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Metabolism of 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) by

Human CYP1B1 Genetic Variants

Jing-Fen Han*, Xiao-Yang He*, Jason S. Herrington, Lori A. White, Jun-Feng Zhang,
and Jun-Yan Hong

School of Public Health/Environmental and Occupational Health Sciences Institute,
University of Medicine and Dentistry of New Jersey, Piscataway, NJ 08854, USA
(J.-F. H., X.-Y. H., J. H., J. Z., J.-Y. H.); Department of Biochemistry and
Microbiology, Rutgers University, New Brunswick, NJ 08901, USA (L. A. W.).

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Running Title

human CYP1B1 genetic variants and PhIP metabolism

Corresponding Author

Dr. Jun-Yan Hong

School of Public Health

University of Medicine and Dentistry of New Jersey

Room 385, 683 Hoes Lane West

Piscataway, NJ 08854, USA

1-732-235-2845

1-732-235-4004 (Fax)

jyhong@ehsi.rutgers.edu

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List of abbreviations

HCA	heterocyclic amines
PhIP	2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine
CYP1B1	cytochrome P450 1B1
HPLC	high performance liquid chromatography
N ² -OH-PhIP	2-hydroxyamino-PhIP
4'-OH-PhIP	4-hydroxy-PhIP

Abstract

Human cytochrome P450 1B1 (CYP1B1) plays a critical role in the metabolic activation of a variety of pro-carcinogens, including 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP). The existence of human CYP1B1 missense genetic variants has been demonstrated but their activities in metabolizing PhIP are unknown. In this study, we expressed 15 naturally-occurring CYP1B1 variants (with either single or multiple amino acid substitutions) and determined their activity changes in metabolizing PhIP to its two major metabolites, 2-hydroxyamino-PhIP (N²-OH-PhIP) and 4'-hydroxy-PhIP (4'-OH-PhIP). While the PhIP-metabolizing activities of four variants (Ala¹¹⁹Ser, Pro³⁷⁹Leu, Ala⁴⁴³Gly, Arg⁴⁸Gly/Leu⁴³²Val) were comparable to that of the expressed wild-type CYP1B1, five variants (Trp⁵⁷Cys, Gly⁶¹Glu, Arg⁴⁸Gly/Ala¹¹⁹Ser, Arg⁴⁸Gly/Ala¹¹⁹Ser/Leu⁴³²Val, Arg⁴⁸Gly/Ala¹¹⁹Ser/Leu⁴³²Val/Ala⁴⁴³Gly) exhibited more than two fold decrease in activity and a reduction in the catalytic efficiency (V_{\max}/K_m) for both N- and 4-hydroxylation of PhIP. Six variants (Gly³⁶⁵Trp, Glu³⁸⁷Lys, Arg³⁹⁰His, Pro⁴³⁷Leu, Asn⁴⁵³Ser, Arg⁴⁶⁹Trp) showed little activity in PhIP metabolism but the molecular mechanisms involved are apparently different. The microsomal CYP1B1 protein level was significantly decreased for the Trp³⁶⁵, Lys³⁸⁷, and His³⁹⁰ variants and was not detectable for the Ser⁴⁵³ variant. In contrast, there was no difference between the Trp⁴⁶⁹ variant and the wild-type in the microsomal CYP1B1 protein level and P450 content but the Trp⁴⁶⁹ variant totally lost its metabolic activity towards PhIP. The Leu⁴³⁷ variant also had a substantial amount of CYP1B1 protein in the microsomes but was a lack of detectable P450 peak and activity. Our results should be useful in selecting appropriate CYP1B1 variants as cancer susceptibility biomarkers for human population studies related to PhIP exposure.

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Introduction

The estimated daily exposure of an individual to heterocyclic amines (HCAs) ranges from nanograms to a few micrograms (Augustsson et al., 1999).

2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) is the most mass abundant heterocyclic amines (HCAs) formed in meat and fish during high-temperature cooking as a result of the pyrolysis of amino acids and proteins (Sugimura et al., 1983). Tobacco smoke is another major source of PhIP (Hecht, 2002). PhIP has been found in the urine of healthy volunteers eating a normal diet (Ushiyama et al., 1991) and PhIP-related DNA adducts have been detected in the urine of smokers (Peluso et al., 1991). These results suggest that humans are ubiquitously exposed to PhIP. PhIP is mutagenic in bacterial and mammalian cells (Edwards et al., 1994; Hammons et al., 1997). It induces colon, mammary gland, and prostate tumors in rats, and T-cell and B-cell lymphoma in mice (Malfatti et al., 1999; Guengerich 2000; Williams et al., 2000; Ochiai et al., 2002; Nakagama et al., 2005). Epidemiological studies suggest that colon cancer risk is increased in persons who consume high levels of mutagens in cooked-meat (Gunter et al., 2005). The National Toxicology Program concludes that PhIP is “reasonably anticipated to be a human carcinogen.”

(<http://www.niehs.nih.gov/oc/news/canceragents.htm>).

As most environmental carcinogens, PhIP requires metabolic activation to exert its carcinogenic effects. The metabolism of PhIP leads to the formation of two major metabolites: 2-hydroxyamino-PhIP (N²-OH-PhIP) and 4'-hydroxy-PhIP (4'-OH-PhIP) (Shirai et al., 1997; King et al., 1999) (Fig. 1). N²-OH-PhIP is highly

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mutagenic in both bacterial and mammalian cells, and its N-acetylated and -sulfated conjugates can form DNA adducts. In contrast, 4'-OH-PhIP is considered to be non-carcinogenic and is eventually excreted through urine after its conjugation with glucuronide or sulfate (Crofts et al., 1997). The metabolic activation of PhIP, i.e. N-hydroxylation, is primarily catalyzed by the enzymes belonging to the cytochrome P450 (CYP) 1 family, including CYP1B1 (Crofts et al., 1997). Human CYP1B1 is constitutively expressed in many extra-hepatic tissues and often over-expressed in a variety of human cancers (Murray et al., 1997). In addition to PhIP, it catalyzes the activation of some other environmental carcinogens including arylarenes, nitroarenes, arylamines, and polycyclic aromatic hydrocarbons (Shimada et al., 1997). Recently, the existence of the genetic variants of human CYP1B1 has been demonstrated (Stoilov et al., 1998; McLellan et al., 2000; Aklillu et al., 2002; Tanaka et al., 2002). Functional characterization of several CYP1B1 missense variants has been conducted with carcinogen substrates estradiol, benzo[α]pyrene, and 7,12-dimethylbenz[α]anthracene (Li et al., 2000; Shimada et al., 2001; Aklillu et al., 2002; Aklillu et al., 2005). However, the impact of these and other reported CYP1B1 missense variations on PhIP metabolism is yet unknown. It is important to emphasize that an alteration in catalytic function, induced by amino acid substitution, of a particular enzyme could be substrate-dependent. For example, while in comparison with the Val⁴³² form of CYP1B1, the Leu⁴³² form was reported to have a threefold increase in the K_m value for estradiol hydroxylation but little effect on benzo[α]pyrene epoxidation (Li et al., 2000).

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In the present study, we used a heterologous system to express a total of 15 naturally-occurring CYP1B1 variant proteins (with either single or multiple amino acid substitutions) (Table 1) and compared them with the wild-type CYP1B1 protein in metabolizing PhIP to its major metabolites, N²-OH-PhIP and 4'-OH-PhIP. Our results are expected to provide direct evidence for the functional significance of these genetic variations in CYP1B1-mediated PhIP metabolism.

Materials and Methods

Materials

PhIP and N²-OH-PhIP were purchased from Toronto Research Chemicals (Toronto, Ontario, Canada). For standard validation, we also obtained N²-OH-PhIP as a gift from NCI Chemical Carcinogen Repository Midwest Research Institute (Kansas, MI). 4'-OH-PhIP was a generous gift from Dr. Kulp KS and Dr. Knize MG (Lawrence Livermore National Laboratory Livermore, CA). Glucose-6-phosphate, glucose-6-phosphate dehydrogenase, NADP⁺, δ -aminolevulinic acid, ferric citrate and HPLC grade methanol were purchased from Sigma-Aldrich (St. Louis, MO). B_{AC}-TO-B_{AC} baculovirus expression system (including pFastbac1 expression vector) and Grace insect cell culture media were obtained from Invitrogen (Carlsbad, CA). NADPH-P450 oxidoreductase was purified from rat liver microsomes as described previously (Yasukochi et al., 1976). A polyclonal rabbit anti-human CYP1B1 antibody was obtained from Genetest Corp. (Boston, MA). Goat anti-rabbit IgG horseradish peroxidase conjugate was obtained from Bio-Rad (Hercules, CA). The ECL enhanced

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chemiluminescence reagents for immunoblotting detection were obtained from Amersham Biosciences Inc. (Piscataway, NJ). KOD Hot Start DNA Polymerase was obtained from Novagen (Madison, WI).

