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RESEARCH ARTICLE

Functional influence of human CYP2D6 allelic variations: P34S, E418K, S486T, and R296C

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Abstract CYP2D6 is responsible for the oxidative metabolism of 20–25 % of clinical drugs and its genetic polymorphisms can significantly influence the drug metabolism. In this study, we analyzed the functional activities of four nonsynonymous single nucleotide polymorphisms from CYP2D6*52 allele, which were recently found, and one found frequently in CYP2D6 alleles. Recombinant variant enzymes of E418K, S486T, and R296C were successfully expressed in *Escherichia coli* and purified. However, a CYP holoenzyme spectrum of P34S variant was not detected in *E. coli* whole cell level. Structural analysis indicated that P34S mutation seemed to perturb a highly conserved proline-rich N-terminus of CYP2D6. Steady state kinetic analyses showed the significant reductions of enzymatic activities in E418K and R296C variants. In the case of bufuralol 1'-hydroxylation, a novel mutant, E418K, showed 32 % decrease in catalytic efficiency (k_{cat}/K_m) mainly due to the decrease of k_{cat} value. R296C showed much greater reduction in the catalytic efficiency (9 % of wild-type) due to both of a decrease of k_{cat} value and an increase of K_m value. In the case of dextromethorphan *O*-demethylation, E418K showed both of a decrease of k_{cat} value and an increase K_m value to result in ~43 % reduction of catalytic efficiency. A highly decreased catalytic efficiency (~6 % of wild-type) in the mutant of R296C also was observed mainly due to the dramatic change of k_{cat} value of dextromethorphan *O*-demethylation. These results suggested that individuals carrying these allelic variants are likely to have the altered metabolic abilities of many clinical drugs therefore, these polymorphisms of CYP2D6 should be much concerned for reliable drug treatment.

Keywords P450 · CYP2D6 · Allelic variants · Polymorphism · Bufuralol · Dextromethorphan

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Introduction

Human genome contains 57 cytochromes P450 (CYPs, P450s) enzymes and 15 among them are considered as xenobiotics metabolic enzymes responsible for the metabolism of most of drugs (Stark and Guengerich 2007). Many adverse drug–drug interactions are attributable to pharmacokinetic problems and can be understood in terms of alterations of CYP-catalyzed reactions (Guengerich 1997). The genetic polymorphisms of drug metabolizing CYPs is of particular interest because it can promise the rational optimization of drug therapy with respect to patient's genotype to ensure maximum efficacy with minimal adverse effects (Lee and Kim 2011).

Genetic polymorphisms in CYP enzymes can cause dramatic differences in the response to specific drugs and the debrisoquine poor-metabolizer phenotype of CYP2D6 is a well-known example (Zhou et al. 2004). Clinically, the

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CYP2D6 has been issues due to the large variation in the genetic polymorphism unlike other CYP enzymes (Lee and Kim 2011). So far, 105 different human CYP2D6 allelic variants have been detected and ~40 nonsynonymous single nucleotide polymorphisms (SNPs) have been reported (http://www.cypalleles.ki.se/) (Lee and Kim 2011). Recently, Lee et al. identified a novel amino acid change (E418K) of CYP2D6*52 allele from Korean populations (Lee et al. 2009). The CYP2D6*52 allele contains P34S and S486T amino acid changes in addition to E418K and its frequency was 0.33 % in Korean populations (Lee et al. 2009). P34S and S486T substitutions were present in the CYP2D6*10 allele with a very high frequency in Asian populations (Johansson et al. 1994).

In this study, we analyzed the drug metabolic activities of four mutants (P34S, E418K, P34S/E418K, and S486T) from a novel CYP2D6*52 allele and one mutant (R296C) highly found in many CYP2D6 alleles. In view of the importance of CYP2D6 in the metabolism of many clinical drugs, we determined the functional outcomes from the novel mutations of CYP2D6.

