The effects of single nucleotide polymorphisms in CYP2A13 on metabolism of 5-methoxypsoralen

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List of nonstandard abbreviations: CYP, cytochrome P450; SNPs, single nucleotide polymorphisms; 5-MOP, 5-methoxypsoralen; 8-MOP, 8-methoxypsoralen.
Abstract

A number of studies have demonstrated that cytochrome P450 converts furanocoumarin derivatives into reactive molecules, which forms covalent bonds to biomolecules. 5-MOP is a natural furanocoumarin from apiaceous plants. In this study, we examined the effect on 5-methoxypsoralen (5-MOP) metabolism of single nucleotide polymorphisms (SNPs) in CYP2A13. We used E. coli-generated recombinant enzymes of wild type CYP2A13*1 and five variants, CYP2A13*4 (R101Q), CYP2A13*5 (F453Y), CYP2A13*6 (R494C), CYP2A13*8 (D158E) and CYP2A13*9 (V323L). In HPLC analyses of 5-MOP metabolic products, CYP2A13*1 converted 5-MOP into 5-MOP dihydrodiol; $K_m$ and $V_{max}$ values of the reaction were 1.44 ± 0.17 µM and 4.23 ± 0.36 nmol/min/nmol P450, respectively. The generation of a dihydrodiol from 5-MOP implies that conversion by CYP2A13 causes toxicity due to the formation of covalent bonds with DNA or proteins. Most of the CYP2A13 variants could metabolize 5-MOP; $K_m$ values for CYP2A13*5, *6, *8 and *9 were 1.63 ± 0.12, 1.36 ± 0.10, 0.85 ± 0.09 and 0.58 ± 0.06 µM, respectively, and $V_{max}$ values were 3.20 ± 0.13, 4.69 ± 0.13, 2.34 ± 0.07 and 1.84 ± 0.09 nmol/min/nmol P450, respectively. The processing of 5-MOP by CYP2A13*4, however, was not detectable. Based on this data, we hypothesize that SNPs within the CYP2A13 gene affect metabolism of 5-MOP in humans.
Introduction

Cytochrome P450 (P450) catalyzed monooxygenation at the first step in metabolism of xenobiotic compounds. In some cases, the catalysis by P450 can generate reactive intermediates that are harmful for human health. Several dietary constituents are converted into toxic reactive metabolites in humans (Zhou et al., 2007). Alkenylbenzens, such as estragole, safrole and methyleugenol, become genotoxic and carcinogenic through mechanisms initiated by P450 proteins (Jeurissen et al., 2007; Jeurissen et al., 2006; Jeurissen et al., 2004). Also, certain metabolic products of capsaicin may be harmful when covalently bonded with biomolecules (Reilly and Yost, 2006). While these studies show potential risks of dietary constituents caused by normal metabolism in human, the risks in foods are generally not investigated as often as the hazards in drugs.

Many beverages and oils derived from fruits and vegetables contain furanocoumarin derivatives. It is well known that hepatic cytochrome P450 2A6 (CYP2A6) is involved in production of reactive intermediates from furanocoumarins. Koenigs and Trager demonstrated that five furanocoumarins, 5-methoxypsoralen (5-MOP), 8-methoxypsoralen (8-MOP), psoralen, 5-hydroxypsoralen and 8-hydroxypsoralen, were converted into reactive intermediates (Koenigs and Trager, 1998). They also showed that efficiency of 8-MOP for the metabolic activation was higher than those of the other derivatives. Cytochrome P450 2A13 (CYP2A13) is 94% identical to CYP2A6 in deduced amino acid sequences and mainly expressed in human respiratory tissues (Zhu et al., 2006; Su et al., 2000; Gu et al., 2000; Keskela et al., 1999). CYP2A13 is involved in the metabolism of coumarin, nicotine, 4-(methylnitrosamo)-1-(3-pyridyl)-1-butanones (NNK), 4-aminobiphenyl, phenacetin, theophylline and aflatoxin B1 (AFB1) (Fukami et al., 2007; Nakajima et al., 2006; He et al., 2006; Bao et al., 2005; von Weymarn and Murphy, 2003; Su et al., 2000). Substrate selectivity of CYP2A13 overlaps that of CYP2A6, for example coumarin, nicotine and NNK are
common substrate of these enzymes. Like CYP2A6, a furanocoumarin derivative 8-MOP was indicated to be converted into metabolic-activated intermediates by CYP2A13 (von Weymarn et al., 2005; Koenigs et al., 1997). Although these studies imply that CYP2A13 is likely to be involved in the metabolism of 5-MOP, there is no article to indentify metabolites or metabolic activity.