Construction of CYP1B1 Variant cDNAs

The wild-type human CYP1B1 cDNA (NM_000104) was used as a template to generate the variant cDNAs by site-directed mutagenesis as described previously (Han et al., 2006). The PCR primer sequences for the mutagenesis were shown in Table 2. Additional rounds of mutagenesis were conducted to generate the variant cDNAs containing more than one nucleotide variations. All the variant cDNAs were sequenced after mutagenesis to ensure there were no extra mutations produced during PCR amplification process.

Heterologous Expression and Microsome Preparation

A B_{AC-TO}-B_{AC} baculovirus expression system was used for the expression of CYP1B1 proteins. Wild-type and variant cDNAs were individually sub-cloned into the expression vector pFASTbac1. The reconstructed bacmid DNAs obtained after DNA transpositions in DH10B_{AC} cells were transfected into Sf9 cells to obtain the recombinant baculovirus particles. Sf9 cells were subsequently infected in large scale with the virus particles for the production of the CYP 1B1 proteins. δ -Aminolevulinic acid and ferric citrate stock solutions (100 mM each, dissolved in the Grace medium) were added to the culture medium for a final concentration of 100 μ M. The infected

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Sf9 cells were harvested 72 hr after infection and re-suspended in the PBS buffer containing 5 mM imidazole, 20% glycerol, and 1 mM freshly added PMSF.

Microsomes were prepared from the cells by sonication and differential centrifugation as previously described (Hong et al., 1999). The microsome preparations were stored at -70°C prior to use. Protein concentrations were determined by the protein assay reagents from Bio-Rad.

Immunoblot Analysis and P450 Content Determination

Microsomal proteins (1 μg) were loaded onto 10% SDS-polyacrylamide gels for electrophoresis and transferred to a nitrocellulose membrane. The membrane was blocked 1-hr using 5% (w/v) evaporated milk in Tris-buffered saline containing 0.0005% (v/v) Tween 20. The membrane was probed with a CYP1B1-specific polyclonal antibody (1:3300 dilution) as the primary antibody for 1 hr and followed by goat anti-rabbit IgG conjugated with horseradish peroxidase (1:5000 dilution), as the secondary antibody for 1 hr. The immunoblot was visualized by ECL reagents according to the manufacture's protocol (Amersham Biosciences Inc., Piscataway, NJ). Microsomal P450 content was determined by reduced CO-difference spectrum using a UV/visible spectrophotometer as previously described (He et al., 2004).

PhIP Metabolism and Kinetic Analysis

The incubation mixture for PhIP metabolism consisted of 100 mM sodium phosphate buffer (pH 7.4), 5 mM MgCl_2 , an NADPH generating system (15 mM

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glucose-6-phosphate, 1 unit of glucose-6-phosphate dehydrogenase, 1 mM NADP⁺), NADPH-P450 oxidoreductase (reductase:P450 = 1:1 in molar ratio), 100 pmol of CYP1B1, and PhIP in a total volume of 1ml. After a 5-min pre-incubation, the reaction was initiated with the NADPH generating system and incubated at 37°C for 30 min. The reaction was terminated by adding one volume of ice-cold methanol. The mixture was then centrifuged at 4°C and filtered. Samples were stored at -70 °C prior to analysis by HPLC within 48 hr after the preparation. For the determination of kinetic parameters, eight different PhIP concentrations, ranging from 0.3 μM to 50 μM, were used for incubation. The selection of substrate concentrations for activity assay and the determination of kinetic parameters was based on literature (Crofts et al., 1997) and our pilot experiments. Commercially available human CYP1B1, CYP1A1 and CYP1A2 (Genetest) were used as positive controls to confirm the formation of PhIP metabolites under our assay conditions. Incubation without CYP enzymes or with pre-inactivated CYP1B1 proteins (adding methanol before initiation of the reaction) was used as a negative control.

HPLC Analysis

PhIP metabolites were analyzed using an HPLC-fluorescence system (Thermo Separation Products Inc., CA) consisting of Spectra Physics P4000 Mobile Phase Pump, Spectra Physics AS3000 Autosampler, Spectra Physics FL2000 Fluorescence Detector, a Waters μ Bondapak C18 column (3.9 × 300 mm, 125 Å, 10 μm), a guard cartridge (μ Bondapak, 125 Å, 10 μm) and software PC1000 for data collection and

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analysis. The metabolites were eluted with 55% methanol in water containing 0.1% diethylamine (pH 4.0 with acetic acid). The isocratic solvent was held at flow rate of 1.25 ml/min for 20 min. The sample injection volume was 100 μ l. The excitation wavelength was 316 nm and the emission wavelength was 370 nm. The analytical protocol was able to clearly resolve N²-OH-PhIP and 4'-OH-PhIP in less than 20 min. Concentrations of the PhIP metabolites were determined by calibration curves prepared using standard solution of N²-OH-PhIP and 4'-OH-PhIP. Under our analytical conditions, the limits of detection were 0.02 pg for N²-OH-PhIP and 3.2 pg for 4'-OH-PhIP. For the validation of HPLC analysis, we used PhIP metabolite standards as well as the positive control and negative control samples from the in vitro metabolism.

Results

Heterologous Expression of the Wild-type and Variant CYP1B1 Proteins

All the variant CYP1B1 cDNAs were successfully constructed and used for protein expression in the B_{AC}-TO-B_{AC} baculovirus/Sf9 insect cell system. For most of the variant proteins, immunoblot analysis of the microsomal proteins detected a single protein band with the same expected molecular weight as the wild-type CYP1B1 (Fig. 2A). There were no detectable CYP1B1 proteins in the microsomes prepared from the Sf9 cells infected with the vector only (containing no CYP1B1 cDNA) or the bacmid containing the Asn⁴⁵³Ser variant cDNA. The microsomal CYP1B1 protein levels for the Gly³⁶⁵Trp, Glu³⁸⁷Lys and Arg³⁹⁰His variants were reproducibly lower than the

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wild-type and most of the variants.

Most of the microsomes containing the expressed recombinant CYP1B1 proteins displayed the characteristic P450 absorption peak in CO-difference spectrum analysis with the average of 219 pmol/mg (range: 118-324 pmol/mg). A significant decrease in P450 content was observed in the microsomes from the cells expressing Gly³⁶⁵Trp, Pro³⁷⁹Leu, and Glu³⁸⁷Lys variants proteins with an average of 39 pmol/mg (range: 24-55 pmol/mg) (Table 3). Microsomes from the cells infected with the Arg³⁹⁰His and Asn⁴⁵³Ser variants cDNAs showed no detectable P450 (data not shown) and little or no detectable CYP1B1 proteins (Fig. 2A). The Pro⁴³⁷Leu variant showed a substantial amount of expressed CYP1B1 protein but no detectable P450 peak (Fig. 2B). All these results were confirmed by 3 to 5 independent expression experiments in the Sf9 cells. For the CYP1B1 variants with significantly lower level of P450 content, the repeated expressions started from the expression vector reconstruction.

Activity of CYP1B1 Variants and Enzyme Kinetics

The formation of N²-OH-PhIP and 4'-OH-PhIP during CYP1B1-mediated PhIP metabolism was determined by a reported HPLC-fluorescence method (Crofts et al., 1997). We modified the original protocol by changing gradient elution (from 33% to 55%) to isocratic elution with 55% methanol. This change resulted in a satisfactory resolution of PhIP, N²-OH-PhIP, and 4'-OH-PhIP in less than 20 min instead of 40 min in the original method, which reduced the possibility of metabolite degradation when a large number of samples were analyzed. As shown in Fig. 3, the retention

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times of N²-OH-PhIP, PhIP, and 4'-OH-PhIP are 4 min, 8 min, and 13 min, respectively.