Materials and methods

Chemicals

Bufuralol, dextromethorphan, dextrorphan, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and $NADP^+$ were purchased from Sigma-Aldrich (St. Louis, MO, USA). 1′-Hydroxybufuralol was kindly provided by Professor F. Peter Guengerich (Vanderbilt University, TN, USA). Ni^{2+}-nitrilotriacetate agarose was purchased from Qiagen (Valencia, CA, USA). Other chemicals were of the highest grade commercially available. *Escherichia coli* DH5α cells were purchased from Invitrogen (Carlsbad, CA, USA).

Construction of expression vectors for CYP2D6 allelic variants

The pCW plasmid containing CYP2D6 gene and 4× His-tag at C-terminus [pCW(CYP2D6)] was used to construct the CYP2D6 allelic variants (Hanna et al. 2001). Site-directed mutagenesis was performed using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA) with the following primers: 5′-TGCACGCTACTCACCA-3′, 5′-TGGTGAGT AGCGTGCA-3′ (P34S); 5′-TTCCACCCCAAACACTTC-3′, 5′-GAAGTGTTTGGGGTGGAA-3′ (E418K); 5′-TTCC TGGTGACCCCATCC-3′, 5′-GGATGGGGTCACCAGGA A-3′ (S486T); 5′-GAGAACCTGTGCATAGTG-3′, 5′-CAC TATGCACAGGTTCTC-3′ (R296C). The P34S/E418K variant was generated using primers for P34S and E418K. The constructed variant clones were confirmed by nucleotide sequencing analysis.

Expression of recombinant CYP2D6 allelic variants in *E. coli*

E. coli strain DH5α was used for transformation of both pCW(CYP2D6) and pGro12 encoding the chaperone protein GroEL/ES (Wu et al. 2009). Single transformed colonies were selected on LB plate media containing ampicillin (50 μg/ml) and kanamycin (20 μg/ml) and then inoculated in 5 ml of LB liquid cultures. After overnight incubation at 37 °C, 220 rpm, the cultures were transferred to 500 ml of Terrific broth (TB) containing ampicillin (50 μg/ml) and kanamycin (20 μg/ml) and incubated at 37 °C, 220 rpm. When the OD_{600} was reached to approximately 0.5, the induction was done with addition of 1.0 mM isopropyl β-D-thiogalactoside (IPTG), 0.5 mM 5-aminolevulinic acid (ALA), 1.0 mM thiamine, trace elements, and 0.1 mg/ml arabinose. Cultures were further incubated for 48 h at 28 °C, 200 rpm, and then harvested by centrifugation.

Purification of CYP2D6 allelic variant proteins

The harvested cells were resuspended in TES buffer (50 mM Tris–acetate, 250 mM sucrose, 0.25 mM EDTA, pH 7.6) containing lysozyme and incubated for 40 min at 4 °C. Cell pellets were centrifuged and then ultra-sonicated in 150 ml of sonication buffer containing 10 mM potassium phosphate (pH 7.4), 20 % glycerol (v/v), 6.0 mM Mg-acetate, and 0.1 mM DTT. After ultracentrifugation, the membrane fractions were prepared and solubilized in 100 mM Tris–HCl (pH 7.4) buffer containing 20 % glycerol (v/v), 1.0 mM DTT, 0.625 % Na-cholate, and 1.25 % Triton N-101 (v/v). After ultracentrifugation, the supernatant containing the solubilized proteins were loaded into Ni^{2+}-nitrilotriacetate agarose column pre-equilibrated with 30 mM potassium phosphate buffer (pH 7.4) containing 20 % glycerol (v/v), 5 mM imidazole, and 0.5 M NaCl. After loading, the columns were washed with 30 mM imidazole, and then, eluted with 500 mM imidazole. The eluted proteins were dialyzed three times in 100 mM potassium phosphate buffer (pH 7.4) containing 20 % glycerol at 4 °C.