Previous studies show that the metabolic activity of P450 enzyme is influenced by single nucleotide polymorphisms (SNPs). Nine kinds of SNPs are known in the CYP2A13 gene, and some alleles show regional differences; the CYP2A13*4 allele is more frequent in French Caucasians (3.8%) than in the Japanese population (0.3%), while the CYP2A13*8 allelic frequency in French Caucasians (1%) is lower than in Japanese (4.9%) and Chinese populations (1.8%) (Wang et al., 2006). Some of the mutations are found in highly-conserved region in P450 enzymes. Arginine 101, which is substituted in the CYP2A13*4, is positioned in putative substrate recognition site 1 and conserved in all human cytochrome P450 subfamily 1 and 2. Valine 323 that is conserved in most xenobiotic P450s (CYP1A1, CYP2A6, 2B6, 2C8/9/18/19, 2D6 and 3A4/5) are substituted in CYP2A13*9 allele. These substitutions can affect substrate specificity or metabolic turnover of CYP2A13 enzyme. However, functional differences in the SNP variants of CYP2A13 are limited; the studies using recombinant proteins expressed in mammalian cell cultures showed that the CYP2A13*4 protein has decreased metabolic activity on NNK and AFB1, and that the CYP2A13*2 protein is less efficient in metabolic activation of NNK than wild type CYP2A13*1 (Wang et al., 2006; Wang et al., 2003; Zhang et al., 2002).

In order to reveal the effects of SNPs within human CYP2A13 on metabolism of 5-MOP, we used recombinant CYP2A13 enzyme variants overexpressed in Escherichia coli. We demonstrate that the CYP2A13 enzyme is involved in the conversion of 5-MOP into a dihydrodiol form, and that the enzymatic activity of the recombinant CYP2A13*4 enzyme is
remarkably decreased. These results show that SNPs within CYP2A13 may affect the pharmacokinetics of 5-MOP.


Materials and Methods

Chemicals

5-Methoxypsoralen (5-MOP) was purchased from Sigma-Aldrich Co. (St. Louis, MO). 7-hydroxycoumarin (7-HC) was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). 5-aminolevulinic acid hydrochloride, isopropyl β-D-thiogalactopyranoside, and glucose 6-phosphate were purchased from Nakalai Tesque Inc. (Kyoto, Japan). Glucose 6-phosphate dehydrogenase was purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). Other chemicals were commercially available.

