Hydrogen bonds, attractive charge interactions, alkyl interactions, and pi-anion interaction are shown in light green, red, purple, and mustard lines respectively.

**Fig. 7.** Diagram showing part of the crystal structures of CYP2B6.1 (left panel) and CYP2B6 variants (right panel). The CYP2B6.1 Gln172 residue and the CYP2B6 His172 variant residue are shown in yellow. The Thr302 residue is shown in green. The amino acid residues forming interactions with either Gln172 or His172 are included in diagram (A). The CYP2B6.1 residue Lys262 and the CYP2B6 variants residue Arg262 are both shown in yellow. Hydrogen bonds and attractive charge interactions are shown with a light green and red line, respectively (B).

**Table 1** CYP2B6 allelic variants characterized in this study.

| Variants | Protein | Nucleotide changes | Amino acid changes |
|---|---|---|---|
| *CYP2B6*1* | CYP2B6.1 | | |
| *CYP2B6*2* | CYP2B6.2 | 64C>T | Arg22Cys |
| *CYP2B6*3* | CYP2B6.3 | 777C>A | Ser259Arg |
| *CYP2B6*4* | CYP2B6.4 | 785A>G | Lys262Arg |
| *CYP2B6*5* | CYP2B6.5 | 1459C>T | Arg487Cys |
| *CYP2B6*6* | CYP2B6.6 | 516G>T; 785A>G | Gln172His; Lys262Arg |
| *CYP2B6*7* | CYP2B6.7 | 516G>T; 785A>G; 1459C>T | Gln172His; Lys262Arg; Arg487Cys |
| *CYP2B6*8* | CYP2B6.8 | 415A>G | Lys139Gln |
| *CYP2B6*9* | CYP2B6.9 | 516G>T | Gln172His |
| *CYP2B6*10* | CYP2B6.10 | 62A>T; 64C>T; 216G>T | Gln21Leu; Arg22Cys |

| | | | |
|---|---|---|---|
| *CYP2B6*11* | CYP2B6.11 | 136A>G | Met46Val |
| *CYP2B6*12* | CYP2B6.12 | 296G>A | Gly99Glu |
| *CYP2B6*13* | CYP2B6.13 | 415A>G; 516G>T; 785A>G | Lys139Gln; Gln172His; Lys262Arg |
| *CYP2B6*14* | CYP2B6.14 | 419G>A | Arg140Gln |
| *CYP2B6*15* | CYP2B6.15 | 1172T>A | Ile391Asn |
| *CYP2B6*16* | CYP2B6.16 | 785A>G; 983T>C | Lys262Arg; Ile328Thr |
| *CYP2B6*17* | CYP2B6.17 | 76A>T; 83A>G; 85T>A; 86G>C | Thr26Ser; Asp28Gly; Arg29Thr |
| *CYP2B6*18* | CYP2B6.18 | 983T>C | Ile328Thr |
| *CYP2B6*19* | CYP2B6.19 | 516G>T; 785A>G; 1006C>T | Gln172His; Lys262Arg; Arg336Cys |
| *CYP2B6*20* | CYP2B6.20 | 503C>T; 516G>T; 785A>G | Thr168Ile; Gln172His; Lys262Arg |
| *CYP2B6*21* | CYP2B6.21 | 1282C>A | Pro428Thr |
| *CYP2B6*23* | CYP2B6.23 | 1375A>G | Met459Val |