Catalytic activity analysis

Catalytic activities of CYP2D6 wild type and variant enzymes in all reactions were determined using the CYP/NADPH-P450 reductase/phospholipid reconstituted system (Han et al. 2009, 2012). The reaction mixtures included 50 pmol of purified CYP2D6 enzymes (wild type and

variants), 100 pmol of rat NPR, 30 μg sonicated 1,2-dilauroyl-*sn*-glycero-3-phosphocholine (DLPC), and specified amount of the substrates in 0.5 ml of 100 mM potassium phosphate buffer (pH 7.4). An NADPH generating system (100 mM glucose-6-phosphate, 10 mM $NADP^+$, glucose-6-phosphate dehydrogenase) was used to initiate the enzymatic reactions.

The reaction metabolites were analyzed using a reversed-phase ODS column (5 μm, 150 × 4.6 mm, Agilent) equipped with YL9100 HPLC system (Young-Lin, Korea). For bufuralol 1′-hydroxylation, buffer A consisted of 20 mM Na-ClO_4 (pH 2.5) and 10 % CH_3CN (v/v), and buffer B consisted of 20 mM Na-ClO_4 (pH 2.5) and 40 % CH_3CN (v/v). The following gradient was used with a flow rate of 1.0 ml/min: from 0 to 15 min, linear gradient from 0 to 50 % B (v/v); from 15 to 20 min, linear gradient to 100 % B; from 20 to 23 min, held at 100 % B; from 23 to 25 min linear gradient to 100 % A; from 25 to 30 min, held at 100 % A. For dextromethorphan *O*-demethylation, the mobile phase consisted of 20 mM Na-ClO_4 (pH 2.5) and MeOH. The following gradient was used with a flow rate 0.6 ml/min: from 0 to 30 min, linear gradient from 30 to 80 % MeOH; from 30 to 33 min, held at 80 % MeOH; from 33 to 35 min, linear gradient from 80 to 100 % MeOH; from 35 to 38 min, held at 100 % MeOH. A_{242} (for bufuralol) and A_{280} (for dextromethorphan) were monitored respectively. The metabolites were identified using authentic standards, and the peaks were integrated. Steady state kinetic parameters were calculated using GraphPad Prism software (La Jolla, CA, USA).

Results

Expression and purification of recombinant CYP2D6 allelic variant enzymes

All of the five CYP2D6 variants were constructed in the pCW expression vectors, and recombinant proteins of CYP2D6 variants were expressed in *E. coli* DH5α. Recombinant CYP2D6 expression levels in the whole-cell cultures were determined by CO-binding spectra (Fig. 1). The expression level of CYP2D6 wild-type was ~140 nmol/l, and the levels of E418K, S486T, and R296C were ranged from 40 to 70 nmol/l. However, no CYP holoenzyme spectrum detected for the P34S and P34S/E418K variants. This result suggests that the P34S substitution may affect to the accurate folding or expression of the holoenzyme. Recombinant proteins of CYP2D6 wild-type and three variants (E418K, S486T, and R296C) were

