DMD Fast Forward. Published on January 28, 2008 as DOI: 10.1124/dmd.107.016824
This article has not been copyedited and formatted. The final version may differ from this version.
DMD Fast Forward. Published on January 28, 2008 as doi:10.1124/dmd.107.016824

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.).

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 17, 2024

Copyright 2008 by the American Society for Pharmacology and Experimental Therapeutics.

DMD Fast Forward. Published on January 28, 2008 as DOI: 10.1124/dmd.107.016824
This article has not been copyedited and formatted. The final version may differ from this version.

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@eohsi.rutgers.edu

Number of text pages	16
Number of tables	4
Number of figures	4
Number of references	36
Abstract	250
Introduction	565
Discussion	1050

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 17, 2024

DMD Fast Forward. Published on January 28, 2008 as DOI: 10.1124/dmd.107.016824
This article has not been copyedited and formatted. The final version may differ from this version.

List of abbreviations

HCAs	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

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 17, 2024

DMD Fast Forward. Published on January 28, 2008 as DOI: 10.1124/dmd.107.016824
This article has not been copyedited and formatted. The final version may differ from this version.

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 (N^2-OH-PhIP) and 4'-hydroxy-PhIP (4'-OH-PhIP). While the PhIP-metabolizing activities of four variants (Ala^{119}Ser, Pro^{379}Leu, Ala^{443}Gly, Arg^{48}Gly/Leu^{432}Val) were comparable to that of the expressed wild-type CYP1B1, five variants (Trp^{57}Cys, Gly^{61}Glu, Arg^{48}Gly/Ala^{119}Ser, Arg^{48}Gly/Ala^{119}Ser/ Leu^{432}Val, Arg^{48}Gly/ Ala^{119}Ser/Leu^{432}Val/Ala^{443}Gly) 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 (Gly^{365}Trp, Glu^{387}Lys, Arg^{390}His, Pro^{437}Leu, Asn^{453}Ser, Arg^{469}Trp) showed little activity in PhIP metabolism but the molecular mechanisms involved are apparently different. The microsomal CYP1B1 protein level was significantly decreased for the Trp^{365}, Lys^{387}, and His^{390} variants and was not detectable for the Ser^{453} variant. In contrast, there was no difference between the Trp^{469} variant and the wild-type in the microsomal CYP1B1 protein level and P450 content but the Trp^{469} variant totally lost its metabolic activity towards PhIP. The Leu^{437} 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.

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 17, 2024

DMD Fast Forward. Published on January 28, 2008 as DOI: 10.1124/dmd.107.016824
This article has not been copyedited and formatted. The final version may differ from this version.

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 (N^2-OH-PhIP) and 4’-hydroxy-PhIP (4’-OH-PhIP) (Shirai et al., 1997; King et al., 1999) (Fig. 1). N^2-OH-PhIP is highly

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 17, 2024

DMD Fast Forward. Published on January 28, 2008 as DOI: 10.1124/dmd.107.016824
This article has not been copyedited and formatted. The final version may differ from this version.

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 Val^{432} form of CYP1B1, the Leu^{432} 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).

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 17, 2024

DMD Fast Forward. Published on January 28, 2008 as DOI: 10.1124/dmd.107.016824
This article has not been copyedited and formatted. The final version may differ from this version.

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, N^2-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 N^2-OH-PhIP were purchased from Toronto Research Chemicals (Toronto, Ontario, Canada). For standard validation, we also obtained N^2-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). $B_{AC\text{-}TO\text{-}}B_{AC}$ baculovirus expression system (including pFastbac1expression 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

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 17, 2024

DMD Fast Forward. Published on January 28, 2008 as DOI: 10.1124/dmd.107.016824
This article has not been copyedited and formatted. The final version may differ from this version.

chemiluminescence reagents for immunoblotting detection were obtained from Amersharm 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 $B_{AC\text{-}TO\text{-}}B_{AC}$ 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 μM. The infected

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 17, 2024

DMD Fast Forward. Published on January 28, 2008 as DOI: 10.1124/dmd.107.016824
This article has not been copyedited and formatted. The final version may differ from this version.

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

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 17, 2024

DMD Fast Forward. Published on January 28, 2008 as DOI: 10.1124/dmd.107.016824
This article has not been copyedited and formatted. The final version may differ from this version.

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 37ºC for 30 min. The reaction was terminated by adding one volume of ice-cold methanol. The mixture was then centrifuged at 4ºC 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 availably 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

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 17, 2024

DMD Fast Forward. Published on January 28, 2008 as DOI: 10.1124/dmd.107.016824
This article has not been copyedited and formatted. The final version may differ from this version.

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 µl. The excitation wavelength was 316 nm and the emission wavelength was 370 nm. The analytical protocol was able to clearly resolve N^2-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 N^2-OH-PhIP and 4'-OH-PhIP. Under our analytical conditions, the limits of detection were 0.02 pg for N^2-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 $B_{AC\text{-}TO\text{-}}B_{AC}$ 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 $Asn^{453}Ser$ variant cDNA. The microsomal CYP1B1 protein levels for the $Gly^{365}Trp$, $Glu^{387}Lys$ and $Arg^{390}His$ variants were reproducibly lower than the

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 17, 2024

DMD Fast Forward. Published on January 28, 2008 as DOI: 10.1124/dmd.107.016824
This article has not been copyedited and formatted. The final version may differ from this version.