Preparation of CYP2A13 variants representing single nucleotide polymorphisms

Full-length cDNA fragments of CYP2A13 gene were amplified by polymerase chain reaction methods from a human liver cDNA library by using gene specific primers. To generate proteins in an E. coli system, the N-terminal transmembrane sequences (Δ2-23) were deleted and several amino acid residue substitutions (\(^{24}\text{WRQRKS}^{30}\) to \(^{24}\text{AKKTSS}^{30}\)) were made in the fragments (Smith et al., 2007). Each amplified fragment was inserted into the NdeI and SalI sites of the pT7Blue vector (Takara Bio Inc., Otsu, Japan). Single nucleotide mutations representing polymorphisms of the CYP2A13 gene were introduced into the fragment using the QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA). Sequences of oligonucleotide primers for mutagenesis were as follows: CYP2A13QC4F, 5’- AGTTCAAGGGCGCTGAGTCGACCT-3’, and CYP2A13QC4R, 5’- GCCTGCTCGCCTGCCCTGGCTGAACT-3’, for the allele CYP2A13*4; CYP2A13QC5F, 5’- GGAGCTTTTCTCTGACCCATTACATGCAGA-3’, and CYP2A13QC5R, 5’- TCTGCAATGTGGGTAGAAGAGAAAGAGCTCC-3’, for the allele CYP2A13*5; CYP2A13QC6F, 5’- GAGCTCCTGCCCTGTGAGTCGACCT-3’, and CYP2A13QC6R, 5’-
AGGTCGACTCAGCAGGGCAGGAAGCTC-3', for the allele CYP2A13*6; CYP2A13QC8F, 5'-GGGCTTCCTCCTAGAGCCCTCC-3', and CYP2A13QC8R, 5'-CGGAGGGCCTCGATGAGGAAGCCC-3', for the allele CYP2A13*8; and CYP2A13QC9F, 5'-TGAAGCACCAGTTGGAGCCAAGGTC-3', and CYP2A13QC9R, 5'-GACCTTGCCCTCAACTCTGGTGCTTCA-3', for the allele CYP2A13*9. The substitutions were confirmed by DNA sequence analyses. Coding regions were cloned into the NdeI and SalI sites of pCWR, the vector for co-expression of the P450 and P450 reductase genes (Iwata et al., 1998). Resulting plasmids were introduced into E. coli JM109. The transformants were grown in TB broth (1.2% tryptone, 2.4% yeast extract, 20% glycerol) with 50 mg/l ampicillin until OD_{600} values of cultures reached 0.2–0.3. Cultures were shaken at 180 rpm at 25°C for 20 h with 0.5 mM 5-aminolevulinic acid hydrochrolide and 1 mM isopropyl β-D-thiogalactopyranoside to induce the expression of the recombinant CYP2A13 enzymes. Cells were collected by centrifugation at 5000g for 15 min at 4 °C, suspended in 100 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA and 20% glycerol, and sonicated. The resulting mixture was centrifuged at 5000g for 30 min at 4 °C to obtain a supernatant fraction. The supernatant was supercentrifuged at 100,000g for 60 min at 4 °C to sediment the homogenized membrane fraction of the E. coli cells. The pellets were resuspended in 6 ml of 100 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA and 20% glycerol. These fractions were stored at –80 °C.

Validation of P450 activity and protein concentration

Protein concentration in the membrane fraction was determined by Bradford’s method using the ProteinAssay Kit (BioRad, CA). A standard curve was constructed from diluted bovine serum albumin (0.2–1.0 mg/ml). Reduced CO difference spectra were measured with a Hitachi UV-visible spectrophotometer U-3300 (Hitachi, Japan), according to the protocol.
published by Imaishi and coworkers (2000). The P450 hemoprotein contents in the membrane suspensions were determined with an extinction coefficient of 91.1 mM\(^{-1}\) cm\(^{-1}\) (Omura and Sato, 1964).

**Western blot analyses**

Production of recombinant CYP2A13 proteins in *E. coli* was detected by immunoblotting with a polyclonal antibody against CYP2A13 (AVIVA Systems Biology LLC, San Diego, CA) diluted 600-fold in TBS-T. The membrane suspensions from *E. coli* containing 0.5 µg of total protein were separated by electrophoresis (10% polyacrylamide gel) and transferred to PVDF membranes by using an iBLot apparatus (Invitrogen Co., Carlsbad, CA). A donkey anti-rabbit IgG conjugated to horseradish peroxidase was used as a secondary antibody (1:10,000). Binding of these antibodies to the PVDF membrane was performed using the SNAP i.d. System (Millipore, Billerica, MA). The immunoblot was visualized with an ECL Detection System (GE Healthcare UK Ltd., Buckinghamshire, England), according to the manufacturer’s protocol.

