

## Functional Significance of Cytochrome P450 1A2 Allelic Variants, P450 1A2\*8, \*15, and \*16 (R456H, P42R, and R377Q)

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### Abstract

P450 1A2 is responsible for the metabolism of clinically important drugs and the metabolic activation of environmental chemicals. Genetic variations of P450 1A2 can influence its ability to perform these functions, and thus, this study aimed to characterize the functional significance of three P450 1A2 allelic variants containing nonsynonymous single nucleotide polymorphisms (P450 1A2\*8, R456H; \*15, P42R; \*16, R377Q). Variants containing these SNPs were constructed and the recombinant enzymes were expressed and purified in *Escherichia coli*. Only the P42R variant displayed the typical CO-binding spectrum indicating a P450 holoenzyme with an expression level of ~ 170 nmol per liter culture, but no P450 spectra were observed for the two other variants. Western blot analysis revealed that the level of expression for the P42R variant was lower than that of the wild type, however the expression of variants R456H and R377Q was not detected. Enzyme kinetic analyses indicated that the P42R mutation in P450 1A2 resulted in significant changes in catalytic activities. The P42R variant displayed an increased catalytic turnover numbers ( $k_{cat}$ ) in both of methoxyresorufin *O*-demethylation and phenacetin *O*-deethylation. In the case of phenacetin *O*-deethylation analysis, the overall catalytic efficiency ( $k_{cat}/K_m$ ) increased up to 2.5 fold with a slight increase of its  $K_m$  value. This study indicated that the substitution P42R in the N-terminal proline-rich region of P450 contributed to the improvement of catalytic activity albeit the reduction of P450 structural stability or the decrease of substrate affinity. Characterization of these polymorphisms should be carefully examined in terms of the metabolism of many clinical drugs and environmental chemicals.

**Key Words:** P450 1A2, Allelic variants, Polymorphism, Methoxyresorufin, Phenacetin

### INTRODUCTION

Human cytochrome P450 (P450, CYP) enzymes are responsible for the oxidative metabolism of xenobiotic chemicals including clinical drugs and environmental chemicals (Guengerich, 2003). P450 1A2 is the only member of the P450 1 family expressed exclusively in human liver and shows large inter-individual variation in activity (Kalow and Tang, 1991). It is the major enzyme involved in the bioactivation of arylamines and heterocyclic amines and metabolizes a variety of important clinical drugs, including clozapine, ropivacaine, olanzapine, theophylline, and terbinafine (Kim and Guengerich, 2005; Lee and Kim, 2011).

Genetic polymorphisms in drug-metabolizing enzymes can cause dramatic differences in the response to specific drugs and therefore the study of these polymorphisms is of particu-

lar importance to optimize drug therapy for maximum efficacy with minimal adverse effects (Han *et al.*, 2012). It was first described in the context of extensive metabolizers (EMs) and poor metabolizers (PMs) in the study of debrisoquinone hydroxylation by a P450 enzyme (Smith *et al.*, 1978). A single nucleotide polymorphism (SNP) of P450 is a DNA sequence variation in the P450 genes between individuals or paired chromosomes. Moreover, a nonsynonymous SNP is a genetic change in which each allele produces a different polypeptide sequence (Lee and Kim, 2011). To date, at least 19 nonsynonymous SNPs of P450 1A2 have been identified (<http://www.cypalleles.ki.se/>) and the functional effects of P450 1A2\*3, \*4, \*5, and \*6 were previously analyzed in our previous studies (Zhou *et al.*, 2004; Lee and Kim, 2011).

In this study, the functional activities of three novel P450 1A2 allelic variants (P450 1A2\*15, \*16, and \*8) containing