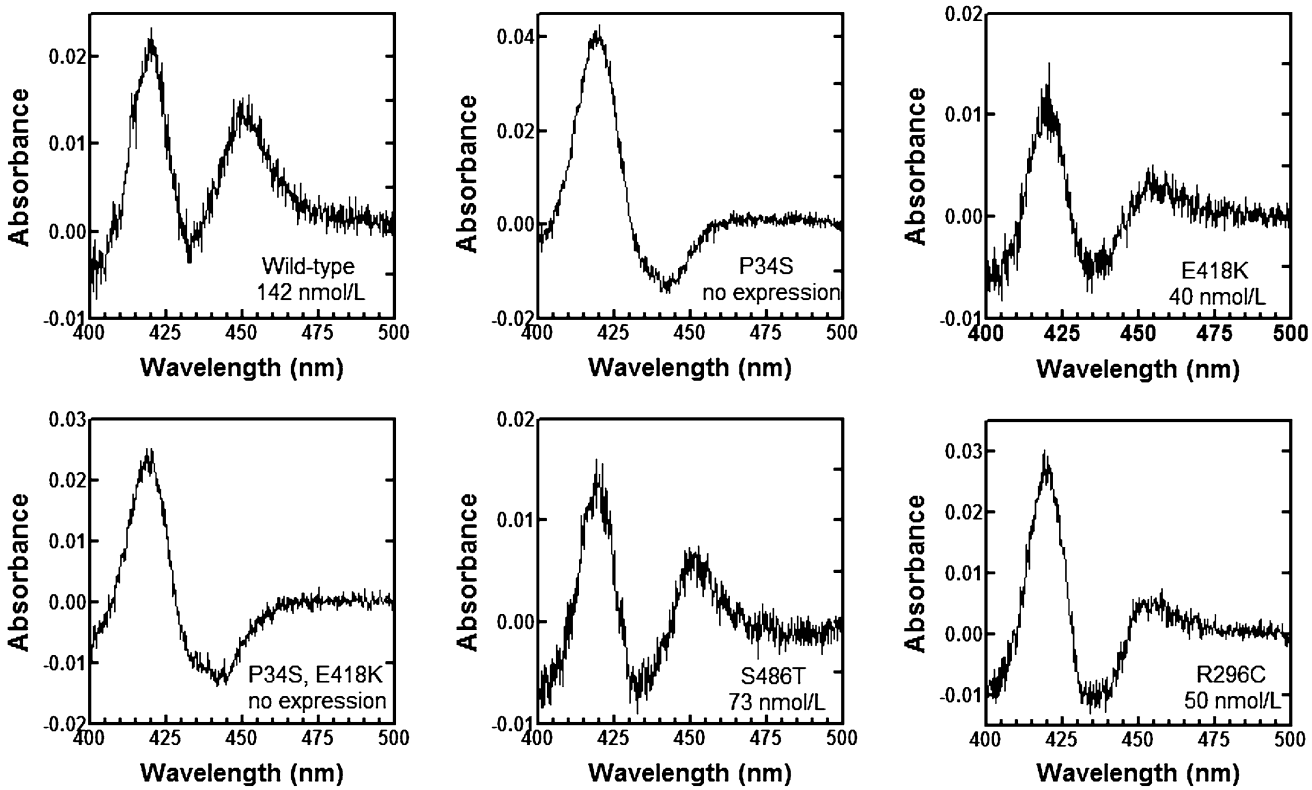

Fig. 1 Expression of recombinant CYP2D6 allelic variant enzymes in *E. coli.* CO-binding spectra of CYP enzymes in *E. coli* whole cells were measured, and the contents of the CYP holoenzyme were calculated

purified using Ni^{2+}-NTA affinity column. The concentrations of all purified CYP2D6 proteins were spectroscopically determined to 0.5–4.6 μM (data not shown).

Bufuralol metabolism by CYP2D6 variants

CYP2D6 converted bufuralol to multiple oxidized products (1′-hydroxybufuralol, 4-hydroxybufuralol, 6-hydroxybufuralol, and Δ1′,2′-bufuralol) (Hanna et al. 2001) (Fig. 2a). In this study, the enzyme reactions by CYP2D6 and variants showed 1′-hydroxybufuralol (retentions time of 6.3 min) was a major metabolite with co-elution of authentic standards (Fig. 3a). Minor products were assigned as 6-hydroxybufuralol (8.4 min), 4-hydroxybufuralol (9.6 min), and Δ1′,2′-bufuralol (11.6 min) based on the previous report (Hanna et al. 2001) (Fig. 3a).