When PhIP was incubated with microsomes containing wild-type CYP1B1, the production of N²-hydroxy PhIP and 4'-hydroxy PhIP was linear up to a 60-min incubation time and up to 120 pmol CYP1B1/ml reaction volume (data not shown). We therefore conducted the PhIP-metabolizing activity assay using 30-min incubation time and 100 pmol CYP1B1/ml reaction volume. For the CYP1B1 variants with no detectable activity, the assay was repeated with increased amount of microsomal proteins (20-fold higher than the wild-type CYP1B1). The activity was determined at both high (10 μ M) and low (1 μ M) substrate concentrations. Fig. 4 shows that at 10 μ M of PhIP, the variants Ala¹¹⁹Ser, Pro³⁷⁹Leu, Ala⁴⁴³Gly and Arg⁴⁸Gly/Leu⁴³²Val displayed almost the same activity as the wild-type CYP1B1 in both N-hydroxylation and 4-hydroxylation of PhIP. The activities of the variants Trp⁵⁷Cys, Gly⁶¹Glu, Arg⁴⁸Gly/Ala¹¹⁹Ser, Arg⁴⁸Gly/Ala¹¹⁹Ser/Leu⁴³²Val, Arg⁴⁸Gly/Ala¹¹⁹Ser/Leu⁴³²Val/Ala⁴⁴³ were lower than the wild-type CYP1B1. There was no detectable PhIP-metabolizing activity in the variants Gly³⁶⁵Trp, Glu³⁸⁷Lys, Arg³⁹⁰His, Pro⁴³⁷Leu, Asn⁴⁵³Ser. It is of interest to note that although the Arg⁴⁶⁹Trp variant showed the same level of CYP1B1 protein and P450 content (190 pmol/mg) as the wild-type, it had a total loss of PhIP-metabolizing activity. This activity profile at 10 μ M substrate concentration was consistent with the result with 1 μ M substrate concentration (data not shown).

We further selected five CYP1B1 variants that showed more than two-fold

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decrease in PhIP-metabolizing activity for detailed kinetic analysis. These included the variants Trp⁵⁷Cys, Gly⁶¹Glu, Arg⁴⁸Gly/Ala¹¹⁹Ser, Arg⁴⁸Gly/Ala¹¹⁹Ser/Leu⁴³²Val, and Arg⁴⁸Gly/Ala¹¹⁹Ser/Leu⁴³²Val/Ala⁴⁴³Gly. The kinetics of PhIP metabolism was determined with eight different substrate concentrations, ranging from 0.3 to 50 μ M. The experiments were always conducted in duplicate and showed less than 10% of assay variations (data not shown). When different concentrations of PhIP were incubated with the microsomes containing the wild-type human CYP1B1, the rate of formation of N²-OH-PhIP and 4'-OH-PhIP was initially linear but reached saturation at the PhIP concentrations above 10 μ M. The values of apparent K_m and V_{max} and catalytic efficiency (V_{max}/K_m) derived from the kinetics curves are shown in Table 4. The K_m and V_{max} values for both N-hydroxylation and 4-hydroxylation of PhIP by wild-type CYP1B1 in our kinetics study are consistent with the reported values (Crofts et al., 1997). The K_m values are 3.6 μ M for N-hydroxylation of PhIP and 4.5 μ M for 4-hydroxylation in our study while the corresponding values reported by Crofts et al. were 5.7 μ M and 2.2 μ M. The V_{max} values for PhIP N-hydroxylation and 4-hydroxylation are 0.115 nmol/min/nmol and 0.120 nmol/min/nmol while the corresponding values by Crofts et al. were 0.40 nmol/min/nmol, and 0.93 nmol/min/nmol. For the CYP1B1 variants, while the K_m for N-hydroxylation of PhIP was nearly unchanged in the Arg⁴⁸Gly/Ala¹¹⁹Ser variant, it was increased in the other four variants (Trp⁵⁷Cys, Gly⁶¹Glu, Arg⁴⁸Gly/Ala¹¹⁹Ser/Leu⁴³²Val, and Arg⁴⁸Gly/Ala¹¹⁹Ser/Leu⁴³²Val/Ala⁴⁴³Gly). The catalytic efficiency (V_{max}/K_m) for PhIP N-hydroxylation in the variant Arg⁴⁸Gly/Ala¹¹⁹Ser/Leu⁴³²Val/Ala⁴⁴³Gly variant

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showed nearly a tenfold decrease because of the substantial increase of K_m value. The effect of the missense changes on the kinetic parameters of N²-OH-PhIP and 4'-OH-PhIP formation appears to be different for different variants. For the Trp⁵⁷Cys variant, the K_m value for N²-OH-PhIP and 4'-OH-PhIP were both increased, and the V_{max} values were both decreased. For the Gly⁶¹Glu variant, the K_m value for N²-OH-PhIP formation was increased; but was unchanged for 4'-OH-PhIP formation. The K_m values of the variants Arg⁴⁸Gly/Ala¹¹⁹Ser/Leu⁴³²Val and Arg⁴⁸Gly/Ala¹¹⁹Ser/Leu⁴³²Val/Ala⁴⁴³Gly for N²-OH-PhIP formation was increased 4-8 fold, while there was no significant change in their K_m values for 4'-OH-PhIP formation. For the Arg⁴⁸Gly/Ala¹¹⁹Ser variant, the K_m values were not changed for the formation of both N²-OH-PhIP and 4'-OH-PhIP formation, but the V_{max} values were decreased.

Discussion

Although previous studies demonstrated that human CYP1A2 is the major enzyme in the metabolic activation of PhIP, it is mainly expressed in the liver (Shimada et al., 1996). In contrast, CYP1B1 is predominantly expressed in extra-hepatic tissues, including prostate, mammary gland and colon (Aklillu et al., 2005). Therefore, metabolic activation of PhIP *in situ* by CYP1B1 and other extra-hepatic enzymes such as CYP1A1 is believed to play a critical role in PhIP-related carcinogenesis in these tissues such as prostate cancer (Holme et al., 1989; Patterson et al., 2002). Since the amino acid substitutions in the CYP1B1

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protein induced by genetic variations may alter the enzyme activity and/or protein stability, the present study characterized the functional significance of 15 CYP1B1 genetic variants in PhIP metabolism. As the result, we identified several CYP1B1 missense variants with changes in protein expression level and/or catalytic activity.

The microsomal CYP1B1 protein level was not detectable for the Asn⁴⁵³Ser variant and was significantly reduced for the Gly³⁶⁵Trp, Glu³⁸⁷Lys and Arg³⁹⁰His variants. These CYP1B1 variants also lost their activity in both N-hydroxylation and 4-hydroxylation of PhIP. Based on a published computer modeling study (Stoilov et al., 1998), Gly³⁶⁵ belongs to the conserved core structures of CYP1B1. Substitution of Gly³⁶⁵ with Trp may interfere with the proper folding and heme-binding of CYP1B1 protein. Both Glu³⁸⁷ and Arg³⁹⁰ residues of CYP1B1 are localized in the conserved helix K, which are one helical turn apart and are predicted to form a salt bridge. Substitutions with Lys and His at these locations probably alter the secondary structure of CYP1B1 protein, which may affect the enzyme in its coupling with NADPH-cytochrome P450 reductase and catalytic function. The lack of detectable Asn⁴⁵³Ser variant protein in our observation is consistent with a recent study in which there was a significant decrease in the level of Asn⁴⁵³Ser variant protein transiently expressed in the COS-1 cells (Bandiera et al., 2005). The same authors also demonstrated that the degradation of the Asn⁴⁵³Ser variant protein by the proteasomal pathway is much faster than the wild-type CYP1B1 protein (Bandiera et al., 2005), suggesting that the substitution of Asn⁴⁵³ with Ser leads to an unstable protein structure. In contrast to a reported finding that the Gly⁶¹Glu variant has a greatly

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diminished protein stability when it was expressed in *E. coli* (Jansson et al., 2001), we did not observe a significant change in protein content of this CYP1B1 variant in our expression system.

It is of interest to observe that both the Pro⁴³⁷Leu and Arg⁴⁶⁹Trp variants showed the same protein expression level in the microsomes as the wild-type CYP1B1 but lost the PhIP-metabolizing activity. However, the involved inactivation mechanisms for these two variants are apparently different. The Pro⁴³⁷Leu variant protein lacks the characteristic P450 absorption peak in CO-difference spectrum analysis, suggesting the amino acid substitution affects the heme binding. This may be explained by the modeling result that Pro⁴³⁷ is localized in the meander region and precedes the heme binding region (Stoilov et al., 1998). On the other hand, the P450 absorption peak was clearly present for the Arg⁴⁶⁹Trp variant, which is consistent with the observation of Jansson et al. that the stability of this variant protein was normal (Jansson et al., 2001). As predicted by the same model (Stoilov et al., 1998), Cys⁴⁷⁰ in CYP1B1 is the cysteine which is completely conserved among all the microsomal cytochrome P450 enzymes and provides the axial heme ligand in the heme-binding region. Arg⁴⁶⁹ immediately precedes Cys⁴⁷⁰ and a replacement with Trp, a larger amino acid residue with a rigid side group, may interfere with the normal conformation of the highly conserved cysteine pocket and catalytic function.