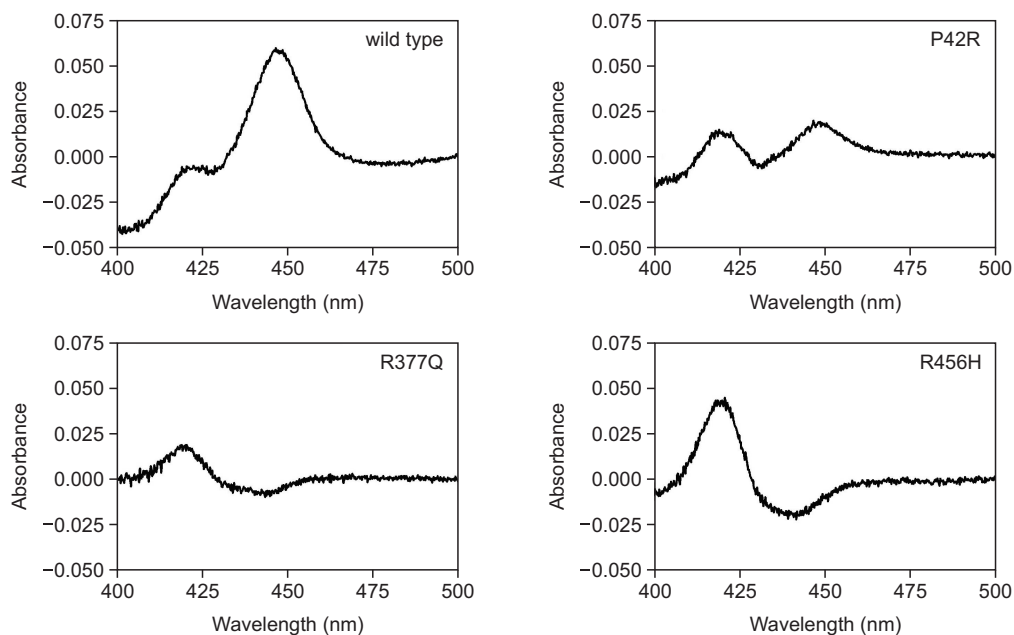
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**Fig. 1.** Expression of recombinant P450 1A2 allelic variants in *E. coli*. CO-binding spectra of P450 wild type and variant enzymes in *E. coli* whole cells were measured.

nonsynonymous SNPs were analyzed using the P450 bicistronic membrane system. These variants were found in Japanese populations at the frequencies of 0.2, 0.2, and 0.4% respectively (Soyama *et al.*, 2005). Given that P450 1A2 has an important role in the metabolic activation of many drugs and environmental chemicals, the prevalence and possible functional outcomes of these allelic mutations should be carefully examined.

## MATERIALS AND METHODS

### Chemicals

Methoxyresorufin, phenacetin, NADP<sup>+</sup>, and Glucose-6-phosphate were purchased from SigmaAldrich (St. Louis, MO, USA). *Escherichia coli* DH5 $\alpha$  cells were purchased from Invitrogen (Carlsbad, CA, USA). Other chemicals were of the highest commercially available grade.

### Construction of P450 1A2 mutants plasmids

The pBluescript plasmid containing of the coding sequence of P450 1A2 was used to construct the expression vectors for P450 1A2 mutants. The general approach has been previously described (Han *et al.*, 2009). Briefly, site-directed mutagenesis was conducted using a QuikChange mutagenesis kit (Stratagene, La Jolla, CA, USA) with the following primers: 5'-GGCAAGCACCGGTGTATC-3', 5'-GATACACCGGTGCTT-GCC-3' (P450 1A2 R456H), 5'-CTGAAAAGTCGACCAGAG-3', 5'-CTCTGGTCGACTTTTCAG-3' (P450 1A2 P42R), 5'-GAGACCTTCCAACACTCC-3', 5'-GGAGTGTTGGAAGGTCTC-3' (P450 1A2 R377Q). The fragments including P450 1A2 mutants in the constructed pBluescript plasmid were purified using the *Nde*I and *Xba*I restriction sites and cloned into pCW bicistronic vector containing NADPH-P450 reductase (NPR). The constructed plasmids were verified by nucleotide sequencing

analysis.

