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13. SUPPLEMENTARY NOTES

The etiology of human prostate cancer is not well understood. Epidemiological studies suggest that exposure to carcinogenic heterocyclic amines (HCA) such as PhIP formed in high-temperature cooked meat/fish is an important risk factor. The Phase I metabolism of PhIP in human is mainly catalyzed by CYP enzymes, which leads to the formation of 2-hydroxyamino-PhIP (*N*-hydroxy PhIP), the carcinogenic metabolite, and 4'-hydroxy PhIP (4'-hydroxy PhIP), the non-carcinogenic metabolite. In the present study, we established a highly sensitive LC/MS method and used it to determine the capability of human prostate tissues (n=31) in metabolizing PhIP and other carcinogenic HCA. Our results indicate that there is no significant *N*-hydroxylation of PhIP, IQ, MeIQ and MeIQx in human prostate microsomes. We also characterized the functional significance of 15 polymorphic variants of CYP1B1, which is a major human enzyme for PhIP metabolic activation in extrahepatic tissues. Results of our study provide important information on the understanding of inter-individual susceptibility to prostate cancer.

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Introduction (modified from previous annual reports)

In the United States, prostate cancer is the most commonly diagnosed cancer in men, and the incidence is rising rapidly. According to a projection by the American Cancer Society, a total of 230,110 new cases of prostate cancer are estimated in the United States in the year 2004 and About 29,900 men will die of this disease (www.cancer.org). They also estimate that African-American men are more likely to have prostate cancer and to die of it than are white or Asian men and the reasons for this are still not known (American Cancer Society, 2003). According to a projection by the Prostate Cancer Charity organization in UK, a total of 542,909 new cases of prostate cancer are diagnosed per year globally and about 204,313 men will die of prostate cancer (<http://www.prostate-cancer.org.uk/learn/bigPicture/index.asp>). Despite extensive efforts, the etiology of prostate cancer and the factors that promote its progression are not well established, although epidemiological evidence shows strong associations with dietary fat intake and red meat consumption (Shirai et al., 2002; Kolonel et al., 1996). The majority of the carcinogenicity/mutagenicity in meat cooked under normal household conditions may be accounted for by a single group of heterocyclic amines (HCAs) such as 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ) and 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) (Fig. 1) (Sugimura et al., 1997). The positive correlation between meat consumption and cancer incidence such as those of the stomach (Stefani et al., 1998), prostate (Norrish et al., 1999), breast (Zheng et al., 1998; Sinha et al., 1999), lung (Sinha et al., 2000) in human populations (Layton et al., 1995) along with carcinogenicity to rats and mice (Archer et al., 2000; Stuart et al., 2000; Bergman et al., 1985; Shirai et al., 1997; Hammons et al., 1997) of these compounds suggest that they may be an important etiological factor in diet-related human cancers. HCAs require bioactivation, which is catalyzed by the CYP enzymes, to exert their carcinogenicity. A critical activation step is *N*-hydroxylation to form the carcinogenic metabolites, *N*-hydroxy HCAs (Ushiyama et al., 1991; Peluso et al., 1991; Edwards et al., 1994; Hammons et al., 1997). The mRNA of HCA-metabolizing CYPs, including CYP1B1, was reported to be present in the human prostate (Williams et al., 2000). The toxicity and carcinogenicity of a given compound in extrahepatic tissues are often linked to its metabolic activation *in situ*, i.e., in the target tissues or organs (Malfatti et al., 1999; Guengerich, 2000; King et al., 1999). In addition, there are often substantial differences between animals and humans in carcinogen metabolic activation. Therefore, it is crucial to directly determine PhIP metabolism in

human prostate tissues for assessing the etiological role of PhIP exposure in the development of prostate cancer. It is also important to understand the impact of genetic variations of major PhIP-metabolizing CYP enzymes on their catalytic activities, which will help us to identify the susceptible individuals for better protection. The present study was conducted to achieve these goals with the following specific aims (adopted from original grant application)

1. To confirm our preliminary finding that a large interindividual variation exists in the PhIP-metabolizing activity of the human prostate with a large number of samples (~100 samples), and to examine the existence of such a variation in the metabolism of other carcinogenic HCAs such as IQ, MeIQ, and MeIQx found in well-done meat.
2. To determine the relationship between the individual variation in prostate HCA-metabolizing activity and the mRNA/protein expression profiles of HCA-metabolizing CYP enzymes (CYP1A2, CYP1A1, and CYP1B1) in the prostate by correlation analysis.
3. To investigate the functional significance of the genetic variants of the human CYP enzymes which are responsible for the metabolism of PhIP and other HCAs in human prostate tissue.