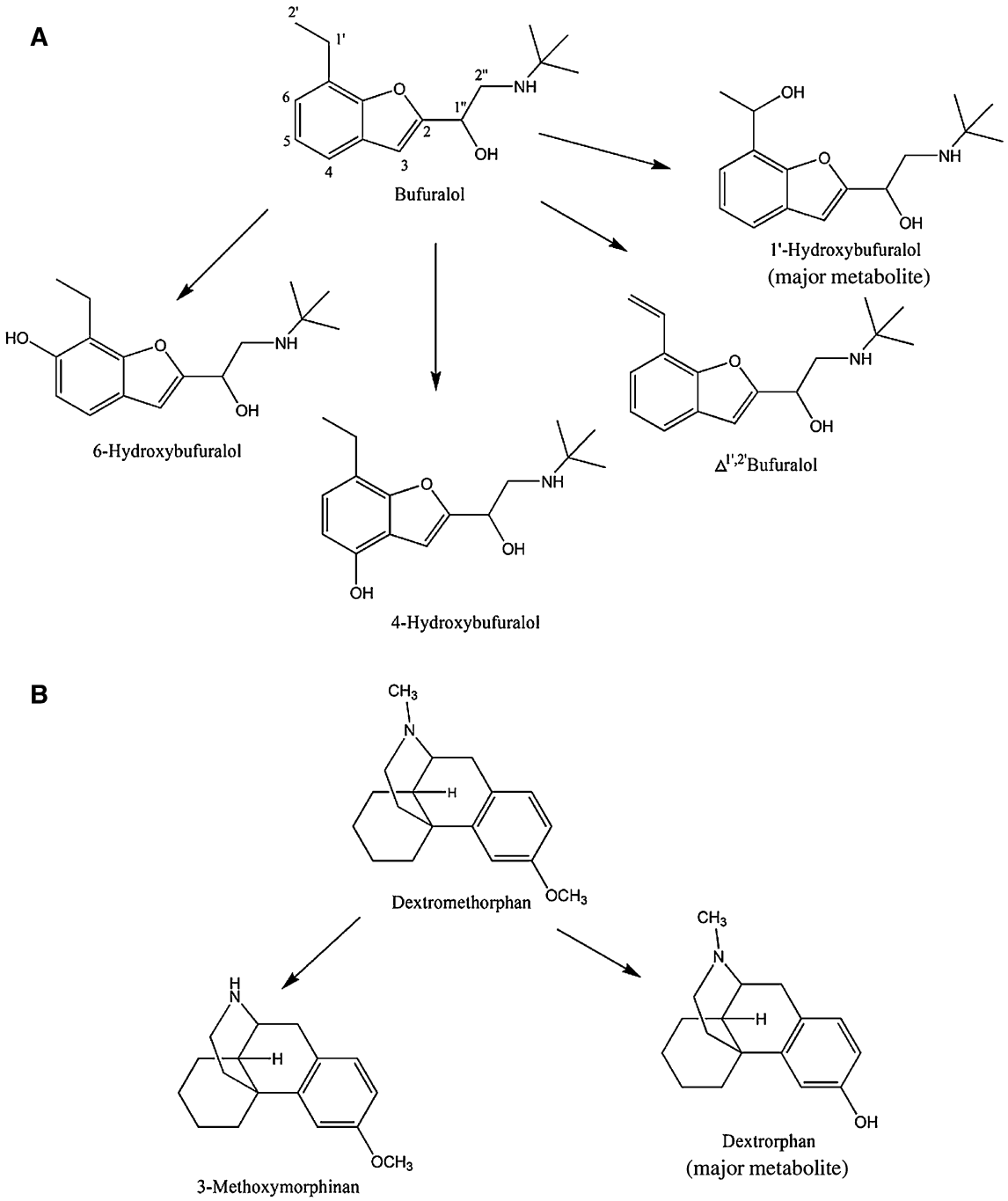

Fig. 2 Metabolism of bufuralol and dextromethorphan by CYP2D6. **a** Oxidation of bufuralol by CYP2D6 (Hanna et al. 2001). **b** Demethylation of dextromethorphan by CYP2D6 (Yu and Haining 2001)