### Expression of recombinant P450 1A2 mutants and membrane preparation

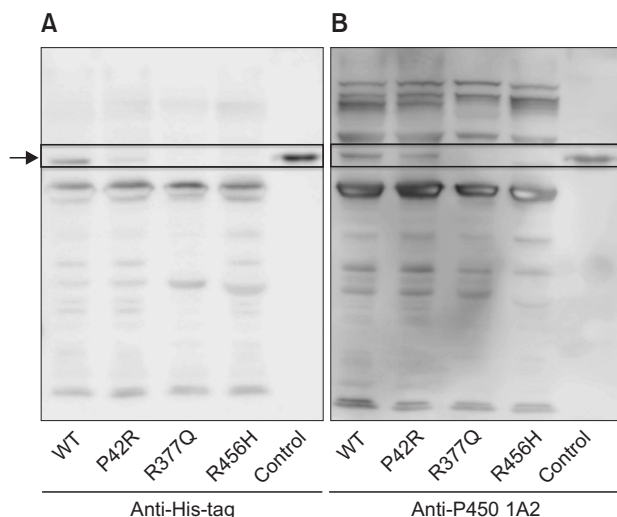
Expression of P450 1A2 wild type and mutant variants were carried out as previously described with some modifications (Choi *et al.*, 2013; Park *et al.*, 2014). The *E. coli* DH5 $\alpha$  strains transformed with pCW bicistronic vectors were inoculated into LB medium containing 50  $\mu$ g/mL ampicillin and pre-cultured overnight at 37°C. LB cultures were then seeded into 500 mL of Terrific broth (TB) expression medium containing 50  $\mu$ g/mL ampicillin. The expression cultures were grown at 37°C with shaking at 250 rpm for 4 h. After the supplements (0.5 mM 5-aminolevulinic acid, 1.0 mM isopropyl  $\beta$ -D-thiogalactoside (IPTG), 1.0 mM thiamine, and trace elements) were added, the expression cultures were further grown at 28°C with shaking at 200 rpm for 21 h. Bicistronic membrane fractions containing P450 1A2 were isolated and prepared from TB expression cultures, as described previously (Kim *et al.*, 2005).

### Western blotting analysis

Expression of P450 1A2 wild type and mutant variants was determined by western blot analysis of whole cell lysates. Cells were harvested and dissolved in sodium dodecyl sulfate (SDS) sample buffer. The cell lysates were boiled for 10 min and then separated on a 12% SDS-PAGE gel and transferred to a polyvinylidene difluoride (PVDF) membrane. Immunoblotting was performed using an anti-CYP1A2 polyclonal antibody and an anti-His-tag antibody (Cell Signaling, Danvers, MA, USA).

### Enzyme catalytic activity assays

Enzyme activities of P450 1A2 wild type and P42R variant were investigated for methoxyresorufin O-demethylation and phenacetin O-deethylation reactions. Reaction mixtures con-



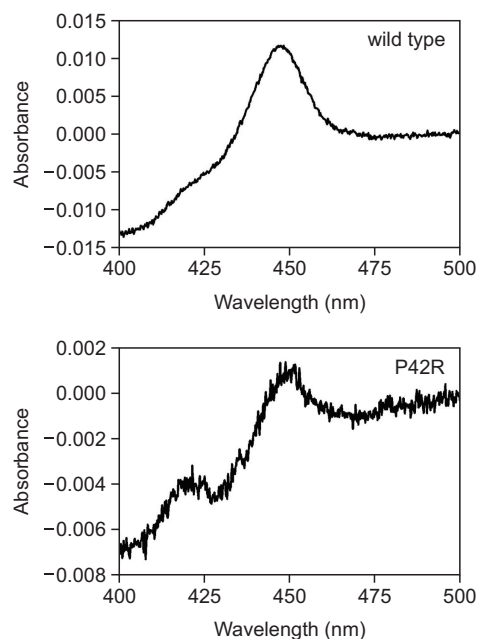
**Fig. 2.** Western blot analysis of P450 1A2 allelic variants. Protein (*E. coli* whole cells) loaded per lane and assessed with an anti-His-tag antibody (A) and an anti-CYP1A2 antibody (B). Lanes represent; P450 1A2 wild type, P42R, R377Q, R456H, and a positive control (purified P450 1A2 wild type enzyme).

sisted of 0.1  $\mu$ M P450 1A2 in bicistronic membranes (including NADPH-P450 reductase), 0.1 M potassium phosphate buffer (pH 7.4), an NADPH-generating system [0.5 mM NADP<sup>+</sup>, 10 mM glucose 6-phosphate, and 1.0 IU/mL glucose 6-phosphate dehydrogenase], and varying concentrations of substrates in a total volume of 0.50 mL. Incubations were terminated after 10 min at 37°C with the addition of 50  $\mu$ l of 2 M HCl and then the reaction mixtures were extracted with two volumes of ethyl acetate. For methoxyresorufin *O*-demethylation assays, the extracted products were transferred to fluorescence microtiter plates, and fluorescence was measured using an excitation wavelength of 544 nm and an emission wavelength of 590 nm. For phenacetin *O*-deethylation activity, the reaction products were analyzed using HPLC. The product (acetaminophen) was isolated from a C18 column (Alltech, Deerfield, IL, USA) with a mobile phase of 10 % (v/v) MeOH including 0.1% formic acid with a flow rate of 1.0 mL/min and monitored at 254 nm. Kinetic parameters were estimated using nonlinear regression analysis with Graph-Pad Prism software (Graph-Pad, San Diego, CA, USA).