**Metabolic assessment of 5-methoxypsoralen and 8-methoxypsoralen**

The reaction mixture was composed of 100 mM potassium phosphate buffer (pH 7.4), 1 mM EDTA, 20% glycerol, 5 mM glucose 6-phosphate, 1 mM NADPH, and 1 unit/ml glucose 6-phosphate dehydrogenase. In all experiments, the membrane suspensions containing approximately 100 µg of protein were added to 1 ml of the reaction mixture. Concentrations of the CYP2A13 variant enzyme in each reaction mixture were estimated as described above (CYP2A13*1, 65.9 pmol/ml; CYP2A13*4, N.D.; CYP2A13*5, 89.4 pmol/ml; CYP2A13*6, 45.1 pmol/ml; CYP2A13*8, 68.6 pmol/ml; CYP2A13*9, 52.7 pmol/ml). In order to acquire time course of 5-MOP dihydrodiol production, 10 µL of 5-MOP solution with
Dimethylsulfoxide (DMSO) was added to the mixtures to reach final concentrations of 5 µM 5-MOP. The reaction mixtures were incubated at 37°C with shaking at 90 rpm for 0, 5, 15, 30 and 60 min. For measurement of the production of 5-MOP dihydrodiol versus initial concentration of 5-MOP, 10 µL of 5-MOP solution with DMSO was added to the mixtures to reach final concentrations of 0.5, 0.75, 1, 2, 10, and 50 µM 5-MOP. Experiments with 8-MOP were performed at a final concentration of 10 µM. The reaction mixtures were incubated at 37°C with shaking at 90 rpm for 5 min. Enzymatic reactions of CYP2A13 variants were stopped by addition of 250 µl of 1 N HCl and 3 ml of ethyl acetate to the reaction mixtures, followed by 10 µL of 0.5 mM 7-hydroxycoumarin (7-HC) as an internal control. The spectrum of 7-HC was nearly identical to that of 5-MOP dihydrodiol (Supplemental data 1). The organic layers were dried by evaporation and the residue was dissolved in 150 µL of methanol. Insoluble matter was removed with a Millex®-LH filter (Millipore), before proceeding with HPLC analysis.

**HPLC analysis**

Twenty microliters of extract was analyzed in an HPLC system (HITACHI High-Technologies Co., Tokyo, Japan) equipped with a TSK-gel® C18 column ODS-80Ts (150 x 4.6 mm) (Tosoh Co., Tokyo, Japan). The mobility phase was changed with a linear gradient from 37.5 to 100% methanol over 15 min and absorbance of metabolites were monitored at 324 nm. The metabolic product was quantified by its ratio to 7-HC.

**Preparation of 5-MOP dihydrodiol standard**

5-MOP dihydrodiol standard was isolated by thin-layer chromatography from metabolites of 5-MOP by human CYP2A13 recombinant proteins. The structure of 5-MOP
Dihydrodiol was determined by liquid chromatography-mass spectrometry (LC-MS) and nuclear magnetic resonance (NMR) analyses.
Results

Expression of human CYP2A13 and its variants

We constructed bacterial expression vectors encoding CYP2A13*1 (wild type) and five SNP variants (CYP2A13*4 (R101Q), CYP2A13*5 (F453Y), CYP2A13*6 (R494C), CYP2A13*8 (D158E) and CYP2A13*9 (V323L)) (Figure 1A). The N-terminal region of each construct was modified to yield recombinant proteins from the *E. coli* expression system (Figure 1A, *italic* letters). Substituted residues in each SNP variant are underlined in Figure 1A and labeled on a structural model of the human CYP2A13 enzyme in Figure 1B. We prepared membrane fractions from *E. coli* transformed with each SNP variant of CYP2A13, and analyzed the membrane fractions by SDS-PAGE and western blot. Major bands were present at the estimated molecular weight of CYP2A13 (54 kDa), and a specific antibody also recognized these bands (Figure 2A, arrowhead). Bands with the same mobility as CYP2A13*1 were present in the membrane fractions of *E. coli* transformed by four variants (CYP2A13*5, CYP2A13*6, CYP2A13*8 and CYP2A13*9). Electrophoretic mobility of the recombinant CYP2A13*4 protein was slightly lower than other recombinant CYP2A13 proteins, but this protein was recognized by the anti-CYP2A13 antibody. These results suggest that protein moieties of the recombinant CYP2A13 enzymes were successfully synthesized in our *E. coli* expression system.