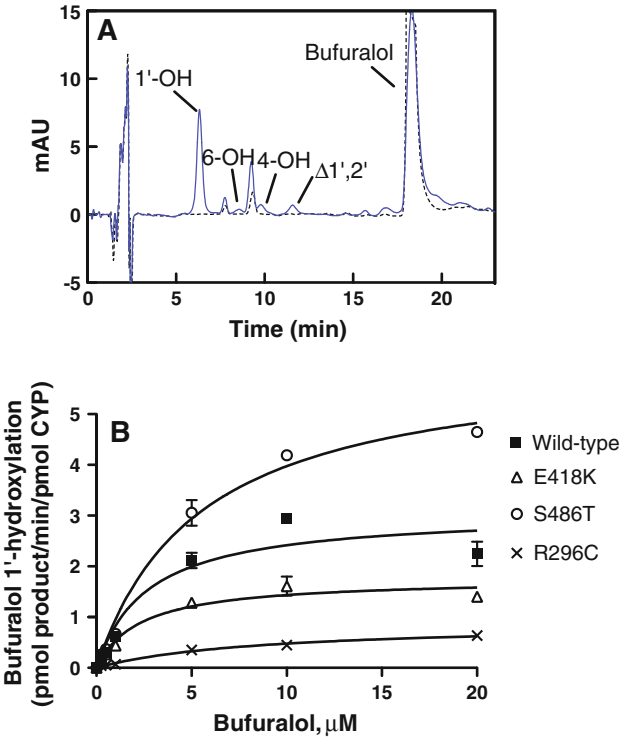

Fig. 3 Oxidation of bufuralol by CYP2D6. **a** HPLC chromatograms of bufuralol oxidation by CYP2D6. 1′-OH, 1′-hydroxybufuralol; 4-OH, 4-hydroxybufuralol; 6-OH, 6-hydroxybufuralol; Δ1′,2', Δ1′, 2′-bufuralol. The *dashed line* indicated the control experiment without NADPH generating system. **b** Steady-state kinetic analysis of bufuralol 1′-hydroxylation by CYP2D6 allelic variant enzymes. Each point presented is a mean ± SD (range) of duplicate assays

Catalytic activities of bufuralol metabolism were determined by measuring the rates of bufuralol 1′-hydroxylation. Steady-state kinetic parameters were determined for wild-type and three mutants (Fig. 3b; Table 1). A novel mutant, E418K, showed 32 % decrease in catalytic efficiency (k_{cat}/K_m) mainly due to the decrease of k_{cat} value (Fig. 3b; Table 1). S486T mutant exhibited increases in both k_{cat} and K_m values, resulting in no significant change in catalytic efficiency (Fig. 3b; Table 1). R296C showed much greater reduction in the catalytic efficiency (9 % of wild-type) due to both of a decrease of k_{cat} value and an increase of K_m value (Fig. 3b; Table 1).

Dextromethorphan metabolism by CYP2D6 variants

CYP2D6 catalyzes the *O*-demethylation reaction of dextromethorphan to produce dextrorphan (Yu and Haining 2001; Lee and Kim 2011) (Fig. 2b). In HPLC analysis, the metabolic products were observed at 14.3 min (dextrorphan), and 17.3 min (3-methoxymorphinan), which was assigned as dextrorphan using an authentic standard and based on the previous report (Yu and Haining 2001) (Fig. 4a). Turnover rate of dextromethorphan *O*-demethylation by CYP2D6 was relatively slower than bufuralol 1′-hydroxylation (Table 1). E418K showed both of a decrease of k_{cat} value and an increase K_m value to result in ~43 % reduction of catalytic efficiency (Table 1; Fig. 4b). S486T mutant showed a small decrease (~24 %) in the catalytic efficiency with small increases in both of k_{cat} and K_m values (Table 1; Fig. 4b). A highly decreased catalytic efficiency (~6 % of wild-type) was observed in the mutant of R296C mainly due to the dramatic change of k_{cat} value of dextromethorphan *O*-demethylation (Table 1; Fig. 4b).