## RESULTS

### Expression of recombinant P450 1A2 variants

All three P450 1A2 variants were successfully constructed in the pCW bicistronic vectors containing the NADPH-P450 reductase (NPR) gene. The recombinant P450 1A2 mutants and NPR proteins were expressed in *E. coli*. Expression levels of P450s were spectrally determined in the whole cell culture after 21 h of IPTG induction (Fig. 1). The expression levels of wild type P450 1A2 reached up to 670 nmol P450 per liter culture medium and the P42R variant (P450 1A2\*15) was found at ~170 nmol P450 holoenzyme per liter culture medium (Fig. 1). However, a P450 holoenzyme spectrum was not observed for R377Q and R456H variants (P450 1A2\*16 and \*8) (Fig. 1).



**Fig. 3.** CO-binding spectra of bicistronic membrane fractions. CO-binding spectra of the prepared bicistronic membrane fractions containing P450 enzymes (P450 1A2 wild type and P42R variant) and NADPH-P450 reductase were measured.

Western blot analysis using an anti-CYP1A2 polyclonal antibody and an anti-His-tag antibody revealed that the levels of expression of the P42R variant was lower than that of wild type P450 1A2 enzyme (Fig. 2). However, variants R377Q and R456H were not detectable (Fig. 2). It should be noted that because western blotting was performed on whole cell lysates, non-specific interactions were extensively observed.

### Preparation of bicistronic membranes containing P450 1A2 wild type and P42R mutant

Bicistronic membrane fractions containing the P450 1A2 P42R variant and NPR were successfully isolated and prepared. CO-binding spectral analysis showed that the wild type membrane fraction displayed 13  $\mu$ M P450 content, while the P42R mutant displayed around 2.2  $\mu$ M P450 content with some P420, indicating possible apoenzyme species (Fig. 3)

### Enzymatic activities of the P450 1A2 P42R variant

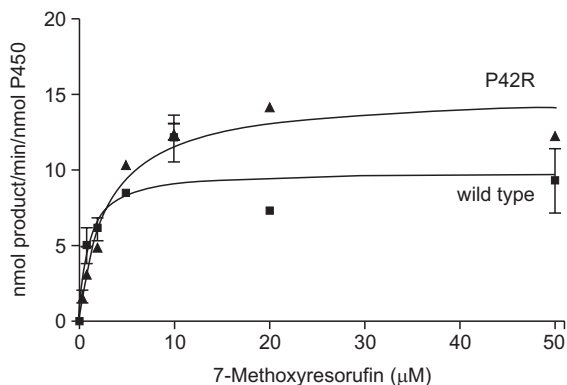
The catalytic activities of the P450 1A2 P42R variant enzyme were determined by measuring the rates of methoxyresorufin 7-*O*-demethylation (MROD) and phenacetin deethylation (PhOD). Steady-state kinetic analysis of MROD indicated that the P42R variant showed an increased catalytic turnover number ( $k_{cat}$ ) while the  $K_m$  value was also increased (Fig. 4). The catalytic turnover of the P42R variant was 150 % of those of the wild type with respect to the MROD reaction (Table 1). However, the overall catalytic efficiency ( $k_{cat}/K_m$ ) decreased for the P42R variant, mainly due to the increase in  $K_m$  value (Table 1).

P450 1A2 oxidizes phenacetin to produce acetaminophen as a major metabolite (Yun *et al.*, 2000). In HPLC analysis, the retention time of this deethylated metabolite was observed at 11.3 min (Fig. 5A). Steady-state kinetic analysis indicated that

the P42R variant exhibited an increase in  $k_{cat}$ , which resulted in a higher catalytic efficiency ( $k_{cat}/K_m$ ) in the PhOD reaction (Fig. 5B, Table 1). The overall catalytic efficiency ( $k_{cat}/K_m$ ) for PhOD increased up to 2.5-fold, with a slight increase of its  $K_m$  value (Table 1).