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 Gly^{365}Trp, Pro^{379}Leu, and Glu^{387}Lys variants proteins with an average of 39 pmol/mg (range: 24-55 pmol/mg) (Table 3). Microsomes from the cells infected with the Arg^{390}His and Asn^{453}Ser variants cDNAs showed no detectable P450 (data not shown) and little or no detectable CYP1B1proteins (Fig. 2A). The Pro^{437}Leu 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 N^2-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, N^2-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

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 17, 2024

DMD Fast Forward. Published on January 28, 2008 as DOI: 10.1124/dmd.107.016824
This article has not been copyedited and formatted. The final version may differ from this version.

times of N^2-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 N^2-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 µM) and low (1 µM) substrate concentrations. Fig. 4 shows that at 10 µM of PhIP, the variants $Ala^{119}Ser$, $Pro^{379}Leu$, $Ala^{443}Gly$ and $Arg^{48}Gly/Leu^{432}Val$ displayed almost the same activity as the wild-type CYP1B1 in both N-hydroxylation and 4-hydroxylation of PhIP. The activities of the variants $Trp^{57}Cys$, $Gly^{61}Glu$, $Arg^{48}Gly/Ala^{119}Ser$, $Arg^{48}Gly/Ala^{119}Ser/Leu^{432}Val$, $Arg^{48}Gly/$ $Ala^{119}Ser/Leu^{432}Val/$ Ala^{443} were lower than the wild-type CYP1B1. There was no detectable PhIP-metabolizing activity in the variants $Gly^{365}Trp$, $Glu^{387}Lys$, $Arg^{390}His$, $Pro^{437}Leu$, $Asn^{453}Ser$. It is of interest to note that although the $Arg^{469}Trp$ 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 µM substrate concentration was consistent with the result with 1 µM substrate concentration (data not shown).

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

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 17, 2024

DMD Fast Forward. Published on January 28, 2008 as DOI: 10.1124/dmd.107.016824
This article has not been copyedited and formatted. The final version may differ from this version.

decrease in PhIP-metabolizing activity for detailed kinetic analysis. These included the variants $Trp^{57}Cys$, $Gly^{61}Glu$, $Arg^{48}Gly/Ala^{119}Ser$, $Arg^{48}Gly/Ala^{119}Ser/Leu^{432}Val$, and $Arg^{48}Gly/$ $Ala^{119}Ser/Leu^{432}Val/$ $Ala^{443}Gly$. The kinetics of PhIP metabolism was determined with eight different substrate concentrations, ranging from 0.3 to 50 μM. The experiments were always conduced 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 N^2-OH-PhIP and 4'-OH-PhIP was initially linear but reached saturation at the PhIP concentrations above 10 μM. 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 μM for N-hydroxylation of PhIP and 4.5 μM for 4-hydroxylation in our study while the corresponding values reported by Crofts et al. were 5.7 μM and 2.2 μM. 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 $Arg^{48}Gly/Ala^{119}Ser$ variant, it was increased in the other four variants ($Trp^{57}Cys$, $Gly^{61}Glu$, $Arg^{48}Gly/Ala^{119}Ser/Leu^{432}Val$, and $Arg^{48}Gly/$ $Ala^{119}Ser/$ $Leu^{432}Val$ / $Ala^{443}Gly$). The catalytic efficiency (V_{max}/K_m) for PhIP N-hydroxylation in the variant $Arg^{48}Gly/Ala^{119}Ser/Leu^{432}Val$ / $Ala^{443}Gly$ variant

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 17, 2024

DMD Fast Forward. Published on January 28, 2008 as DOI: 10.1124/dmd.107.016824
This article has not been copyedited and formatted. The final version may differ from this version.

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 N^2-OH-PhIP and 4'-OH-PhIP formation appears to be different for different variants. For the $Trp^{57}Cys$ variant, the K_m value for N^2-OH-PhIP and 4'-OH-PhIP were both increased, and the V_{max} values were both decreased. For the $Gly^{61}Glu$ variant, the K_m value for N^2-OH-PhIP formation was increased; but was unchanged for 4'-OH-PhIP formation. The *K*m values of the variants $Arg^{48}Gly/Ala^{119}Ser/Leu^{432}Val$ and $Arg^{48}Gly/Ala^{119}Ser/Leu^{432}Val/Ala^{443}Gly$ for N^2-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 $Arg^{48}Gly/Ala^{119}Ser$ variant, the K_m values were not changed for the formation of both N^2-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

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 17, 2024

DMD Fast Forward. Published on January 28, 2008 as DOI: 10.1124/dmd.107.016824
This article has not been copyedited and formatted. The final version may differ from this version.