According to the CYP1B1 model (Stoilov et al., 1998), Trp⁵⁷ is located in a hinge region and precedes the last proline residue of the hinge. As one of the most conserved amino acid residues in the same region, Gly⁶¹ is also very close to the

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proline. Previous studies revealed that substitution of the corresponding proline residue in CYP2C11 and CYP2C2 could influence heme incorporation and reduce enzymatic activity (Yamazaki et al., 1993; Chen et al., 1996). Substitution of non-polar and hydrophobic tryptophan with polar cysteine or replacement of non-charged glycine with charged glutamic acid in this hinge region may interfere with the proper folding of the CYP1B1 protein and result in decreased enzymatic activity as observed in PhIP metabolism.

The CYP1B1 variants Arg⁴⁸Gly/Ala¹¹⁹Ser (CYP1B1.2), Arg⁴⁸Gly/Ala¹¹⁹Ser/Leu⁴³²Val (CYP1B1*6), and Arg⁴⁸Gly/Ala¹¹⁹Ser/Leu⁴³²Val/Ala⁴⁴³Gly (CYP1B1*7) exist as common haplotypes in several different populations (Aklillu et al., 2002; Aklillu et al., 2005). During the enzyme kinetic study of the CYP1B1 variants with reduced PhIP-metabolizing activity, the variants Arg⁴⁸Gly/Ala¹¹⁹Ser/Leu⁴³²Val (CYP1B1.6) and Arg⁴⁸Gly/Ala¹¹⁹Ser/Leu⁴³²Val/Ala⁴⁴³Gly (CYP1B1.7) were found to have the most significant change in the K_m values for N²-OH-PhIP formation (14.8 and 30.1 μ M, respectively, versus 3.6 μ M in wild-type CYP1B1) but not in the 4'-OH-PhIP formation. Because the Arg⁴⁸Gly/Ala¹¹⁹Ser variant (CYP1B1.2) showed no K_m value change for PhIP N-hydroxylation and in the metabolism of other substrates (Aklillu et al., 2002; Aklillu et al., 2005), it is reasonable to speculate that the K_m value changes in the variants Arg⁴⁸Gly/Ala¹¹⁹Ser/Leu⁴³²Val (CYP1B1*6) and Arg⁴⁸Gly/Ala¹¹⁹Ser/Leu⁴³²Val/Ala⁴⁴³Gly (CYTP1B1*7) are mainly due to the inclusion of a common Leu⁴³²Val substitution. Ala⁴⁴³ is conserved in the mouse, rat and human CYP1B1. Inclusion of an additional Ala⁴⁴³Gly substitution in the

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CYP1B1.7 variant resulted in a further increase in the K_m value for PhIP N-hydroxylation, suggesting that the substitution also affects substrate binding. In addition, the alteration of enzymatic function in CYP1B1 variants is clearly substrate-dependent. While the capacity for N-hydroxylation of PhIP was significantly decreased in both CYP1B1.6 and CYP1B1.7 variants, a significantly decreased capacity for oxidation of benzo[α]pyrene was only observed in CYP1B1.7 variant but not in CYP1B1.6 variant (Aklillu et al., 2005).

In summary, the present study demonstrates that several genetic variants of human CYP1B1 have significant changes in the protein expression level and PhIP-metabolizing activity. These results appear to be consistent with the prediction of a published CYP1B1 protein model (Stoilov et al., 1998). Our study provides new information on the role of involved amino acid residues in protein stability and catalysis of CYP1B1. In addition, identification of these functional CYP1B1 variants should help understand the inter-individual variations in PhIP metabolism and their potential impact on the risk to human cancers related to PhIP exposure.

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References

- Aklillu E, Oscarson M, Hidestrand M, Leidvik B, Otter C and Ingelman-Sundberg M (2002) Functional analysis of six different polymorphic CYP1B1 enzyme variants found in an Ethiopian population. *Mol Pharmacol* 61: 586-594.
- Aklillu E, Ovrebo S, Botnen I V, Otter C and Ingelman-Sundberg M (2005) Characterization of common CYP1B1 variants with different capacity for benzo[a]pyrene-7,8-dihydrodiol epoxide formation from benzo[a]pyrene. *Cancer Res* 65: 5105-5111.
- Augustsson K, Skog K, Jagerstad M, Dickman P W and Steineck G (1999) Dietary heterocyclic amines and cancer of the colon, rectum, bladder, and kidney: a population-based study. *Lancet* 353: 703-707.
- Bandiera S, Weidlich S, Harth V, Broede P, Ko Y and Friedberg T (2005) Proteasomal degradation of human CYP1B1: effect of the Asn453Ser polymorphism on the post-translational regulation of CYP1B1 expression. *Mol Pharmacol* 67: 435-443.
- Chen C D and Kemper B (1996) Different structural requirements at specific proline residue positions in the conserved proline-rich region of cytochrome P450 2C2. *J Biol Chem* 271: 28607-28611.
- Crofts F G, Strickland P T, Hayes C L and Sutter T R (1997) Metabolism of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) by human cytochrome P4501B1. *Carcinogenesis* 18: 1793-1798.
- Edwards R J, Murray B P, Murray S, Schulz T, Neubert D, Gant T W, Thorgeirsson S S, Boobis A R and Davies D S (1994) Contribution of CYP1A1 and CYP1A2 to the activation of heterocyclic amines in monkeys and human. *Carcinogenesis* 15: 829-836.
- Guengerich F P (2000) Metabolism of chemical carcinogens. *Carcinogenesis* 21: 345-351.
- Gunter M J, Probst-Hensch N M, Cortessis V K, Kulldorff M, Haile R W and Sinha R (2005) Meat intake, cooking-related mutagens and risk of colorectal adenoma in a sigmoidoscopy-based case-control study. *Carcinogenesis* 26: 637-642.
- Hammons G J, Milton D, Stepps K, Guengerich F P, Tukey R H and Kadlubar F F (1997) Metabolism of carcinogenic heterocyclic and aromatic amines by recombinant human cytochrome P450 enzymes. *Carcinogenesis* 18: 851-854.
- Han J F, Wang S L, He X Y, Liu C Y and Hong J Y (2006) Effect of genetic variation

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on human cytochrome p450 reductase-mediated paraquat cytotoxicity. *Toxicol Sci* 91: 42-48.

- He X Y, Shen J, Ding X, Lu A Y and Hong J Y (2004) Identification of critical amino acid residues of human CYP2A13 for the metabolic activation of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, a tobacco-specific carcinogen. *Drug Metab Dispos* 32: 1516-1521.
- Hecht S S (2002) Tobacco smoke carcinogens and breast cancer. *Environ Mol Mutagen* 39: 119-126.
- Holme J A, Wallin H, Brunborg G, Soderlund E J, Hongslo J K and Alexander J (1989) Genotoxicity of the food mutagen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP): formation of 2-hydroxyamino-PhIP, a direct acting genotoxic metabolite. *Carcinogenesis* 10: 1389-1396.
- Hong J Y, Wang Y Y, Bondoc F Y, Lee M, Yang C S, Hu W Y and Pan J M (1999) Metabolism of methyl tert-butyl ether and other gasoline ethers by human liver microsomes and heterogously expressed human cytochrome P450: identification of CYP2A6 as a major catalyst. *Toxicol Appl Pharmacol.* 160: 43-48.
- Jansson I, Stoilov I, Sarfarazi M and Schenkman J B (2001) Effect of two mutations of human CYP1B1, G61E and R469W, on stability and endogenous steroid substrate metabolism. *Pharmacogenetics* 11: 793-801.
- King R S, Teitel C H, Shaddock J G, Casciano D A and Kadlubar F F (1999) Detoxification of carcinogenic aromatic and heterocyclic amines by enzymatic reduction of the N-hydroxy derivative. *Cancer Lett* 143: 167-171.
- Li D N, Seidel A, Pritchard M P, Wolf C R and Friedberg T (2000) Polymorphisms in P450 CYP1B1 affect the conversion of estradiol to the potentially carcinogenic metabolite 4-hydroxyestradiol. *Pharmacogenetics* 10: 343-353.
- Malfatti M A, Kulp K S, Knize M G, Davis C, Massengill J P, Williams S, Nowell S, MacLeod S, Dingley K H, Turteltaub K W, Lang N P and Felton J S (1999) The identification of [2-(14)C]2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine metabolites in humans. *Carcinogenesis* 20: 705-713.
- McLellan R A, Oscarson M, Hidestrand M, Leidvik B, Jonsson E, Otter C and Ingelman-Sundberg M (2000) Characterization and functional analysis of two common human cytochrome P450 1B1 variants. *Arch Biochem Biophys* 378: 175-181.