**Locations of the mutated residues in P450 1A2 variants**

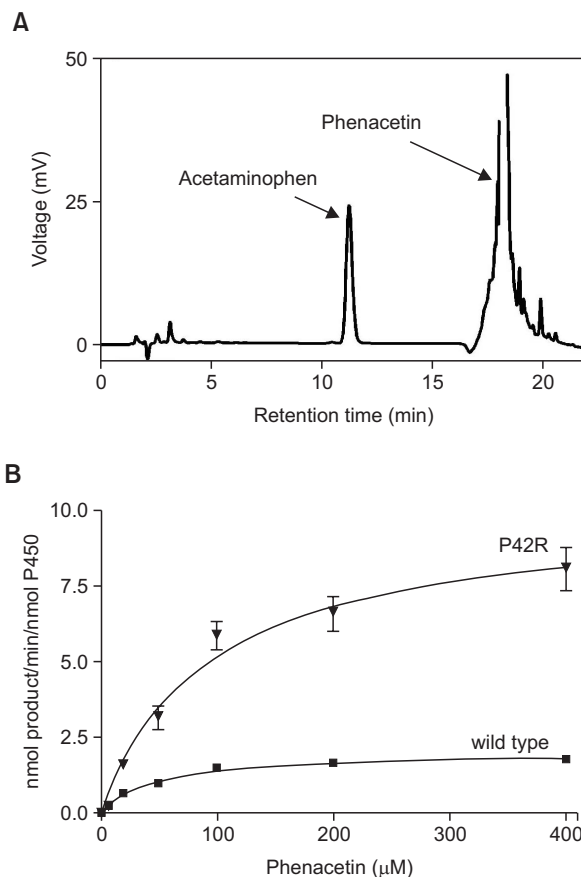
The X-ray crystal structure of P450 1A2 in complex with the inhibitor  $\alpha$ -naphthoflavone (PDB, entry code 2HI4) was used to locate the mutated residues in the structure of P450 1A2 (Fig. 6)(Sansen *et al.*, 2007). The R456H mutation in P450 1A2 (P450 1A2\*8) is found near the proximal Cys residue in the signature region (FXGXRXCXG) of the P450 enzyme. The Arg residue in this signature region has been well conserved throughout P450 enzymes and the mutation of this residue may interrupt the fundamental role of the proximal Cys residue to coordinate heme in the P450 enzyme, and therefore results in the structural instability of the P450 holoenzyme (Fig. 1). Arg377 of P450 1A2 is located in the K-helix (Fig. 6), and it seems to interact with the flexible loop region (Sansen *et al.*, 2007). The size of Arg377 in P450 1A2 may be critical for the stable folding of the P450, and therefore, substitution of Arg377 to Gln (P450 1A2\*16) could result in a nonfunctional apoenzyme. Pro42 is located in the highly conserved N-terminal proline-rich region (PPEPWGWPLLGH) of the P450 enzyme (Fig. 6). This region was proposed as a hydrophobic knob to be inserted into the membrane (Kemper, 2004). Therefore, substitution Pro42 to Arg (P450 1A2\*15) may reduce the proper folding of the enzyme and thereby result in the decrease of P450 holoenzyme expression.



**Fig. 4.** Steady-state kinetic analysis of methoxyresorufin 7-O-demethylation by P450 1A2 wild type and P42R variant. Each point represents the mean  $\pm$  SD (range) of duplicate assays. The steady-state kinetic parameters are shown in Table 1.

**DISCUSSION**

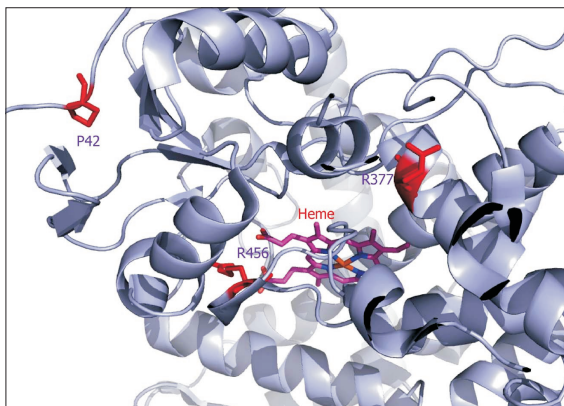
Human P450 1A2 enzyme has very diverse metabolic reactions including the oxidation of caffeine, 7-ethoxy- and 7-methoxyresorufin, and heterocyclic aromatic amines as well as many clinically important drugs (Lee and Kim, 2011). Polymorphisms in P450 1A2 have attracted the greatest interest given its preferred catalytic activity towards carcinogen bioactivation reactions, such as polycyclic aromatic hydrocarbon epoxidation and aromatic/heterocyclic amine *N*-hydroxylation (Kim and Guengerich, 2005). Previously, four allelic variants of P450 1A2 (D348W, I386F, C406Y, and R431W) were characterized in terms of its mutagenic activity for imidazoquino-



**Fig. 5.** Phenacetin *O*-deethylation by P450 1A2 wild type and P42R variant. (A) HPLC chromatogram of phenacetin *O*-deethylation reaction. Phenacetin and the product, acetaminophen are indicated. (B) Steady-state kinetic analysis of Phenacetin *O*-deethylation. Each point presented is a mean  $\pm$  SD of triplicate assays. The steady-state kinetic parameters are shown in Table 1.