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 $Asn^{453}Ser$ variant and was significantly reduced for the $Gly^{365}Trp$, $Glu^{387}Lys$ and $Arg^{390}His$ 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), Gly^{365} belongs to the conserved core structures of CYP1B1. Substitution of Gly^{365} with Trp may interfere with the proper folding and heme-binding of CYP1B1 protein. Both Glu^{387} and Arg ^{390} 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 $Asn^{453}Ser$ variant protein in our observation is consistent with a recent study in which there was a significant decrease in the level of $Asn^{453}Ser$ variant protein transiently expressed in the COS-1 cells (Bandiera et al., 2005). The same authors also demonstrated that the degradation of the $Asn^{453}Ser$ variant protein by the proteasomal pathway is much faster than the wild-type CYP1B1 protein (Bandiera et al., 2005), suggesting that the substitution of Asn^{453} with Ser leads to an unstable protein structure. In contrast to a reported finding that the $Gly^{61}Glu$ variant has a greatly

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 17, 2024

DMD Fast Forward. Published on January 28, 2008 as DOI: 10.1124/dmd.107.016824
This article has not been copyedited and formatted. The final version may differ from this version.

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 Pro^{437}Leu and Arg^{469}Trp 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 Pro^{437}Leu 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 Pro^{437} 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 Arg^{469}Trp variant，which is consistent with the observation of Jansson et al. that the stability of this variant protein was nomal (Jansson et al., 2001). As predicted by the same model (Stoilov et al., 1998), Cys^{470} 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. Arg^{469} immediately precedes Cys^{470} 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), Trp^{57} 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, Gly^{61} is also very close to the

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 17, 2024

DMD Fast Forward. Published on January 28, 2008 as DOI: 10.1124/dmd.107.016824
This article has not been copyedited and formatted. The final version may differ from this version.

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 $Arg^{48}Gly/Ala^{119}Ser$ (CYP1B1.2), $Arg^{48}Gly/Ala^{119}Ser/Leu^{432}Val$ (CYP1B1*6), and $Arg^{48}Gly/Ala^{119}Ser/Leu^{432}Val/Ala^{443}Gly$ (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 $Arg^{48}Gly/Ala^{119}Ser/ Leu^{432}Val$ (CYP1B1.6) and $Arg^{48}Gly/Ala^{119}Ser/ Leu^{432}Val/Ala^{443}Gly$ (CYP1B1.7) were found to have the most significant change in the K_m values for N^2-OH-PhIP formation (14.8 and 30.1 μM, respectively, versus 3.6 μM in wild-type CYP1B1) but not in the 4'-OH-PhIP formation. Because the $Arg^{48}Gly/Ala^{119}Ser$ 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 $Arg^{48}Gly/Ala^{119}Ser/ Leu^{432}Val$ (CYP1B1*6) and $Arg^{48}Gly/Ala^{119}Ser/ Leu^{432}Val/Ala^{443}Gly$ (CYTP1B1*7) are mainly due to the inclusion of a common $Leu^{432}Val$ substitution. Ala^{443} is conserved in the mouse, rat and human CYP1B1. Inclusion of an additional $Ala^{443}Gly$ substitution in the

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 17, 2024

DMD Fast Forward. Published on January 28, 2008 as DOI: 10.1124/dmd.107.016824
This article has not been copyedited and formatted. The final version may differ from this version.

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.

Acknowledgement

We thank Yi-Hua Jan for help in the enzyme kinetics study and Emerson Liu for editing the manuscript.

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 17, 2024

DMD Fast Forward. Published on January 28, 2008 as DOI: 10.1124/dmd.107.016824
This article has not been copyedited and formatted. The final version may differ from this version.

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

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 17, 2024

DMD Fast Forward. Published on January 28, 2008 as DOI: 10.1124/dmd.107.016824
This article has not been copyedited and formatted. The final version may differ from this version.

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. Carcinogeneis. 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.

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 17, 2024

DMD Fast Forward. Published on January 28, 2008 as DOI: 10.1124/dmd.107.016824
This article has not been copyedited and formatted. The final version may differ from this version.

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, Watanable J, Guengerich F P, Inoue K and Gillam E M J (2001) Specificity of 17ß-oestradiol and benzo[α]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

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 17, 2024

DMD Fast Forward. Published on January 28, 2008 as DOI: 10.1124/dmd.107.016824
This article has not been copyedited and formatted. The final version may differ from this version.

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 propeties of a detergent-solubilized NADPH-cytochrome c (cytochrome P-450) reductase purified by biospecific affinity chromatography. . J Biol Chem. 251: 5337-5344.

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 17, 2024

DMD Fast Forward. Published on January 28, 2008 as DOI: 10.1124/dmd.107.016824
This article has not been copyedited and formatted. The final version may differ from this version.

Footnotes

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

This work was supported by the US Army Medical Research Prostate Cancer Program DAMD 17-02-0250 (J-Y Hong).

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 17, 2024

DMD Fast Forward. Published on January 28, 2008 as DOI: 10.1124/dmd.107.016824
This article has not been copyedited and formatted. The final version may differ from this version.