DMD #16824

- Murray G I, Taylor M C, McFadyen M C, McKay J A, Greenlee W F, Burke M D and Melvin W T (1997) Tumor-specific expression of cytochrome P450 CYP1B1. *Cancer Res* 57: 3026-3031.
- Nakagama H, Nakanishi M and Ochiai M (2005) Modeling human colon cancer in rodents using a food-borne carcinogen, PhIP. *Cancer Sci.* 96: 627-631.
- Ochiai M, Imai H, Sugimura T, Nagao M and Nakagama H (2002) Induction of intestinal tumors and lymphomas in C57BL/6N mice by a food-borne carcinogen, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine. *Jpn J Cancer Res* 93: 478-483.
- Patterson L H and Murray G I (2002) Tumour cytochrome P450 and drug activation. *Curr Pharm Des* 8: 1335-1347.
- Peluso M, Castegnaro M, Malaveille C, Friesen M, Garren L, Hautefeuille A, Vineis P, Kadlubar F and Bartsch H (1991) 32P Postlabelling analysis of urinary mutagens from smokers of black tobacco implicates 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) as a major DNA-damaging agent. *Carcinogenesis* 12: 713-717.
- Shimada T, Gillam E M, Sutter T R, Strickland P T, Guengerich F P and Yamazaki H (1997) Oxidation of xenobiotics by recombinant human cytochrome P450 1B1. *Drug Metab Dispos* 25: 617-622.
- Shimada T, Watanabe J, Guengerich F P, Inoue K and Gillam E M J (2001) Specificity of 17 β -oestradiol and benzo[*a*]pyrene oxidation by polymorphic human cytochrome P450 1B1 variants substituted at residue 48, 119, and 432. *Xenobiotica* 31: 163-176.
- Shimada T, Yamazaki H, Mimura M, Wakamiya N, Ueng Y F, Guengerich F P and Inui Y (1996) Characterization of microsomal cytochrome P450 enzymes involved in the oxidation of xenobiotic chemicals in human fetal liver and adult lungs. *Drug Metab Dispos* 24: 515-522.
- Shirai T, Sano M, Tamano S, Takahashi S, Hirose M, Futakuchi M, Hasegawa R, Imaida K, Matsumoto K, Wakabayashi K, Sugimura T and Ito N (1997) The prostate: a target for carcinogenicity of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) derived from cooked foods. *Cancer Res* 57: 195-198.
- Stoilov I, Akarsu A N, Alozie I, Child A, Barsoum-Homsy M, Turacli M E, Or M, Lewis R A, Ozdemir N, Brice G, Aktan S G, Chevrette L, Coca-Prados M and Sarfarazi M (1998) Sequence analysis and homology modeling suggest that

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primary congenital glaucoma on 2p21 results from mutations disrupting either the hinge region or the conserved core structures of cytochrome P4501B1. *Am J Hum Genet* 62: 573-584.

Sugimura T and Sato S (1983) Mutagens-carcinogens in foods. *Cancer Res* 43: 2415s-2421s.

Tanaka Y, Sasaki M, Kaneuchi M, Shiina H, Igawa M and Dahiya R (2002) Polymorphisms of the CYP1B1 gene have higher risk for prostate cancer. *Biochem Biophys Res Commun* 296: 820-826.

Ushiyama H, Wakabayashi K, Hirose M, Itoh H, Sugimura T and Nagao M (1991) Presence of carcinogenic heterocyclic amines in urine of healthy volunteers eating normal diet, but not of inpatients receiving parenteral alimentation. *Carcinogenesis* 12: 1417-1422.

Williams J A, Martin F L, Muir G H, Hewer A, Grover P L and Phillips D H (2000) Metabolic activation of carcinogens and expression of various cytochromes P450 in human prostate tissue. *Carcinogenesis* 21: 1683-1689.

Yamazaki S, Sato K, Suhara K, Sakaguchi M, Mihara K and Omura T (1993) Importance of the proline-rich region following signal-anchor sequence in the formation of correct conformation of microsomal cytochrome P-450s. *J Biochem (Tokyo)* 114: 652-657.

Yasukochi Y and Masters B S (1976) Some properties of a detergent-solubilized NADPH-cytochrome c (cytochrome P-450) reductase purified by biospecific affinity chromatography. *J Biol Chem.* 251: 5337-5344.

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Footnotes

* The first two authors made the same contributions to this work.

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Figures Legends

FIG. 1 *Metabolic pathways of PhIP and its two major metabolites.*

FIG. 2. *Heterologous expression of human CYP1B1 cDNAs in Sf9 cells.* Microsomal proteins were prepared from the Sf9 cells infected for 72 hrs with recombinant bacmids containing either wild-type or variant CYP1B1 cDNAs. **A.** Immunoblot analysis. Microsomal proteins (1 μ g) of each sample were used for electrophoresis. The membrane was probed with a CYP1B1-specific polyclonal antibody as the primary antibody, followed by goat anti-rabbit IgG conjugated with horseradish peroxidase as the secondary antibody for 1 hr. Microsomes from the Sf9 cells transfected with the pFASTBAC1 vector alone (without CYP1B1 cDNA) were used as a negative control (-). **B.** Representative CO-difference spectra. The assay solution containing microsomal proteins (2 μ g/ μ l) was bubbled with CO for 60 sec followed by addition of dithionite into the test cuvette and gentle mixing. The absorption spectrum was recorded from 500nm to 400nm. Microsomes prepared from the Sf9 cells expressing wild-type CYP1B1 and the Ser¹¹⁹ and Trp⁴⁶⁹ variants showed the characteristic P450 absorption peak, while the Leu⁴³⁷ only displayed a major absorption peak at 420nm.

FIG. 3. *HPLC chromatograms of PhIP and its metabolites.* The metabolites were eluted with 55% methanol in water containing 0.1% diethylamine (pH 4.0 with acetic acid). The isocratic solvent was held at flow rate of 1.25 ml/min for 20 min. The sample injection volume was 100 μ l. The excitation wavelength was 316nm and the

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emission wavelength was 370nm. **A.** Mixture of the PhIP metabolite standards: 4'-OH-PhIP (5 pg) and N²-OH-PhIP (5 pg). **B.** Metabolites produced during the incubation of PhIP (0.63 μM) with microsomes containing expressed wild-type human CYP1B1 enzyme.

FIG. 4. *PhIP N-hydroxylation and 4-hydroxylation activity of heterologously expressed wild-type and variant CYP1B1.* The activity was determined at 10 μM PhIP. The incubation mixture for PhIP metabolism consisted of 100 mM sodium phosphate buffer (pH 7.4), 5 mM MgCl₂, an NADPH generating system. After a 5-min pre-incubation, the reaction was initiated with the NADPH generating system and NADPH-P450 oxidoreductase incubated at 37°C for 30 min. The reaction was terminated by adding one volume of ice-cold methanol. The results are the average of duplicated experiments with less than 10% variations. **A.** PhIP N-hydroxylation activity. **B.** PhIP 4-hydroxylation activity. There were no detectable activities in both N-hydroxylation and 4-hydroxylation of PhIP for the CYP1B1 variants Trp³⁶⁵, Lys³⁸⁷, His³⁹⁰, Leu⁴³⁷, Ser⁴⁵³ and Trp⁴⁶⁹.