| | | | |
|---|---|---|---|
| *CYP2B6*24* | CYP2B6.24 | 1427G>A | Gly476Asp |
| *CYP2B6*25* | CYP2B6.25 | 1454A>T | Gln485Leu |
| *CYP2B6*26* | CYP2B6.26 | 499C>G; 516G>T; 785A>G | Pro167Ala; Gln172His; Lys262Arg |
| *CYP2B6*27* | CYP2B6.27 | 593T>C | Met198Thr |
| *CYP2B6*28* | | (917C>G); 1132C>T | (Thr306Ser); Arg378Ter |
| *CYP2B6*31* | CYP2B6.31 | 937C>A | Leu313Ile |
| *CYP2B6*32* | CYP2B6.32 | 1219G>A | Ala407Thr |
| *CYP2B6*33* | CYP2B6.33 | 1459C>A | Arg487Ser |
| *CYP2B6*34* | CYP2B6.34 | 516G>T; 785A>G; 1459C>A | Gln172His; Lys262Arg; Arg487Ser |
| *CYP2B6*35* | CYP2B6.35 | 329G>T; 341T>C; 444G>T; 593T>C; 835G>C | Gln110Val; Ile114Thr; Glu148Asp; Met198Thr; Ala279Pro |
| *CYP2B6*37* | CYP2B6.37 | 516G>T; 548T>G; 785A>G | Gln172His; Val183Gly; Lys262Arg |

| | | |
|---|---|---|
| Novel variant 1 | 103C>T | Arg35Cys |
| Novel variant 2 | 433C>T | Arg145Trp |
| Novel variant 3 | 472C>G | Arg158Gly |
| Novel variant 4 | 1017G>C | Glu339Asp |
| Novel variant 5 | 1138T>C | Tyr380His |
| Novel variant 6 | 1145T>A | Ile382Asn |
| Novel variant 7 | 1327C>T | Arg443Cys |

**Table 2** PCR primers used to amplify sequences of the human CYP2B6 gene to confirm the novel allelic variants observed in 1070 Japanese subjects.

| Nucleotide change | Primer (5′ –3′) | | Product length (bp) |
|---|---|---|---|
| | Forward | Reverse | |
| 103C>T | GTGCAGGGCAGTCAGACCAG | CAAGGCAAGAAGCAGCTCAC | 243 |
| 433C>T | CAGGTGTGATCTTTGCCAATG | GATGGTGTCTCCAGTTTCGTC | 280 |
| 472C>G | | | |
| 1017G>C | CCACCTCAACCTCCAAAATTG | GATATTGCCATTCCCTCCAAC | 454 |
| 1138T>C | | | |
| 1145T>A | | | |
| 1327C>T | GCAGTGGACATTTGTGTCTGG | CTCACTTGCAATGTGACCTCAG | 441 |

**Table 3** Kinetic parameters of EFZ 8-hydroxylation

| Variants | $K_m$ (μM) | $V_{max}$ (pmol/min/pmol CYP2B6) | $CL_{int}$ ($V_{max}/K_m$) (μL/min/pmol CYP2B6) | % of wild-type $CL_{int}$ |
|---|---|---|---|---|
| CYP2B6.1 | 4.09 ± 0.15 | 0.35 ± 0.03 | 0.086 ± 0.004 | |
| CYP2B6.2 | 4.53 ± 0.42 | 0.37 ± 0.02 | 0.081 ± 0.003 | 95% |
| CYP2B6.3 | 4.57 ± 1.22 | 0.28 ± 0.02 | 0.064 ± 0.013 | 75% |
| CYP2B6.4 | 3.70 ± 0.66 | 0.38 ± 0.00 | 0.105 ± 0.020 | 122% |
| CYP2B6.5 | 4.13 ± 0.44 | 0.38 ± 0.06 | 0.094 ± 0.018 | 109% |
| CYP2B6.6 | 4.34 ± 0.20 | 0.99 ± 0.06* | 0.228 ± 0.006*** | 266% |
| CYP2B6.7 | 4.82 ± 0.22 | 0.69 ± 0.05 | 0.143 ± 0.007* | 166% |
| CYP2B6.8 | N.D. | N.D. | N.D. | |
| CYP2B6.9 | 5.31 ± 0.27 | 0.78 ± 0.04 | 0.148 ± 0.016 | 172% |