Figures Legends

FIG. 1 M*etabolic 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 µg) 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 µg/µl) 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 Ser^{119} and Trp^{469} variants showed the characteristic P450 absorption peak, while the Leu^{437} 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 µl. The excitation wavelength was 316nm and the

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 17, 2024

DMD Fast Forward. Published on January 28, 2008 as DOI: 10.1124/dmd.107.016824
This article has not been copyedited and formatted. The final version may differ from this version.

emission wavelength was 370nm. **A.** Mixture of the PhIP metabolite standards: 4'-OH-PhIP (5 pg) and N^2-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 $MgCl_2$, 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 37ºC 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 Trp^{365}, Lys^{387}, His^{390}, Leu^{437}, Ser^{453} and Trp^{469}.

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 17, 2024

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

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

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

DMD Fast Forward. Published on January 28, 2008 as DOI: 10.1124/dmd.107.016824
This article has not been copyedited and formatted. The final version may differ from this version.

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 17, 2024

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

Variants	Direction	Sequences (5'→3')
Arg48Gly	Forward	GGCGGCAGCTCGGGTCCGCGCCCC
	Reverse	GGGGCGCGGACCCGAGCTGCCGCC
Trp57Cys	Forward	CCCGTTTGCGTGCCCACTGATCGGAAAC
	Reverse	GTTTCCGATCAGTGGGCACGCAAACGGG
Gly61Glu	Forward	GGCCACTGATCGAAAACGCGGCGGCG
	Reverse	CGCCGCCGCGTTTTCGATCAGTGGCC
Ala119Ser	Forward	CCGACCGGCCGTCCTTCGCCTCCT
	Reverse	AGGAGGCGAAGGACGGCCGGTCGG
Gly365Trp	Forward	GGATCAGGTCGTGTGGAGGGACCGTCTG
	Reverse	CAGACGGTCCCTCCACACGACCTGATCC
Pro379Leu	Forward	CAGCCCAACCTGCTCTATGTCCTGGCCT
	Reverse	AGGCCAGGACATAGAGCAGGTTGGGCTG
Glu387Lys	Forward	CCTGGCCTTCCTTTATAAAGCCATGCGCTTCTCC
	Reverse	GGAGAAGCGCATGGCTTTATAAAGGAAGGCCAGG
Arg390His	Forward	CTTTATGAAGCCATGCACTTCTCCAGCTTTGTGC
	Reverse	GCACAAAGCTGGAGAAGTGCATGGCTTCATAAAG-3'
Leu432Val	Forward	GTGAATCATGACCCAGTGAAGTGGCCTAAC
	Reverse	GTTAGGCCACTTCACTGGGTCATGATTCAC
Pro437Leu	Forward	GTGAAGTGGCCTAACCTGGAGAACTTTGATCCAG
	Reverse	CTGGATCAAAGTTCTCCAGGTTAGGCCACTTCAC
Ala443Gly	Forward	GAGAACTTTGATCCAGGTCGATTCTTGGACAAGG
	Reverse	CCTTGTCCAAGAATCGACCTGGATCAAAGTTCTC
Asn453Ser	Forward	GGATGGCCTCATCAGCAAGGACCTGACCAG
	Reverse	CTGGTCAGGTCCTTGCTGATGAGGCCATCC
Arg469Trp	Forward	CAGTGGGCAAAAGGTGGTGCATTGGCGAAG
	Reverse	CTTCGCCAATGCACCACCTTTTGCCCACTG

DMD Fast Forward. Published on January 28, 2008 as DOI: 10.1124/dmd.107.016824
This article has not been copyedited and formatted. The final version may differ from this version.

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 17, 2024

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

DMD Fast Forward. Published on January 28, 2008 as DOI: 10.1124/dmd.107.016824
This article has not been copyedited and formatted. The final version may differ from this version.