**Table 1.** Steady state kinetic parameters of P450 1A2 wild type and P42R variant

P450 1A2 allele	Variation	Methoxyresorufin 7-O-demethylation			Phenacetin O-deethylation		
		$k_{cat}$ , min <sup>-1</sup>	$K_m$ , $\mu$ M	$k_{cat}/K_m$	$k_{cat}$ , min <sup>-1</sup>	$K_m$ , $\mu$ M	$k_{cat}/K_m$
P450 1A2*1	wild type	9.9 $\pm$ 0.9	1.0 $\pm$ 0.4	10.1	2.0 $\pm$ 0.1	46.2 $\pm$ 4.6	0.04
P450 1A2*15	P42R	15.0 $\pm$ 0.7	3.0 $\pm$ 0.5	5.0	10.1 $\pm$ 0.7	94.8 $\pm$ 18.7	0.11



**Fig. 6.** Locations of the mutated amino acid residues in P450 1A2 allelic variants. The three-dimensional locations of the P42R, R377Q, and R456H mutations are indicated with red colors in the ribbon diagram of P450 1A2 X-ray crystal structure (PDB entry code: 2HI4).

line mutagens (Zhou *et al.*, 2004). The R431W variant (P450 1A2\*6) was enzymatically inactive due to its structural instability, while the remaining three variants showed decreased bioactivation and metabolic activities as compared to the P450 1A2 wild type enzyme (Zhou *et al.*, 2004).

The initial characterization of P450 1A2\*8, \*15, and \*16 variants studied here was previously performed using Chinese hamster V79 cells (Saito *et al.*, 2005). This previous analysis reported that all three variants exhibited reduced protein expression levels and decreased 7-ethoxyresorufin *O*-deethylation activities as compared to those of the wild type enzyme, without any change in mRNA expression levels (Saito *et al.*, 2005). However, our study showed no 450 nm peak (indicating a functional P450 enzyme) for the R456H and R377Q variants as observed in CO-difference analysis, indicating that Arg377 and Arg456 are critical amino acids for the accurate folding of functional P450 1A2 holoenzymes. As previously mentioned in the structural analysis, these two residues are located in or near the signature sequences of the P450 enzyme (Fig. 6). The Arg456 residue is in the signature region of the P450 enzyme containing the proximal Cys and Arg377 is the invariant glutamic acid residue within the conserved EXXR motif in the K-helix in most P450s (Ravichandran *et al.*, 1993). A previous study indicated that EXXR is critical for correct P450 folding, and therefore, mutagenesis of Arg365 in CYP19A1 led to the production of an inactive and misfolded protein (Chen and Zhou, 1992). The expression analysis of the P42R variant clearly indicated that it was a functional P450 holoenzyme, although its expression level was lower than the P450 1A2 wild type enzyme (Fig. 1, 3). Moreover, the P42R variant enzyme activities were also altered in comparison to the wild type activities (Fig. 4, 5, Table 1). It should be noted that there was an inconsistency in the expression and catalytic activity for P42R with the previous report (Saito *et al.*, 2005), and our current speculation is that it can be attributed to the difference in the expression and assay system (whole cell culture versus bicistronic membrane system).

It is a very interesting finding that the P42R allelic variant possessing a nonsynonymous SNP displayed an increased catalytic turnover as compared to the wild type enzyme. Previ-

ously, the ultrarapid metabolizer (UM) was found in P450 2D6 but this UM phenotype was speculated to be due to a gene duplication, with up to 13 copies present in some individuals (Lundqvist *et al.*, 1999; Lee and Kim, 2011). Therefore, the P42R variant is unique in that the naturally found polymorphism induced the intrinsic improvement in P450 1A2 catalytic activity.

In conclusion, we constructed four allelic variants of P450 1A2 and examined their expression and functional alternations towards substrates 7-methoxyresorufin and phenacetin. One of these variants, P42R showed a reduced expression level but highly enhanced catalytic activities. The other two P450 1A2 variants contained polymorphisms that were detrimental to the P450 tertiary structure.

## ACKNOWLEDGMENTS

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