| | | | | |
|---|---|---|---|---|
| CYP2B6.10 | 6.04 ± 1.13 | 0.26 ± 0.03 | 0.044 ± 0.005* | 51% |
| CYP2B6.11 | N.D. | N.D. | N.D. | |
| CYP2B6.12 | N.D. | N.D. | N.D. | |
| CYP2B6.13 | N.D. | N.D. | N.D. | |
| CYP2B6.14 | 7.27 ± 1.95* | 0.13 ± 0.01 | 0.019 ± 0.004*** | 22% |
| CYP2B6.15 | N.D. | N.D. | N.D. | |
| CYP2B6.16 | N.D. | N.D. | N.D. | |
| CYP2B6.17 | 4.11 ± 0.42 | 0.30 ± 0.00 | 0.073 ± 0.007 | 85% |
| CYP2B6.18 | N.D. | N.D. | N.D. | |
| CYP2B6.19 | 7.09 ± 1.13* | 0.22 ± 0.01 | 0.032 ± 0.006* | 37% |
| CYP2B6.20 | 1.51 ± 0.58 | 0.41 ± 0.03 | 0.311 ± 0.139 | 363% |
| CYP2B6.21 | N.D. | N.D. | N.D. | |

| | | | | |
|---|---|---|---|---|
| CYP2B6.23 | 3.74 ± 0.24 | 0.38 ± 0.02 | 0.103 ± 0.007 | 120% |
| CYP2B6.24 | N.D. | N.D. | N.D. | |
| CYP2B6.25 | 2.85 ± 0.66 | 0.20 ± 0.01 | 0.072 ± 0.013 | 85% |
| CYP2B6.26 | 2.74 ± 0.16 | 0.43 ± 0.01 | 0.157 ± 0.011* | 183% |
| CYP2B6.27 | 1.73 ± 0.46 | 0.11 ± 0.01 | 0.067 ± 0.015 | 79% |
| Arg378Ter | N.D. | N.D. | N.D. | |
| CYP2B6.31 | 5.35 ± 0.87 | 0.63 ± 0.07 | 0.118 ± 0.006 | 137% |
| CYP2B6.32 | 3.63 ± 0.20 | 0.56 ± 0.01 | 0.153 ± 0.005*** | 179% |
| CYP2B6.33 | 4.81 ± 0.46 | 0.68 ± 0.05 | 0.142 ± 0.004*** | 165% |
| CYP2B6.34 | 4.85 ± 0.59 | 0.71 ± 0.04 | 0.147 ± 0.009* | 171% |
| CYP2B6.35 | N.D. | N.D. | N.D. | |
| CYP2B6.37 | N.D. | N.D. | N.D. | |

| | | | | |
|---|---|---|---|---|
| Arg35Cys | 3.77 ± 0.54 | 0.23 ± 0.02 | 0.062 ± 0.011 | 73% |
| Arg145Trp | N.D. | N.D. | N.D. | |
| Arg158Gly | 5.95 ± 1.75 | 0.15 ± 0.02 | 0.026 ± 0.005*** | 30% |
| Glu339Asp | 4.05 ± 0.17 | 0.20 ± 0.00 | 0.049 ± 0.002** | 57% |
| Tyr380His | 5.52 ± 0.89 | 0.10 ± 0.01* | 0.019 ± 0.002*** | 22% |
| Ile382Asn | N.D. | N.D. | N.D. | |
| Arg443Cys | N.D. | N.D. | N.D. | |

These data represent the mean ± SD of three independently performed catalytic assays.

*$P < 0.05$, **$P < 0.01$, and ***$P < 0.005$ compared with CYP2B6.1. N.D. represents not determined.

The kinetic parameters of EFZ 8-hydroxylation regarding 15 variants could not be determined because the enzymatic activity of CYP2B6 was not detected at the highest substrate concentration assayed (20 μM EFZ).