Table 1 Amino Acid Changes and Distribution Frequency of the Reported Fifteen CYP1B1 Missense Variants

Allele Name	Nucleotide Changes	Missense Mutations	Allelic Frequency	References
<i>CYP1B1</i> *2	142C>G; 355G>T	Arg ⁴⁸ Gly/Ala ¹¹⁹ Ser	36.7% (Ethiopian), 25.5% (Spanish)	(Akiyllu et al., 2002; Akiyllu et al., 2005)
<i>CYP1B1</i> *4	1358A>G	Asn ⁴⁵³ Ser	2% (Ethiopian)	(Stoilov et al., 1998)
<i>CYP1B1</i> *5	142C>G; 1294C>G	Arg ⁴⁸ Gly/Leu ⁴³² Val	0.7% (Ethiopian)	(Akiyllu et al., 2002)
<i>CYP1B1</i> *6	142C>G; 355G>T; 1294C>G	Arg ⁴⁸ Gly/Ala ¹¹⁹ Ser/Leu ⁴³² Val	6.3% (Ethiopian), 0.4% (Spanish)	(Akiyllu et al., 2002; Akiyllu et al., 2005)
<i>CYP1B1</i> *7	142C>G; 355G>T; 1294C>G; 1328C>G	Arg ⁴⁸ Gly/Ala ¹¹⁹ Ser/Leu ⁴³² Val/Ala ⁴⁴³ Gly	7% (Ethiopian), 2.6% (Spanish)	(Akiyllu et al., 2002; Akiyllu et al., 2005)
<i>CYP1B1</i> *11	171G>C	Trp ⁵⁷ Cys	3.57% (Hispanic)	(Stoilov et al., 1998)
<i>CYP1B1</i> *12	182G>A	Gly ⁶¹ Glu	17.85% (Turkish)	(Stoilov et al., 1998)
-	355G>T	Ala ¹¹⁹ Ser	50% (Seveso)	(Tanaka et al., 2002)
<i>CYP1B1</i> *18	1093G>T	Gly ³⁶⁵ Trp	3.57% (U.S.)	(Stoilov et al., 1998)
<i>CYP1B1</i> *19	1136C>T	Pro ³⁷⁹ Leu	3.57% (Turkish)	(Stoilov et al., 1998)
<i>CYP1B1</i> *20	1159G>A	Glu ³⁸⁷ Lys	7.14% (Hispanic, French Canadian)	(Stoilov et al., 1998)
<i>CYP1B1</i> *21	1169G>A	Arg ³⁹⁰ His	3.57% (Pakistani)	(Stoilov et al., 1998)
<i>CYP1B1</i> *23	1310C>T	Pro ⁴³⁷ Leu	3.57% (Turkish)	(Stoilov et al., 1998)
-	1328C>G	Ala ⁴⁴³ Gly	7% (Ethiopian)	(Akiyllu et al., 2002)
<i>CYP1B1</i> *25	1405C>T	Arg ⁴⁶⁹ Trp	14.29% (British, Turkish)	(Stoilov et al., 1998)

<http://www.cypalleles.ki.se/cyp1b1.htm>

Table 2 Sequence of the Primers Used for Site-directed Mutagenesis

Variants	Direction	Sequences (5' → 3')
Arg ⁴⁸ Gly	Forward	GGCGGCAGCTC <u>GGG</u> TCCGCGCCCC
	Reverse	GGGGCGCGGAC <u>CC</u> GAGCTGCCGCC
Trp ⁵⁷ Cys	Forward	CCCGTTTGC <u>TGCC</u> CACTGATCGGAAAC
	Reverse	GTTTCCGATCAGTGG <u>GCA</u> CGCAAACGGG
Gly ⁶¹ Glu	Forward	GGCCACTGATCG <u>AAA</u> ACGCGGCGGCG
	Reverse	CGCCGCCGCGT <u>TTC</u> GATCAGTGGCC
Ala ¹¹⁹ Ser	Forward	CCGACCGGCCG <u>TC</u> CTTCGCCTCCT
	Reverse	AGGAGGCGA <u>AGG</u> ACGGCCGGTCCG
Gly ³⁶⁵ Trp	Forward	GGATCAGGTCGT <u>TGG</u> AGGGACCGTCTG
	Reverse	CAGACGGTCCCT <u>CCA</u> CACGACCTGATCC
Pro ³⁷⁹ Leu	Forward	CAGCCCAACCTG <u>CT</u> TATGTCCTGGCCT
	Reverse	AGGCCAGGACATAG <u>AG</u> CAGGTTGGGCTG
Glu ³⁸⁷ Lys	Forward	CCTGGCCTTCCTTTAT <u>AAA</u> GCCATGCGCTTCTCC
	Reverse	GGAGAAGCGCATGGC <u>TTT</u> ATAAAGGAAGGCCAGG
Arg ³⁹⁰ His	Forward	CTTTATGAAGCCATG <u>CA</u> CTTCTCCAGCTTTGTGC
	Reverse	GCACAAAGCTGGAGA <u>AGT</u> GCATGGCTTCATAAAG-3'
Leu ⁴³² Val	Forward	GTGAATCATGACCCAG <u>TGA</u> AGTGGCCTAAC
	Reverse	GTTAGGCCACTT <u>CA</u> CTGGGTCATGATTCAC
Pro ⁴³⁷ Leu	Forward	GTGAAGTGGCCTAAC <u>CT</u> GGAGAACTTTGATCCAG
	Reverse	CTGGATCAAAGTTCT <u>CC</u> AGGTTAGGCCACTTCAC
Ala ⁴⁴³ Gly	Forward	GAGAACTTTGATCCAG <u>GT</u> CGATTCTTGGACAAGG
	Reverse	CCTTGTCCAAAGAATCG <u>ACC</u> TGGATCAAAGTTCTC
Asn ⁴⁵³ Ser	Forward	GGATGGCCTCATCAG <u>CA</u> AGGACCTGACCAG
	Reverse	CTGGTCAGGTCCTT <u>GCT</u> GATGAGGCCATCC
Arg ⁴⁶⁹ Trp	Forward	CAGTGGGCAAAAGG <u>TGG</u> TGCATTGGCGAAG
	Reverse	CTTCGCCAATGCAC <u>CA</u> CCTTTTGCCCACTG

Table 3 Expression Level of CYP1B1 Variant Proteins in the Microsomes

Missense Mutations	Expression Level (pmol/μg)
Trp ⁵⁷ Cys	0.105
Gly ⁶¹ Glu	0.295
Ala ¹¹⁹ Ser	0.157
Gly ³⁶⁵ Trp	0.038
Pro ³⁷⁹ Leu	0.055
Glu ³⁸⁷ Lys	0.024
Arg ³⁹⁰ His	-
Pro ⁴³⁷ Leu	-
Ala ⁴⁴³ Gly	0.263
Asn ⁴⁵³ Ser	-
Arg ⁴⁶⁹ Trp	0.19
Arg ⁴⁸ Gly/Ala ¹¹⁹ Ser	0.261
Arg ⁴⁸ Gly/Leu ⁴³² Val	0.118
Arg ⁴⁸ Gly/Ala ¹¹⁹ Ser/Leu ⁴³² Val	0.281
Arg ⁴⁸ Gly/Ala ¹¹⁹ Ser/Leu ⁴³² Val/Ala ⁴⁴³ Gly	0.324

Table 4 Kinetic Parameters for PhIP N-hydroxylation and 4-Hydroxylation by Human CYP1B1

CYP1B1 Variants	N ² -OH-PhIP			4'-OH-PhIP		
	<i>K_m</i>	<i>V_{max}</i>	<i>K_{cat}</i> (<i>V_{max}</i> / <i>K_m</i>)	<i>K_m</i>	<i>V_{max}</i>	<i>K_{cat}</i> (<i>V_{max}</i> / <i>K_m</i>)
	<i>μM</i>	nmol/min/nmol		<i>μM</i>	nmol/min/nmol	
wild-type CYP1B1	3.6	0.115	0.032	4.5	0.120	0.026
Trp ⁵⁷ Cys	8.5	0.045	0.005	10.4	0.085	0.008
Gly ⁶¹ Glu	7.5	0.086	0.011	3.9	0.056	0.014
Arg ⁴⁸ Gly/Ala ¹¹⁹ Ser	3.2	0.057	0.018	4.1	0.049	0.012
Arg ⁴⁸ Gly/Ala ¹¹⁹ Ser/Leu ⁴³² Val	14.8	0.153	0.010	6.0	0.100	0.017
Arg ⁴⁸ Gly/Ala ¹¹⁹ Ser/Leu ⁴³² Val/Ala ⁴⁴³ Gly	30.1	0.210	0.007	2.7	0.057	0.021

The values are the average of duplicated experiments with less than 10% variation.