Locations of the mutated amino acids in CYP2D6 variants

The X-ray crystal structure of a CYP2D6 (PDB-entry code, 2F9Q) was used to locate the positions of the mutated residues in the CYP2D6 enzyme (Rowland et al. 2006) (Fig. 5). The P34S substitution is located in a highly conserved proline-rich region at N-terminus of CYP2D6 enzyme (Fig. 5). The proline residue in this conserved region of CYP2D6 seems critical for the stability of protein and the mutation of this residue is likely to produce a structurally unstable enzyme. The E418K substitution seems to be located in K''-helix region and the S486T is found in β-4-strands of the fourth sheet region (Fig. 5). We also found that the R296C substitution is located in N-terminus of I-helix of CYP2D6 structure (Fig. 5).

Table 1 Kinetic parameters of 1′-hydroxylation of bufuralol and *O*-demethylation of dextromethorphan by purified wild-type and variants of CYP2D6

Mutations	1′-Hydroxylation of bufuralol			*O*-Demethylation of dextromethorphan		
	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m
WT	3.1 ± 0.3	2.7 ± 1.0	1.14	0.11 ± 0.01	16.1 ± 2.4	0.0068
E418K	1.8 ± 0.1	2.3 ± 0.6	0.78	0.08 ± 0.01	21.1 ± 4.4	0.0038
S486T	6.1 ± 0.3	5.5 ± 0.8	1.12	0.07 ± 0.01	12.6 ± 1.9	0.0056
R296C	0.9 ± 0.1	8.6 ± 1.4	0.10	0.008 ± 0.001	20.2 ± 11.0	0.0004

Results are presented as mean ± SE

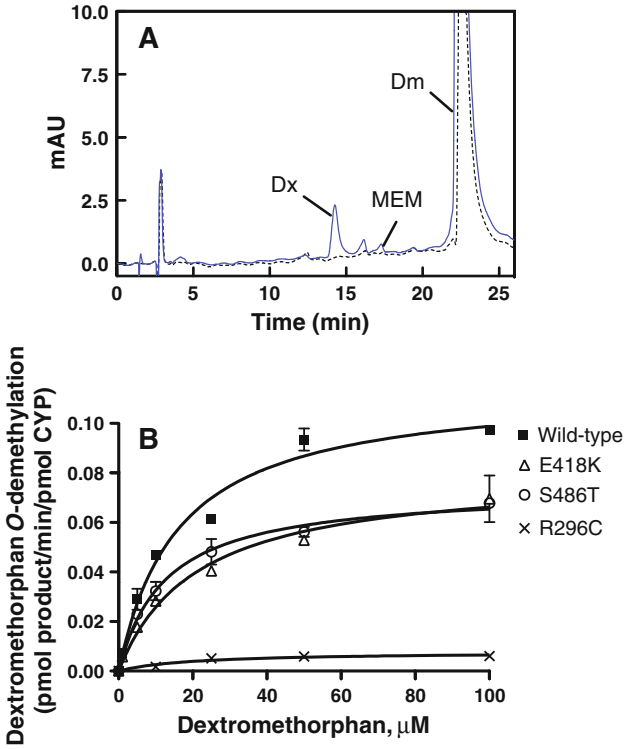

Fig. 4 Demethylation of dextromethorphan by CYP2D6. **a** HPLC chromatograms of dextromethorphan demethylation by CYP2D6. *Dm*, dextromethorphan; *Dx*, dextrorphan; *MEM*, 3-methoxymorphinan. The *dashed line* indicated the control experiment without NADPH generating system. **b** Steady-state kinetic analysis of *O*-demethylation of dextromethorphan by CYP2D6 allelic variant enzymes. Each point presented is a mean ± SD (range) of duplicate assays