Body

Aim 1 was to determine the activity of human prostate tissue in metabolizing PhIP and other carcinogenic HCAs then to examine if there is a large inter-individual difference in such metabolic activity. Because the content of CYP enzymes and the related metabolic activities are usually very low in extrahepatic tissues, a highly sensitive analytical method is essential for this aim. We have conducted extensive studies to improve and validate our original LC/MS method (Prabhu et al., 2001), which are described in detail as follows.

Reagents

PhIP, IQ, MeIQ, MeIQx, *N*-hydroxy PhIP, *N*-hydroxy IQ, *N*-hydroxy MeIQ were purchased from the Toronto Research Chemicals Canada. 4-Hydroxy PhIP was received as a generous gift from Dr. M.G. Knize (Lawrence Livermore National Laboratory, LLNL) and Dr. Fred F. Kadlubar (National Center for Toxicological Research, NCTR). NADPH was obtained from Sigma Chemical Co. (St. Louis, MO). The protein assay reagent was purchased from Bio-Rad Laboratories (Hercules, CA).

HPLC grade solvents were purchased from Burdick and Jackson (Muskegon, MI). Other reagents were of the highest grades available commercially. Recombinant cDNA-expressed human CYP1A1, CYP1A2 and CYP1B1 and human liver microsomes were obtained from Gentest (Woburn, MA).

Human Prostate tissues and microsome preparation

Human prostate tissues were obtained from the Cooperative Human Tissue Network (Philadelphia, PA) and National Disease Research Interchange (Philadelphia, PA) after prior approval of the Institutional Review Board of University of Medicine and Dentistry of New Jersey. The tissues were collected from 31 different individuals undergoing the prostate surgery and the details of the subjects for the prostate tissue are in Table 1. All samples were snap-frozen within 1-hour post surgery and shipped to our lab in a dry ice container. Prostate tissues were stored at -70°C until the preparation of microsomal fractions.

Prostate microsomes were prepared after a small modification as described in (Haaparanta et al., 1983; Gemzik et al., 1992; Philpot et al., 1991). The frozen tissues were briefly thawed to 4°C, weighed and homogenized for three 15 seconds intervals with a Polytron steel blade homogenizer followed by a three complete strokes in a Potter-Elvehjem homogenizer in a 250 mM sucrose containing 5 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), 10 mM KCl; 0.1 mM EDTA, 1 mM MgCl₂ pH 7.2 and 0.1 mM phenylmethyl sulfonyl fluoride, 1mM dithiothreitol was added just before the preparation. The homogenate was subject to centrifugation at 9000 g for 20 min and the resulting pellet was discarded. The supernatant fractions were subjected to centrifugation at 100,000 g for 90 min. The microsomal pellet was suspended in 250 mM sucrose in Tris buffer (pH 7.2) as concentrated suspension and stored in -70°C. All the procedures were essentially carried out at 4°C. Protein concentrations were determined immediately after microsome preparation.

The overall study for LC/MS method establishment is divided into the following parts:

- *Incubation and Assay Conditions*
- *Determination of Selective MS Conditions*
- *Analytical Conditions and Sample Analysis*
- *Data Processing and Review*

Incubation and Assay Conditions

A typical incubation mixture (0.23 ml final volume) consisted of 100 mM sodium phosphate buffer (pH 7.4), prostate microsomal proteins (0.6 – 1 mg/ml) or liver microsomal proteins (0.02 mg/ml), and 200 μ M of the HCA substrate (either of PhIP, IQ, MeIQ, MeIQx). The reaction was initiated by the addition of the 1 mM NADPH after 3 min preincubation at 37°C and terminated at the end of 30 min by the addition of an equal volume of ice-cold methanol. After a brief vortex mixing and centrifugation at 4°C for 30 min the supernatant was filtered using PTFE 0.4 μ m syringe filters (Sartorius, Germany). Negative control experiments consisted of incubation of HCA with liver and prostate microsomes inactivated by addition of ice-cold methanol prior to incubation. For positive control, liver and human recombinant microsomes were used. Due to insufficient amount of the prostate tissues and microsomes, we have used different amount of human liver microsomes to optimize the PhIP metabolism incubation conditions. With a low amount of human liver microsomal protein (5 μ g) for the incubation at different time points to determine the linear range of the metabolite formation. The *N*-hydroxy PhIP metabolite formation was linear up to 45 min (Figure 2). Based on this results all the prostate microsomes were incubated for 30 min.