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 17, 2024

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

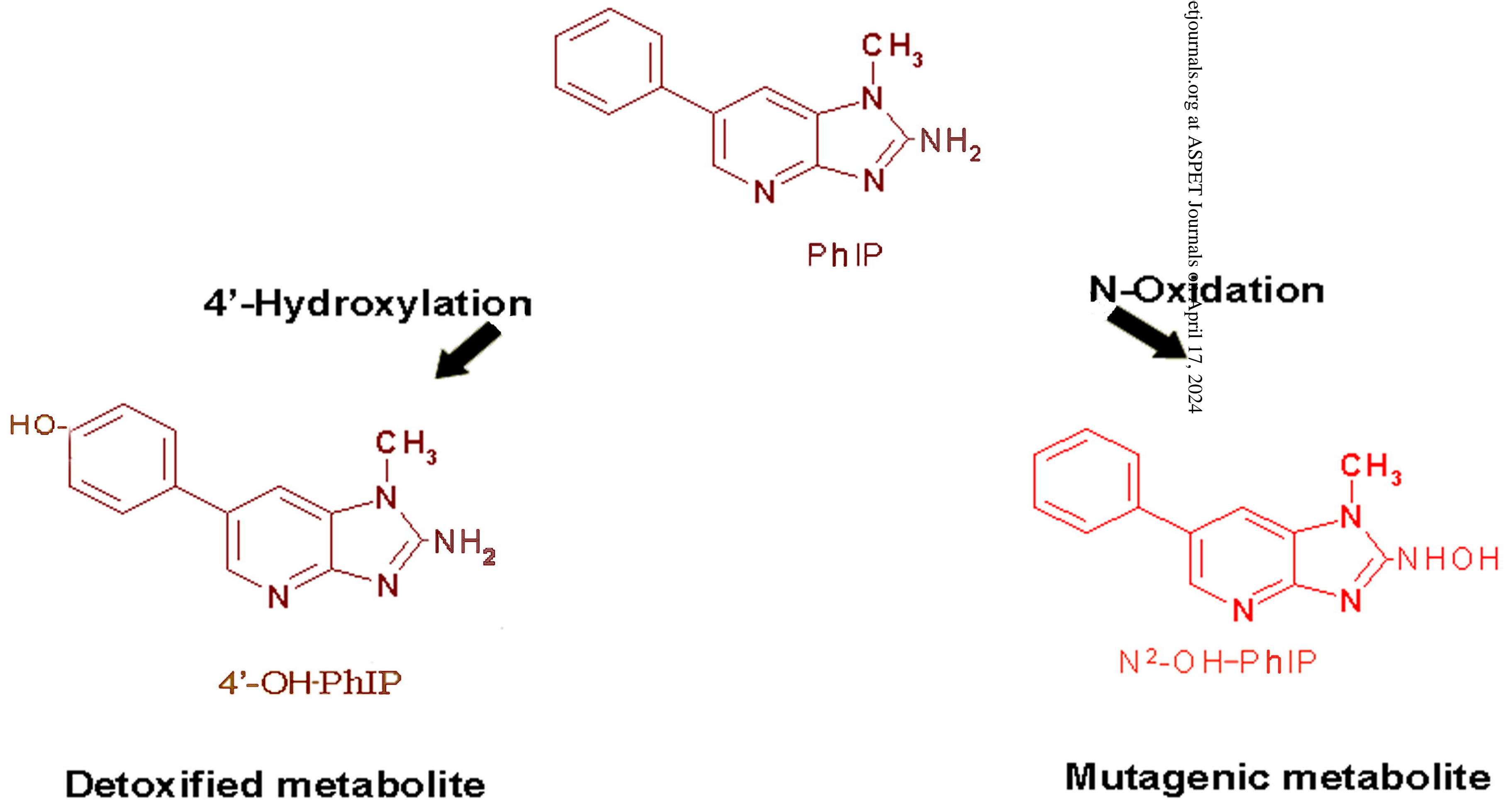
CYP1B1 Variants	N^2-OH-PhIP			4'-OH-PhIP		
	K_m	V_{max}	K_{cat} (V_{max}/K_m)	K_m	V_{max}	K_{cat} (V_{max}/K_m)
	μM	**nmol/min/nmol**		μM	**nmol/min/nmol**	
wild-type CYP1B1	3.6	0.115	0.032	4.5	0.120	0.026
$Trp^{57}Cys$	8.5	0.045	0.005	10.4	0.085	0.008
$Gly^{61}Glu$	7.5	0.086	0.011	3.9	0.056	0.014
$Arg^{48}Gly/Ala^{119}Ser$	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.

DMD Fast Forward. Published on January 28, 2008 as DOI: 10.1124/dmd.107.016824
This article has not been copyedited and formatted. The final version may differ from this version.

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 17, 2024

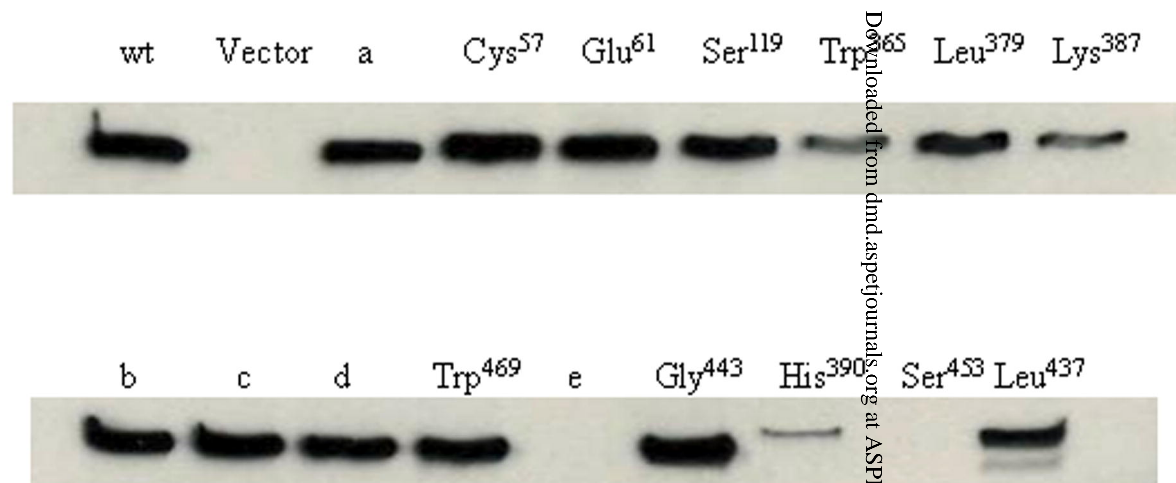
Fig. 1



Downloaded from dmd.aspetjournals.org at ASPET Journals on April 17, 2024

Fig. 2

A. Immunoblot analysis:



a -- $Gly^{48}Val^{432}$, b – $Gly^{48}Ser^{119}Val^{432}Gly^{443}$, c – $Gly^{48}Ser^{119}Val^{432}$, d – $Gly^{48}Ser^{119}$, e – Protein Size Marker