**Table 4** Kinetic parameters of 7-ETC *O*-deethylation

| Variants | $K_m$ (μM) | $V_{max}$ (pmol/min/pmol CYP2B6) | $CL_{int}$ ($V_{max}/K_m$) (μL/min/pmol CYP2B6) | % of wild-type $CL_{int}$ |
|---|---|---|---|---|
| CYP2B6.1 | 7.58 ± 1.50 | 2.11 ± 0.44 | 0.279 ± 0.005 | |
| CYP2B6.2 | 14.90 ± 3.84 | 2.85 ± 0.82 | 0.190 ± 0.010 | 68% |
| CYP2B6.3 | 19.07 ± 7.54 | 1.36 ± 0.07 | 0.082 ± 0.042 | 30% |
| CYP2B6.4 | 5.80 ± 0.84 | 2.61 ± 0.33 | 0.451 ± 0.031 | 162% |
| CYP2B6.5 | 7.65 ± 1.20 | 2.88 ± 0.19 | 0.380 ± 0.038 | 137% |
| CYP2B6.6 | 7.85 ± 0.71 | 3.53 ± 0.24 | 0.453 ± 0.054 | 163% |
| CYP2B6.7 | 16.22 ± 3.39 | 6.73 ± 1.10* | 0.419 ± 0.040 | 150% |
| CYP2B6.9 | 14.24 ± 3.03 | 3.56 ± 0.33 | 0.255 ± 0.040 | 92% |
| CYP2B6.10 | 15.78 ± 0.17 | 1.60 ± 0.38 | 0.101 ± 0.025 | 36% |

| | | | | |
|---|---|---|---|---|
| CYP2B6.14 | 24.34 ± 3.64 | 1.09 ± 0.12 | 0.045 ± 0.004 | 16% |
| CYP2B6.17 | 24.92 ± 4.36 | 0.85 ± 0.06 | 0.035 ± 0.003 | 12% |
| CYP2B6.19 | 45.34 ± 10.94 | 1.59 ± 0.60 | 0.034 ± 0.006 | 12% |
| CYP2B6.20 | 8.55 ± 2.12 | 4.70 ± 0.24* | 0.571 ± 0.132 | 205% |
| CYP2B6.23 | 6.79 ± 1.44 | 3.17 ± 0.26 | 0.476 ± 0.067 | 171% |
| CYP2B6.25 | 13.83 ± 2.06 | 2.62 ± 0.07 | 0.192 ± 0.023 | 69% |
| CYP2B6.26 | 9.10 ± 2.46 | 4.11 ± 0.57 | 0.464 ± 0.070 | 167% |
| CYP2B6.27 | 3.30 ± 0.42 | 1.24 ± 0.03 | 0.378 ± 0.044 | 136% |
| CYP2B6.31 | 6.62 ± 1.34 | 2.94 ± 0.20 | 0.454 ± 0.077 | 163% |
| CYP2B6.32 | 5.57 ± 0.10 | 3.27 ± 0.13 | 0.587 ± 0.033 | 211% |
| CYP2B6.33 | 7.96 ± 3.24 | 3.33 ± 0.58 | 0.472 ± 0.204 | 169% |
| CYP2B6.34 | 12.14 ± 1.61 | 5.53 ± 0.21* | 0.460 ± 0.043 | 165% |

| | | | | |
|---|---|---|---|---|
| Arg35Cys | 10.28 ± 1.03 | 2.37 ± 0.13 | 0.232 ± 0.012 | 83% |
| Arg158Gly | 15.18 ± 2.51 | 1.59 ± 0.04 | 0.106 ± 0.016 | 38% |
| Glu339Asp | 26.34 ± 6.16 | 2.12 ± 0.11 | 0.084 ± 0.022 | 30% |
| Tyr380His | 28.67 ± 1.29*** | 1.14 ± 0.09 | 0.040 ± 0.004 | 14% |

These data represent the mean ± SD of three independently performed catalytic assays.

*$P < 0.05$, and ***$P < 0.005$ compared with CYP2B6.1.

The kinetic parameters of 7-ETC *O*-deethylation regarding 15 variants (CYP2B6.8, .11–.13, .15, .16, .18, .21, .24, Arg378Ter, .35, .37, Arg145Trp, Ile382Asn, and Arg443Cys) could not be determined because the enzymatic activity of CYP2B6 was not detected at the highest substrate concentration assayed (50 μM 7-ETC).