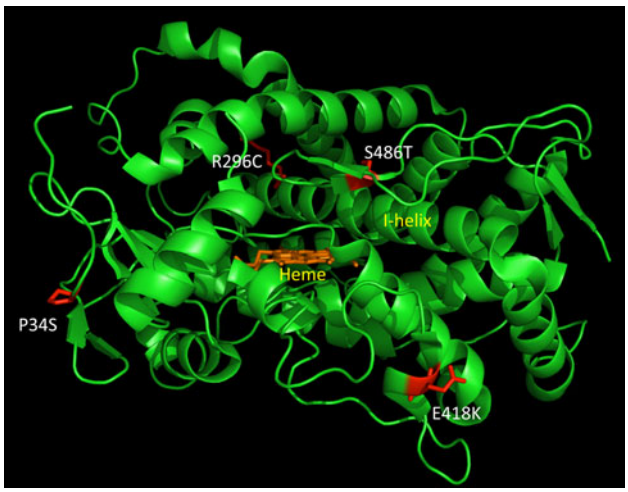

Fig. 5 Positions of the mutated amino acid residues in CYP2D6. The positions of the P34S, E418K, S486T, and R296C mutations are indicated with *colors* in the ribbon diagram of CYP2D6 X-ray crystal structure (PDB entry code: 2F9Q). Heme molecule is shown in *orange* and the mutated residues in *red*. (Color figure online)

Discussion

Previously, we studied the expression of CYP2D6 P34S, E418K, and P34S/E418K variants in COS-7 mammalian cells using CYP2D6 specific polyclonal antibodies (Lee et al. 2011). The expression levels of P34S and P34S/E418K variants showed 4–12 % of wild-type CYP2D6 (Lee et al. 2011). This study indicated that the substitution of P34S induces the very low level of protein production, either as a full-length protein or as proteolytic fragments and this instability of enzyme variant is not due to the expression system.

In the crystal structure of CYP2D6 (Rowland et al. 2006), the P34S substitution is found in a highly conserved region encoding the proline-rich N-terminus (Fig. 5). Structural perturbation of this region seemed to cause expression of an unstable gene product and reduction of enzymatic activity as previously proposed by Johansson et al. (1994). As a result, CYP2D6*52 allele including P34S mutation is likely to produce an unstable and non-functional protein regardless of two other substitutions. This P34S mutation was frequently found in various CYP2D6 alleles (CYP2D6*10, *14, *36, *47, *49, *54, *57, and *72) and the significant decreases of their in vivo activities were consistently reported in other studies (Sakuyama et al. 2008; Gaedigk et al. 2006; Matsunaga et al. 2009). Our current study indicates that the loss of functions in these alleles can be attributed to P34S mutation, therefore resulting in the production of non-functional enzyme.

The K''-helix region, where E418K substitution is located, is presumably expected to bind to the reductase partner (Rowland et al. 2006) (Fig. 5). Therefore, we can speculate that the substitution of the acidic residue to the basic induces the perturbation of electron transfer to CYP, therefore resulting in the decrease of enzymatic activity. The S486T seems to be located in β-4-strands of the fourth sheet region, but the change of serine to the alcohol containing threonine did not affect on the structural or functional basis of the mutant enzyme (Fig. 5). The R296C substitution in N-terminus of I-helix of CYP2D6 structure was found in more than thirty CYP2D6 allelic variants (http://www.cypalleles.ki.se/). Although many of residues in I-helix control the substrate specificity, the Arg296 seems not to interact with substrate directly due to the outward direction and the alienation from the binding site (Fig. 5). In our study, the yield of the final purified protein of R296C was more than five times lower than those of wild-type and the other mutants (data not shown). This result suggests R296C mutation may induce the unstable structure of CYP enzyme.

In conclusion, we have expressed four allelic variants of CYP2D6 and analyzed their catalytic activities. CYP2D6*52 containing the P34S substitution was observed to be inactive in the recombinant expression system that we used, and E418 K and R296C showed the

significant contribution of the reduced metabolic activities of CYP2D6. Due to the importance of CYP2D6 enzyme in the metabolism of many clinical drugs, the possible effects of these polymorphisms on the therapeutic efficacy and toxicity of the drugs should be carefully examined.

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