After establishing the validity of our expression system, we measured reduced CO difference spectra from membrane fractions of CYP2A13 variants (Figure 2B). The wavelength showing maximum increase of absorption after CO treatment ($\lambda_{\text{max}}$) for the CYP2A13*1 enzyme was 450.8 nm, indicating the presence of functional cytochrome P450 enzyme. Reduced CO difference spectrum analyses also show the $\lambda_{\text{max}}$ values for CYP2A13*5, CYP2A13*6, CYP2A13*8, and CYP2A13*9 variants are 449.5 nm, 450.6 nm, 449.9 nm and 450.2 nm respectively (Figure 2B). The expression levels of CYP2A13*1,
CYP2A13*5, CYP2A13*6, CYP2A13*8 and CYP2A13*9 were 1.3 ± 0.53, 1.1 ± 0.49, 1.0 ± 0.49, 1.2 ± 0.50 and 0.79 ± 0.34 nmol/mg total proteins (mean ± standard deviation), respectively. Conversely, the reduced CO difference spectrum of CYP2A13*4 shows that absorption did not increase around 450 nm but that the peak position is 420 nm (Figure 2B). We estimate from SDS-PAGE analyses that the expression of CYP2A13*4 was about 5% of the total protein, which is similar to the other variants.

Metabolism of 5-MOP by human CYP2A13

We performed HPLC analyses to validate the creation of 5-MOP metabolites by CYP2A13. Retention times of 7-hydroxycoumarin and of 5-MOP were 6.3 and 11.3 min, respectively (Figure 3). A peak with a retention time of 5.2 min appeared in the metabolic assay in membrane fractions that included CYP2A13*1 enzymes (Figure 3A). In the absence of NADPH, the peak disappeared from the HPLC chromatogram (Figure 3B), and there was no peak at 5.2 min in the HPLC chromatogram using membrane fractions from *E. coli* transformed with an empty vector (Figure 3C). Therefore, the peak with a retention time of 5.2 min was a main metabolic product of 5-MOP that is generated by CYP2A13 enzymes. We isolated the peak with a retention time of 5.2 min and identified the peak as 5-MOP dihydrodiol by LC-MS and NMR structural analyses (data not shown). Koenigs and Trager proposed that 5-MOP dihydrodilol is a product of 5-MOP oxidation by CYP2A6 (Koenigs and Trager, 1998). We also added CYP2A13*1 enzymes to 8-MOP and analyzed the resulting metabolites of 8-MOP by HPLC. No peaks that signify a metabolite of 8-MOP were detectable, leading us to the hypothesis that the metabolic activity of CYP2A13 on 5-MOP may be higher than that on 8-MOP (Figure 3D). 5-MOP dihydrodiol was also produced in the experiments with the variants CYP2A13*5, CYP2A13*6, CYP2A13*8, and CYP2A13*9 (Figure 3F–I). In metabolic experiments with CYP2A13*4, there were no chromatogram
peaks that could be ascribed to 5-MOP dihydrodiol or other metabolites, which demonstrates that the CYP2A13*4 protein has no ability to metabolize 5-MOP (Figure 3E).

We next determined the kinetic parameters of 5-MOP metabolism by CYP2A13 variant enzymes. The reactions with CYP2A13*1, CYP2A13*5, CYP2A13*6, CYP2A13*8, and CYP2A13*9 enzymes followed Michaelis-Menten kinetics (Figure 4). As shown in Table 1, $K_m$ and $V_{\text{max}}$ of CYP2A13*1 enzymes were $1.44 \pm 0.17 \mu\text{M}$ and $4.23 \pm 0.36 \text{nmol/min/nmol P450}$, respectively. The kinetic parameters of CYP2A13*6 ($K_m = 1.36 \pm 0.10 \mu\text{M}$, $V_{\text{max}} = 4.69 \pm 0.13 \text{nmol/min/nmol P450}$) were almost the same as those for CYP2A13*1. The CYP2A13*5 variant had a similar $K_m$ value ($1.63 \pm 0.12 \mu\text{M}$) to that of CYP2A13*1, and a $V_{\text{max}}$ value ($3.20 \pm 0.13 \text{nmol/min/nmol P450}$) that was 25% less than the $V_{\text{max}}$ value of CYP2A13*1. $K_m$ values of CYP2A13*8 ($0.85 \pm 0.09 \mu\text{M}$) and CYP2A13*9 ($0.58 \pm 0.06 \mu\text{M}$) were lower than that of CYP2A13*1; these variants also had lower $V_{\text{max}}$ values ($2.34 \pm 0.07$ and $1.84 \pm 0.09 \text{nmol/min/nmol P450}$, respectively) than did CYP2A13*1. From these data, the catalytic efficiency for 5-MOP metabolism ($V_{\text{max}}/K_m$) of CYP2A13*1, CYP2A13*5, CYP2A13*6, CYP2A13*8, and CYP2A13*9 was $2.98 \pm 0.17$, $1.99 \pm 0.13$, $3.47 \pm 0.17$, $2.81 \pm 0.21$, and $3.22 \pm 0.23$, respectively. The maximum $V_{\text{max}}/K_m$ was less than twice the minimum. These results show that the catalytic efficiency of CYP2A13*5, CYP2A13*6, CYP2A13*8, and CYP2A13*9 do not greatly differ from that of CYP2A13*1; however, CYP2A13*4 was unable to produce 5-MOP dihydrodiol, so the kinetic parameters for this variant cannot be determined (Figure 4 and Table 1). We conclude that the CYP2A13*4 recombinant protein is the product of a loss-of-function allele.
Discussion

