Biochemical Pharmacology 156 (2018) 420–430

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

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

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ARTICLE INFO

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)

Keywords:
Cytochrome P450
CYP2B6
Genetic polymorphisms
Efavirenz
Pharmacogenetics
Drug metabolism

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.

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.

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https://doi.org/10.1016/j.bcp.2018.09.010
Received 19 July 2018; Accepted 6 September 2018
Available online 08 September 2018

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