Fig. 1

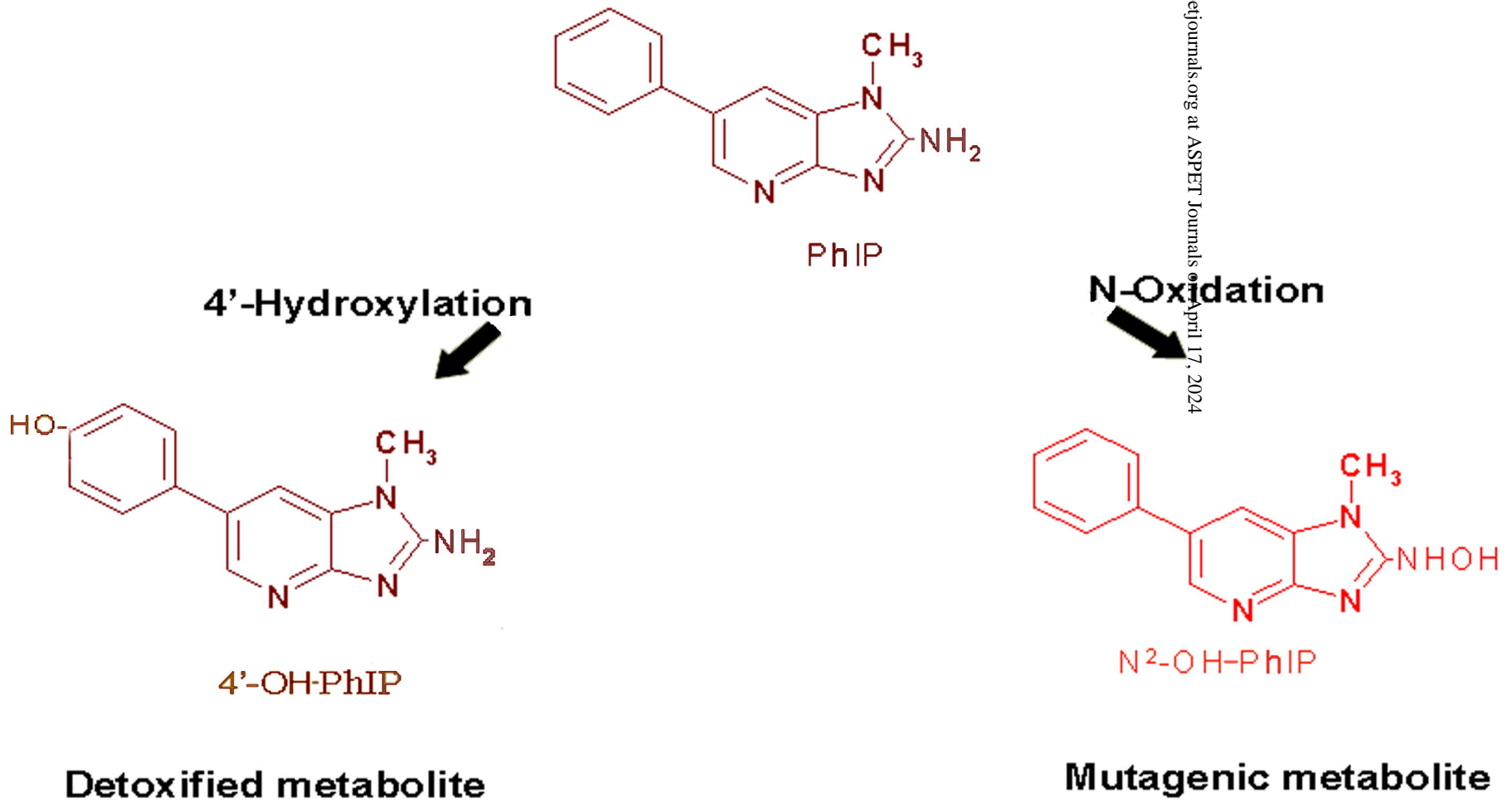
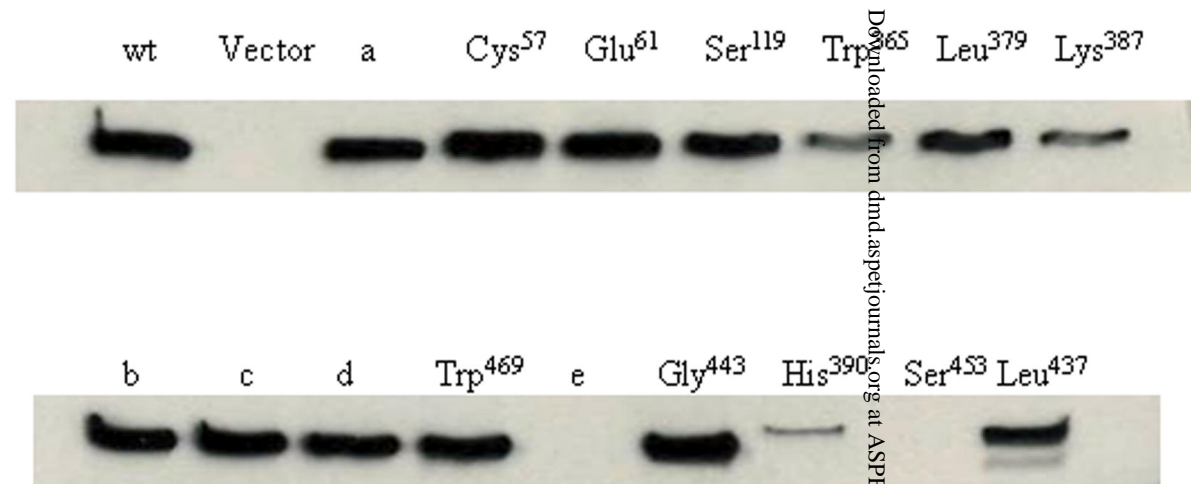


Fig. 2

A. Immunoblot analysis:
CYP1B1



a -- Gly⁴⁸Val⁴³², b -- Gly⁴⁸Ser¹¹⁹Val⁴³²Gly⁴⁴³, c -- Gly⁴⁸Ser¹¹⁹Val⁴³², d -- Gly⁴⁸Ser¹¹⁹, e -- Protein Size Marker