We used heterologously-expressed enzymes in *E. coli* to investigate the effects of CYP2A13 polymorphisms on 5-MOP metabolism. There are several advantages to expressing cytochrome P450 enzymes in *E. coli* for analysis of their function and structure. In general, bacterial expression is more convenient and the yield is higher than in eukaryotic expression systems. Furthermore, because the *E. coli* genome contains no cytochrome P450 genes, the cytochrome P450 of interest can be analyzed without background activity. Despite these advantages, there is the possibility that the system may not be functional. The characteristics of the recombinant *E. coli* cytochrome P450 proteins need to be the same as native cytochrome P450 proteins, because modification of the membrane-binding region at the N-terminus is essential for expression of the human P450 gene in *E. coli* (Yun et al., 2006). In our experiments, CYP2A13*4 enzymes were drastically inactivated, while other recombinant CYP2A13 enzymes metabolized 5-MOP. Wang and coworkers performed the *in vitro* expression of CYP2A13 variants in mammalian CHO cells and insect sf9 cells (Wang et al., 2006). They proposed that the substitution in CYP2A13*4 led to a nonfunctional and an unstable protein, which were consistent with our study (Figures 3 and 4). All of the N-terminal-modified CYP2A13 variants, including CYP2A13*4, were useful for analyzing the corresponding native proteins.

We examined expression of the recombinant CYP2A13 enzymes by Western blot assay. The CYP2A13*4 recombinant enzyme was expressed at levels equal to those of wild type and other variants. The amino acid substitution in CYP2A13*4, R101Q, occurs at a distance of less than 3Å from the heme-activation center, and this substitution seems to influence stability and lead to a remarkable decrease in activity (Smith et al., 2007) (Figure 1B). Surprisingly, though the CYP2A13*4 variant differs from the wild type in only one residue, its electrophoretic mobility was lower. No other examples of a difference in electrophoretic
mobility among SNPs of CYP2A13 have been reported in previous studies. Thus, this study clarifies some important characteristics of the CYP2A13*4 enzyme, including its low electrophoretic mobility, non-functional reduced CO difference spectrum, and loss of ability to metabolize 5-MOP. The relationship between the low electrophoretic mobility and the functional failure of the CYP2A13*4 enzyme is still unknown, and remains a question to be examined in future work.