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 17, 2024

B. CO-difference spectra

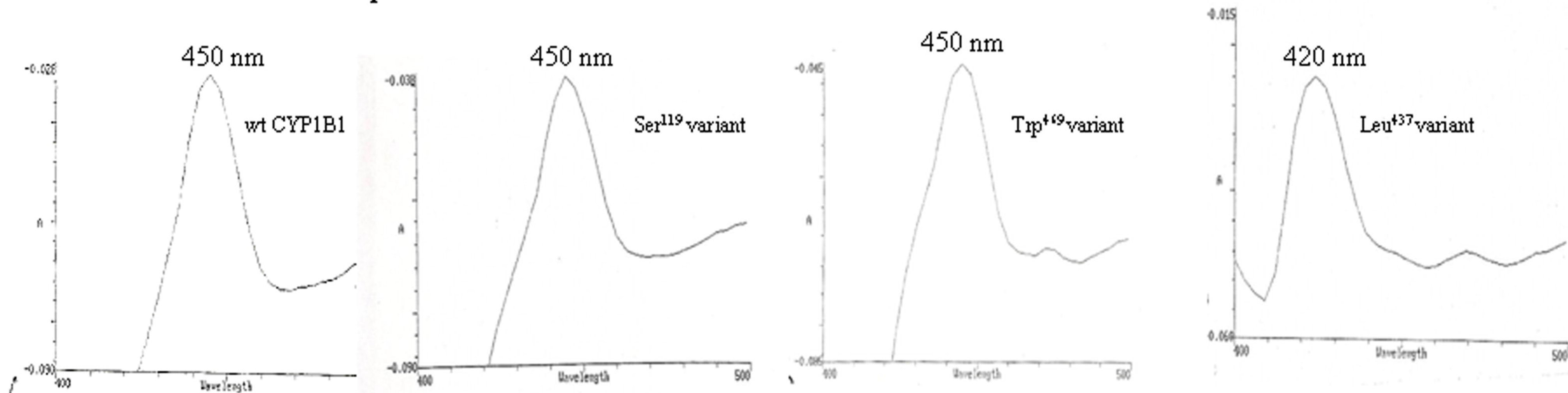
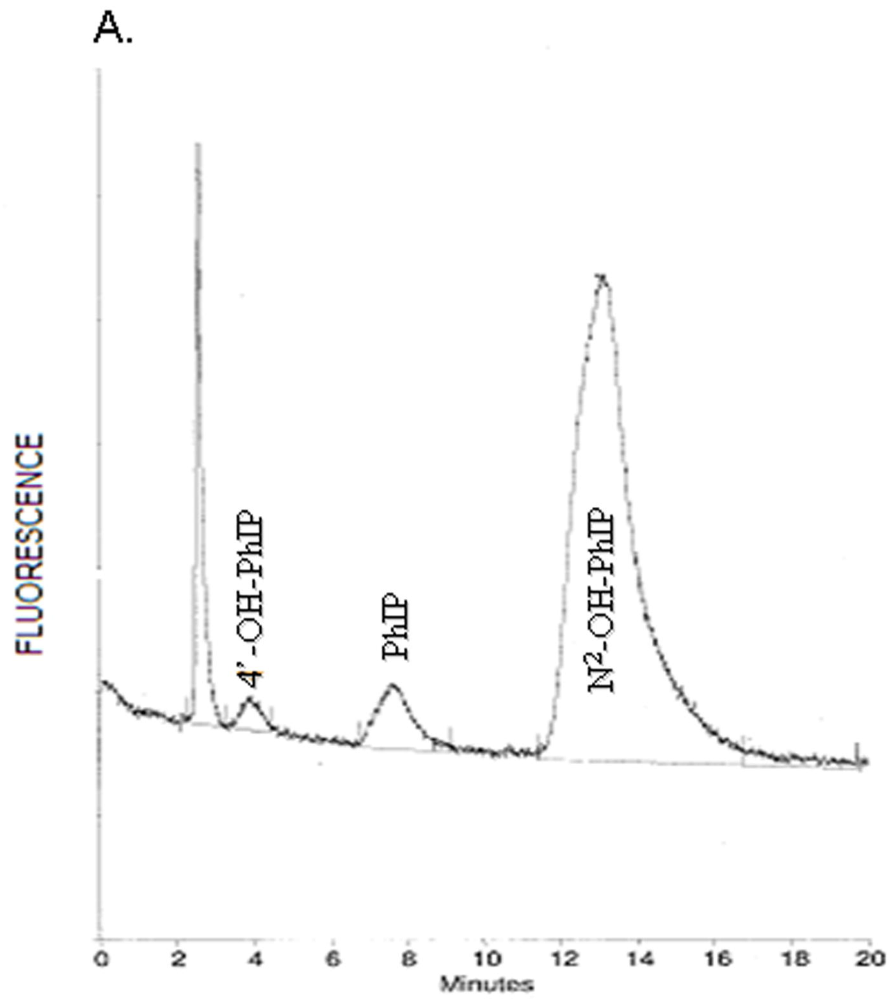
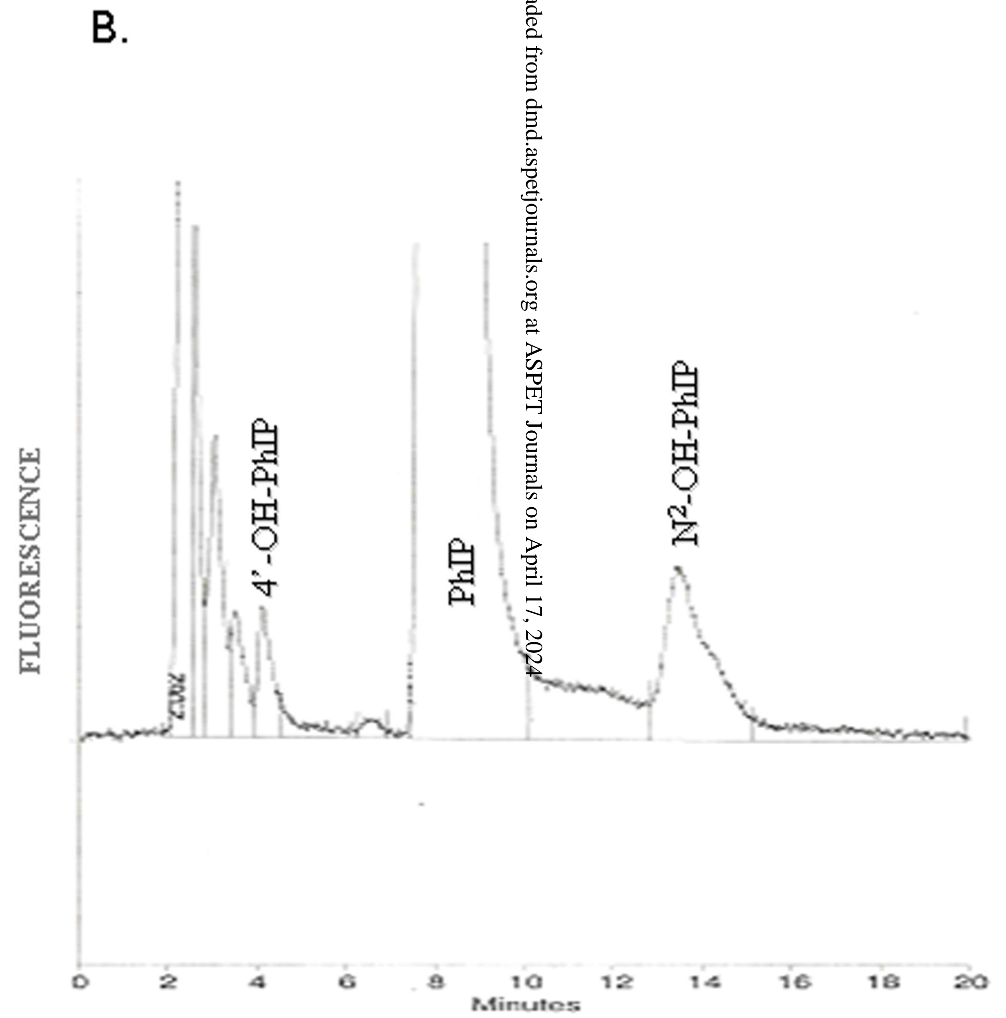