B. CO-difference spectra

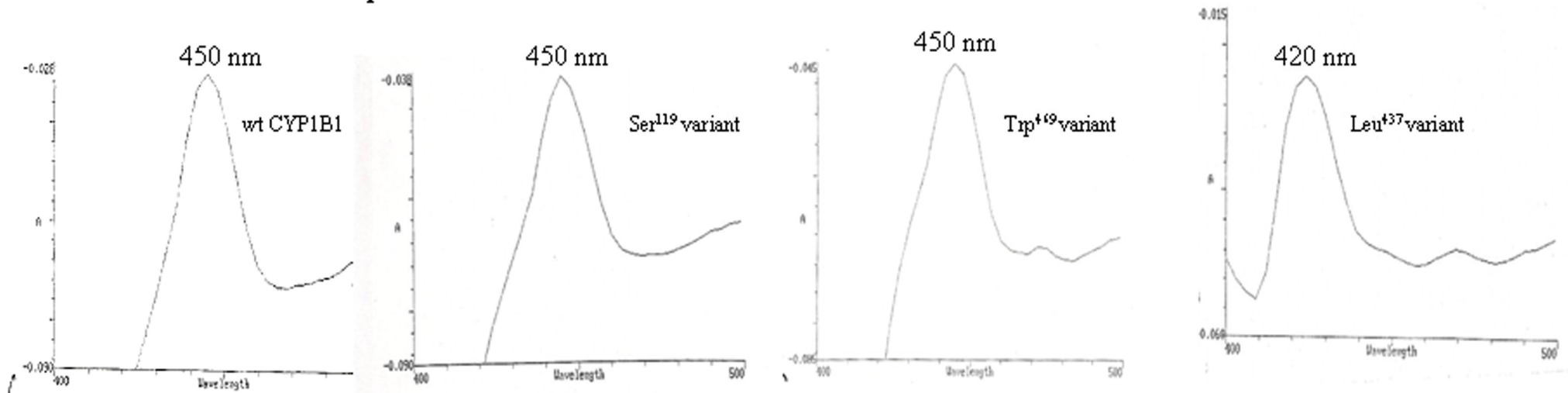


Fig. 3

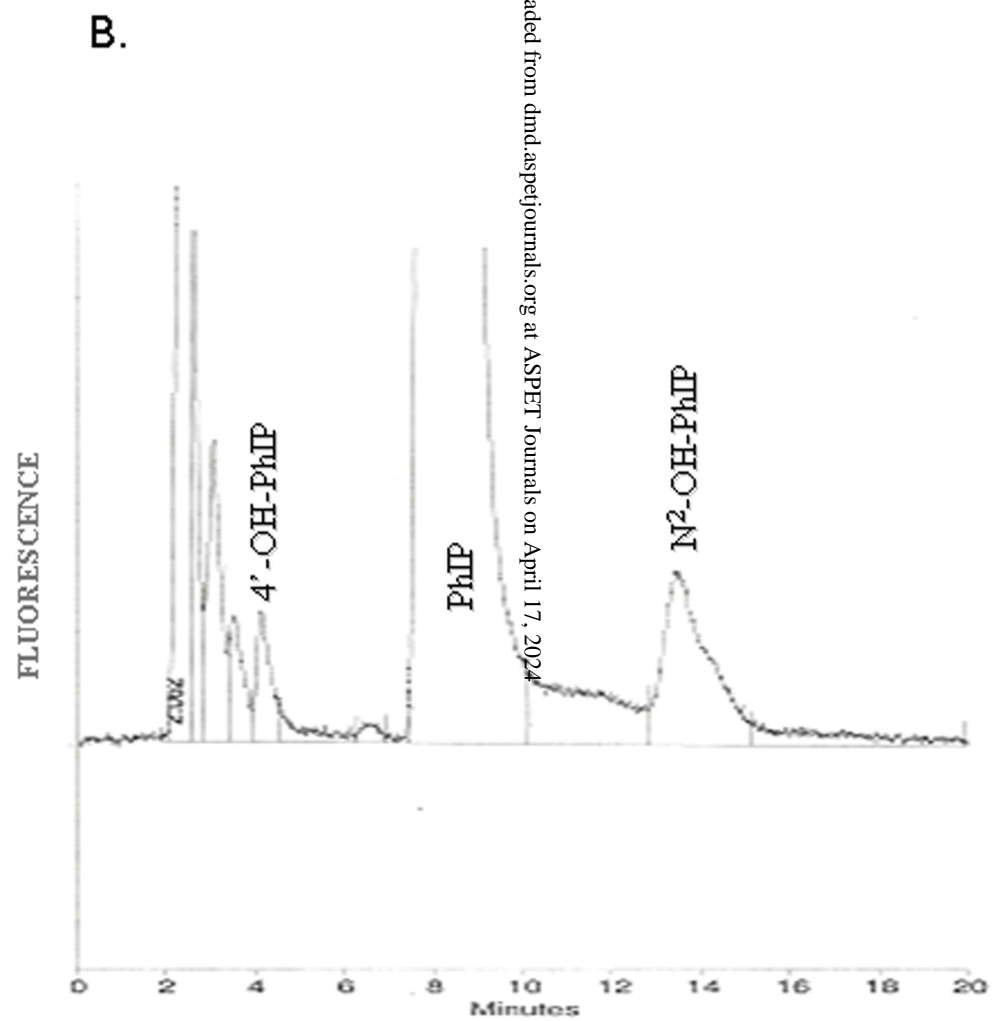
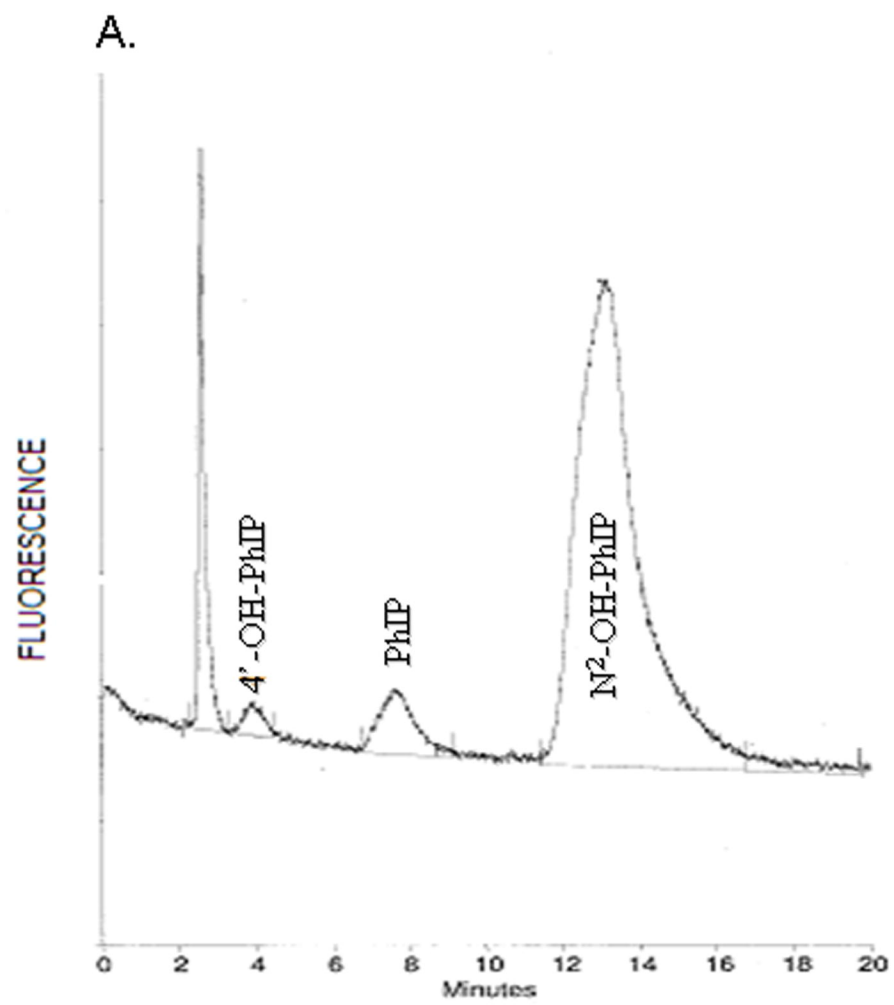
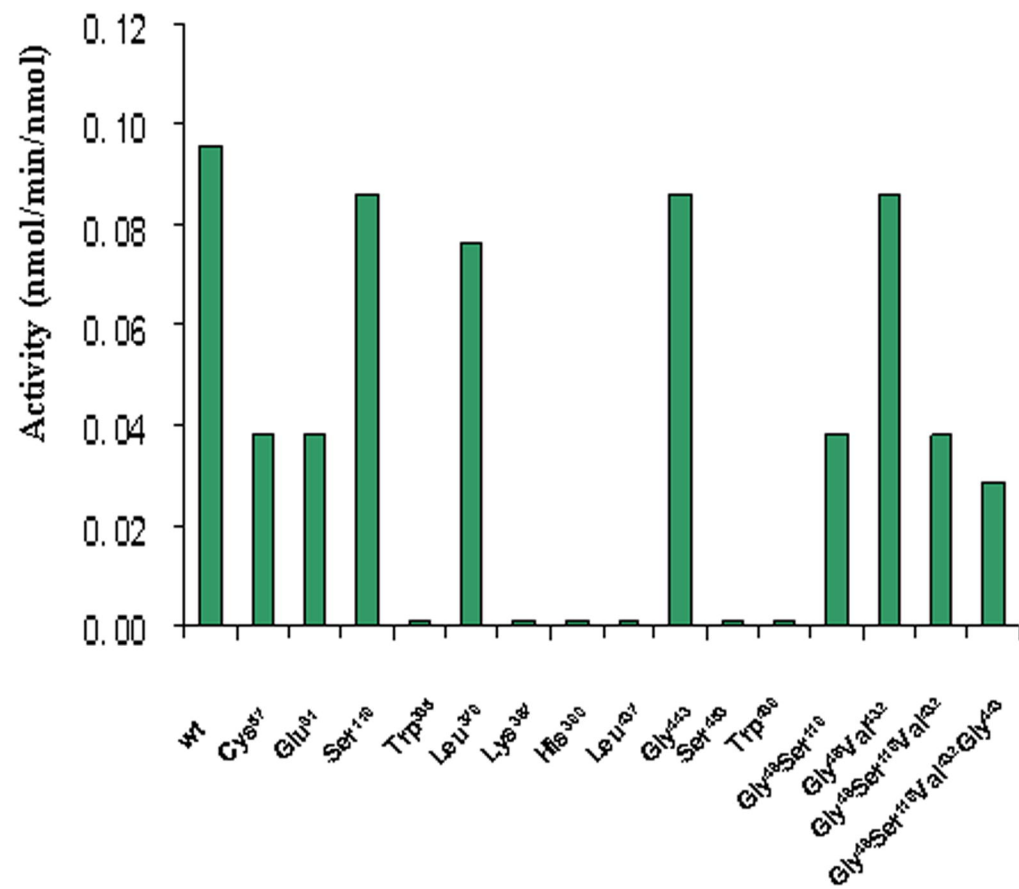


Fig. 4

A. N²-OH-PhIP formation



B. 4'-OH-PhIP formation