This study demonstrated for the first time that CYP2A13 is involved in conversion of 5-MOP into its dihydrodiol form. Natural furanocoumarins, 5-MOP and 8-MOP, are useful food constituents, which are applied to skin or taken orally in combination with UV irradiation for therapy of some skin diseases (e.g. dermatoses, psoriasis and vitiligo) (Tzaneva et al., 2009; Wackernagel et al., 2006; McNeely and Goa, 1998). According to the previously postulated metabolic pathway of 5-MOP in humans, formation of 5-MOP dihydrodiol, which was identified as metabolite of 5-MOP in this study, is the result of two reactions: 1) oxidation of 5-methoxypsoralen to epoxide by CYP2A13 and 2) hydrolysis of epoxide to dihydrodiol either spontaneously or by epoxide hydrolase (John et al., 1992) (Figure 5). Similarly, Koenigs and Trager found that generation of the furanoepoxide and $\gamma$-ketoenal species of 5-MOP, which are capable of forming covalent adducts with proteins and DNA, was mediated by CYP2A6 (Koenigs and Trager, 1998). They also postulated that the binding of these species to CYP2A6 apoprotein caused the inhibition of the CYP2A6 function. Although the furanoepoxide and $\gamma$-ketoenal species of 5-MOP were not directly detected in this study, these intermediates were probably generated during 5-MOP metabolism by CYP2A13 and were hydrolyzed to dihydrodiol spontaneously. The tissue distribution of CYP2A13 in vivo and its metabolic activity led us to the hypothesis that the human CYP2A13 is a major player in the production of mutagenic intermediates from NNK (Zhang et al., 2007; He et al., 2004; Jalas et al., 2003). Our findings indicate the possibility that reactive intermediates from 5-MOP are
produced in respiratory organs by CYP2A13, as well as in liver by other P450s. As 8-MOP are expected to suppress production of carcinogens from NNK (von Weymarn et al., 2005; Sellers et al., 2003; Koenigs et al., 1997), 5-MOP has an effect to prevent activation of procarcinogens.

In conclusion, this study shows that one base mutation in CYP2A13 gene has a role in metabolism of 5-methoxypsoralen. Most substitutions of amino acid simulating SNPs on CYP2A13 enzyme have minor effect, but the alteration at position 101 from arginine to glutamine, simulating CYP2A13*4, inactivates the function of CYP2A13 enzyme. This fact suggested that same tendency among CYP2A13 variants will be also observed in metabolisms of other compounds.
Acknowledgments

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References


of methoxsalen on nicotine and 4-(methylnitrosamo)-1-(3-pyridyl)-1-butanone (NNK) metabolism in vivo. *Nicotine Tob Res* 5:891-899


Footnotes

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Figures Legends

Figure 1. (A) Amino acid sequence alignment of CYP2A13 variants. Modified residues at N-termini are in *italics* and substituted nucleotides are underlined. (B) Positions of the amino acid substitutions performed in this study are displayed on wild type CYP2A13. The basic structure was determined by Smith and coworkers (Smith et al., 2007). Grey sticks, heme; black sticks, side chains of the residue substituted.

Figure 2. Heterologous expression of human CYP2A13 variants representing polymorphisms in *E. coli*. (A) SDS-PAGE (left) and western blot (right) analyses of membrane preparation from transformed *E. coli* cells. The recombinant proteins of CYP2A13 polymorphisms were successfully expressed. The contents of each lane are as follows: lane 1, non-expressed control using the empty pCW vector; lane 2, CYP2A13*1; lane 3, CYP2A13*4; lane 4, CYP2A13*5; lane 5, CYP2A13*6; lane 6, CYP2A13*8; lane 7, CYP2A13*9. (B) Reduced CO difference spectra of membrane preparations. An increase in the absorbance near 450 nm was not observed in the spectrum of CYP2A13*4.

Figure 3. Typical HPLC chromatograms of the membrane fractions of *E. coli* obtained from 5-MOP (A–C, E–H) or 8-MOP (D) metabolic experiments. The retention time of 5-MOP dihydrodiol, 7-hydroxycoumarin, 8-MOP, and 5-MOP were 5.2, 6.3, 10.4, and 11.3 min, respectively. Metabolic reactions were performed for 5 min and the 324 nm absorptions were monitored. 5-MOP (A–C, E–H) or 8-MOP (D) metabolites by membrane fraction from (A) CYP2A13*1, (B) CYP2A13*1 in the absence of NADPH, (C) pCW, (D) CYP2A13*1, (E) CYP2A13*4, (F) CYP2A13*5, (G) CYP2A13*6, (H) CYP2A13*8 and (I) CYP2A13*9.
Figure 4. Productions of 5-MOP dihydrodiol mediated by CYP2A13 variants versus reaction time (A) and initial concentration of 5-MOP (B). Open circle, CYP2A13*1 (65.9 pmol/ml); open square, CYP2A13*4 (N.D.); open triangle, CYP2A13*5, (89.4 pmol/ml); closed circle, CYP2A13*6 (45.1 pmol/ml); closed square, CYP2A13*8 (68.6 pmol/ml); closed triangle, CYP2A13*9 (52.7 pmol/ml). The concentrations of CYP2A13 enzyme in the reaction mixture, quantified as shown in the Material and Methods section, are written in the parentheses. Standard deviations are represented by vertical bars on the symbols. (C) Lineweaver-Burk plot of the kinetic data. Meanings of symbols with vertical bars are same as in panel A and B.