Fig. 3

A.



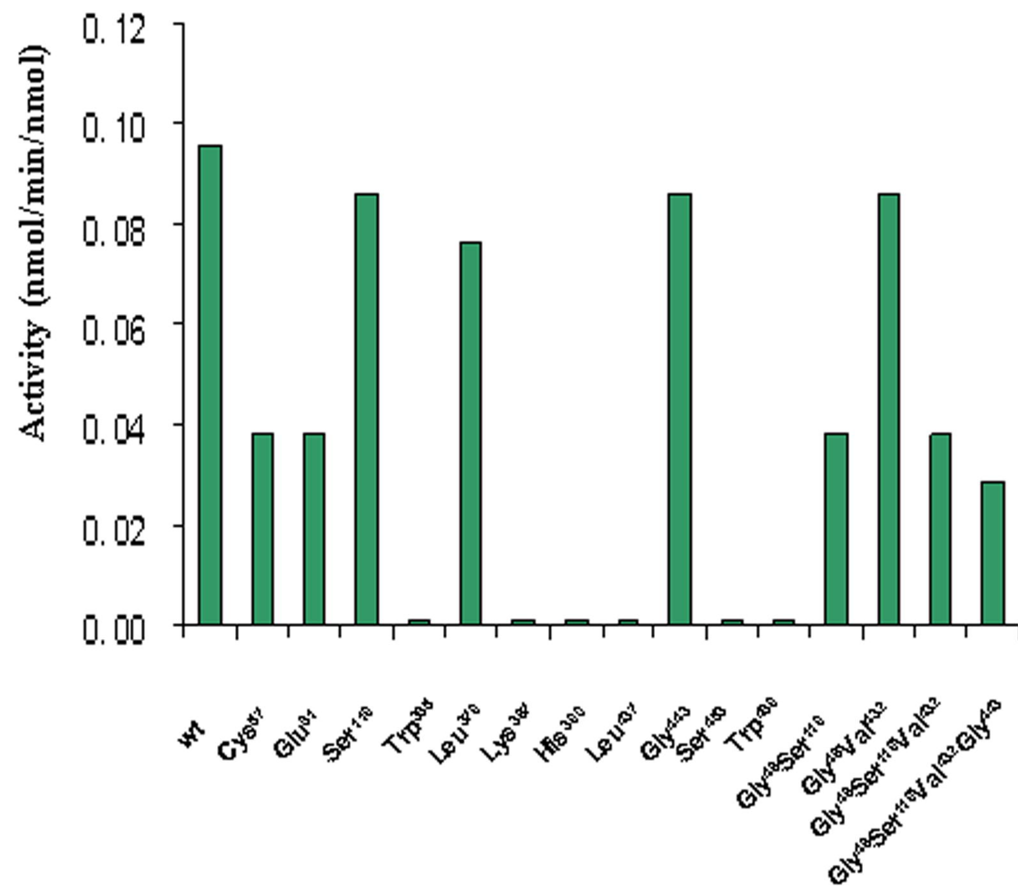
B.



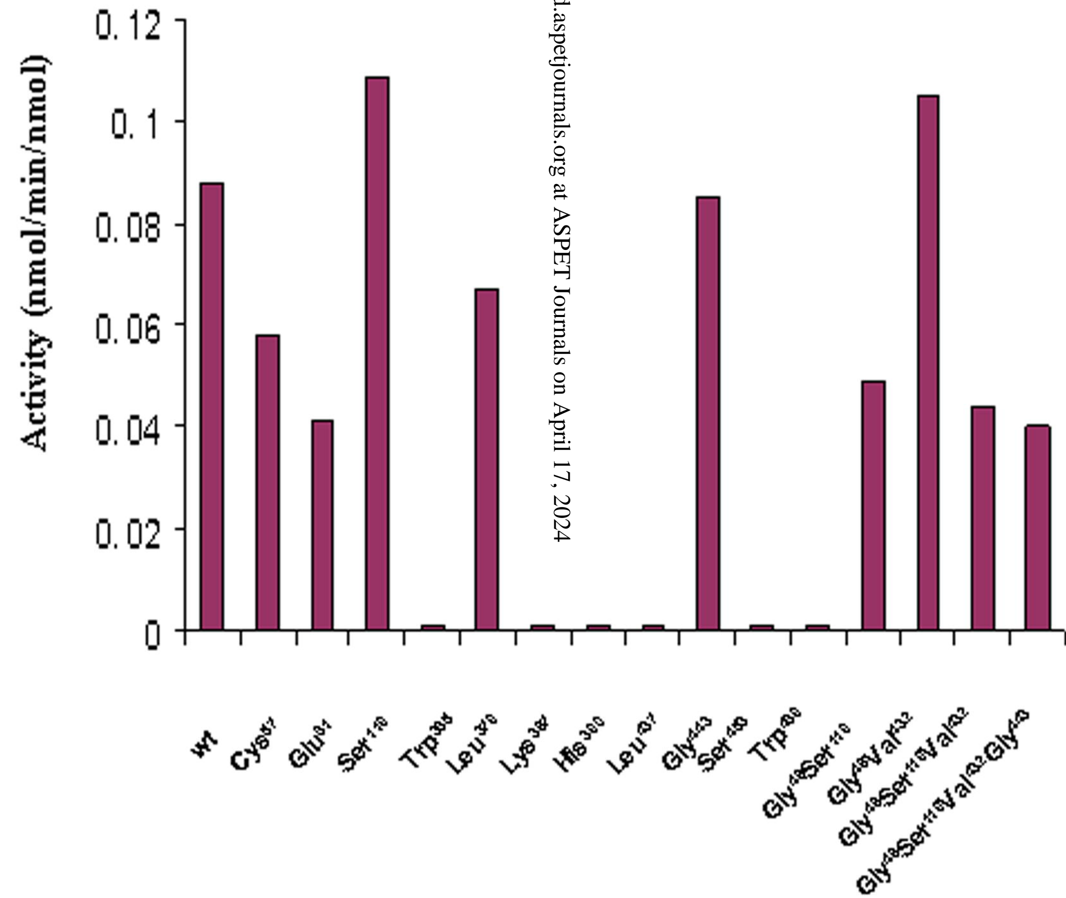
Downloaded from dmd.aspetjournals.org at ASPET Journals on April 17, 2024

Fig. 4

A. N^2-OH-PhIP formation



B. 4'-OH-PhIP formation



Downloaded from dmd.aspetjournals.org at ASPET Journals on April 17, 2024