Figure 5. Involvement of the CYP2A13 enzyme in the biotransformation of 5-MOP. The schematic of 5-MOP metabolism in humans is drawn in reference to the study by John and coworkers (1992). The hypothesized reaction pathway involving CYP2A13 from our study is represented in the parallelogram.

Supplemental data 1. Comparison of typical absorption spectra of 5-methoxypsoralen (5-MOP) (dashed line, $\lambda_{\text{max}}$= 312nm), 5-MOP dihydrodiol (solid line, $\lambda_{\text{max}}$= 322nm) and 7-hydroxycoumarin (7-HC) (dotted line, $\lambda_{\text{max}}$= 322nm). The spectra are normalized with $\lambda_{\text{max}}$ values and overlaid. The spectrum of 7-HC was nearly identical to that of 5-MOP dihydrodiol.
Table 1

Enzymatic parameters of CYP2A13 variants.

<table>
<thead>
<tr>
<th>Variant</th>
<th>$K_m$ (µM)</th>
<th>$V_{max}$ (nmol/min/nmol P450)</th>
<th>$V_{max}/K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2A13*1</td>
<td>1.44 ± 0.17</td>
<td>4.23 ± 0.36</td>
<td>2.98 ± 0.17</td>
</tr>
<tr>
<td>CYP2A13*4</td>
<td>N. D.</td>
<td>N. D.</td>
<td>N. D.</td>
</tr>
<tr>
<td>CYP2A13*5</td>
<td>1.63 ± 0.12</td>
<td>3.20 ± 0.13</td>
<td>1.99 ± 0.13</td>
</tr>
<tr>
<td>CYP2A13*6</td>
<td>1.36 ± 0.10</td>
<td>4.69 ± 0.13</td>
<td>3.47 ± 0.17</td>
</tr>
<tr>
<td>CYP2A13*8</td>
<td>0.85 ± 0.09</td>
<td>2.34 ± 0.07</td>
<td>2.81 ± 0.21</td>
</tr>
<tr>
<td>CYP2A13*9</td>
<td>0.58 ± 0.06</td>
<td>1.84 ± 0.09</td>
<td>3.22 ± 0.23</td>
</tr>
</tbody>
</table>

$^a$Kinetic parameters are presented as means ± standard error determined from three (CYP2A13*4, CYP2A13*6) or four (CYP2A13*1, CYP2A13*5, CYP2A13*8, CYP2A13*9) independent experiments.

$^b$Kinetic parameters could not be determined, since the amount of the enzyme was not quantified from reduced CO difference spectrum.
Figure 2

(A) [Image of gel electrophoresis with molecular weight markers.]

(B) [Graph showing absorbance differences for different CYP2A13 variants.]

- CYP2A13*1
- CYP2A13*4
- CYP2A13*5
- CYP2A13*6
- CYP2A13*8
- CYP2A13*9

Wavelength (nm)
Figure 3

(A) Absorbance vs. Retention time (min)
(B) Absorbance vs. Retention time (min)
(C) Absorbance vs. Retention time (min)
(D) Absorbance vs. Retention time (min)
(E) Absorbance vs. Retention time (min)
(F) Absorbance vs. Retention time (min)
(G) Absorbance vs. Retention time (min)
(H) Absorbance vs. Retention time (min)
(I) Absorbance vs. Retention time (min)
Figure 4

(A) 5-MOP dihyrodiol (nmol/min) vs. Reaction time (min)

(B) 5-MOP dihyrodiol (nmol/min) vs. 5-MOP (μM)

(C) 1/V vs. 1/[S]
Figure 5

5-MOP → Dihydro-5-MOP → Metabolite X

5-MOP 2',3'-epoxide → 5-MOP dihydrodiol

5-MOP dihydrodiol → Metabolite A

5-MOP dihydrodiol → Metabolite B

5-MOP dihydrodiol → Metabolite C