Determination of Selective MS Conditions

In addition to the carcinogenic metabolite *N*-hydroxy PhIP, microsomal incubation system may generate other isomeric and oxidative metabolites such as 2-hydroxy PhIP, 4'-hydroxy PhIP, and 5-hydroxy PhIP. It is necessary to validate selectively the metabolites of interest from such complex isomeric mixtures to avoid interference during the quantitation. The ion trap was operated in selected ion monitoring (SIM) mode and selected reaction monitoring (SRM) mode. The first injection was SIM or SRM 0% collision energy in the positive ion mode to determine the optimal precursor ion. The second injection alternatively switches between several collision energies to determine the optimal product ion and collision energy for each precursor ion found from the first injection. Finer optimization of the collision energy was accomplished manually by incorporating additional collision energy to the computer-generated value.

Analytical Conditions and Sample Analysis

Analysis was performed on a HPLC system comprising of a Spectra P4000 separation module consisting a quaternary pump equipped with a Spectra UV600LP photodiode array UV detector, auto-sampler (AS 3000) maintained at 2°C and Thermo Finnigan

LCQ Decca mass spectrometer (Thermo, San Jose, CA). The mass spectrometer equipped with an electrospray ionization (ESI) source and an ion trap mass analyzer (IT). The LC flow was introduced into the ESI following the detection by UV absorption from 200 to 400 nm. The chromatography was performed on a Symmetry C₁₈ reversed-phase column 2.1 x 100 mm column, Waters, (Milford, MA). The analytical column was protected by a C₁₈ Security Guard system from Phenomenex (Torrance, CA). The separation of HCAs and metabolites were carried out using a isocratic elution program of a binary mobile phase at a flow-rate 100 µl/min composed by 0.5 mM ammonium acetate buffer containing 19% methanol, 0.1% acetic acid and 0.5% tetrahydrofuran (Solvent A), 70% Methanol (Solvent B) and 0.1% acetic acid, 0.1% methanol in 5mM ammonium acetate (Solvent C). In the analysis of PhIP and *N*-hydroxy PhIP 50:50 ratio of A:B were used and for the analysis of IQ, MeIQ, MeIQx and their hydroxy metabolites 70:30 of A:C were used. In the analysis of 4-hydroxy PhIP the mass spectrometer having dual segments in single reaction monitoring (SRM) or a full scan multiple reactions monitoring (MRM) of the parent ion $m/z=241$ with the range of collision energy from 30% - 45% in the IT reduced the detection sensitivity at low level detection of *N*-hydroxy PhIP. Also the relative detector response for 4'-hydroxy PhIP was much higher than the *N*-hydroxy PhIP and the 4'-hydroxy PhIP did not form fragments with 30% collision energy, hence these to metabolites were selectively analyzed separately in SRM mode for *N*-hydroxy PhIP and 4'-hydroxy PhIP.

The voltage on the ESI interface was maintained at +5 kV in the positive ion mode. High-purity nitrogen gas served both as sheath gas with an operating pressure of 90 psi and as auxiliary gas with a flow-rate of 2.6 arb. The heated capillary temperature and voltage were maintained at 250°C and 3.1 V, respectively. The tube lens offset voltage was set at -16.2 V. The ion trap was operated in selected ion monitoring (SIM) (MS) mode and selected reaction monitoring (SRM) (MS/MS) mode. To prevent MS contamination when running MS, a divert valve was used, the first 4 minutes fractions from the column was sent to waste. A solution of caffeine, L-methionyl-arginyl-phenylalanyl-alanine acetate, and Ultramark 1621 prepared in mixture of acetonitrile : methanol : water (2:1:1) containing 1% acetic acid was used for the calibration of mass spectrometer. Data acquisition and system control was performed using Xcalibur software.

After sample preparation the incubation mixture was loaded on HPLC column through the auto sampler (with a constant injection volume of 20 μ l), the column eluent containing phosphates and salts from the incubation medium is directed to waste for a short time 3-4 min, after which the valve switches and the mobile phase directed through the column, causing the analyte elution and goes for the mass spectrometric detection. In the present method, the chromatography system serves to desalt the sample. For reliable and reproducible quantitation, at the column dimension and flow rates used it is necessary to flush and recondition the column after injection of every 5 samples in order to maintain the column in ideal condition. Additionally, standards samples and quality control (QC) samples were injected in the beginning and end of each run as well as intermittently. The precision and accuracy of the assay were determined by replicate analyses of QC samples.

Calibration standards were prepared in triplicates (1 to 50 ng/ml) (Fig. 3). The assay was validated for reliable, repeatable quantitation of *N*-hydroxy PhIP and the linearity of *ioN*-trap response. The *N*-hydroxy metabolite formation was comfortably quantified (Fig. 5) in PhIP incubation containing 1 μ g of human liver microsomal protein (0.004 mg/ml final protein concentration). The assay takes <15 min with isocratic elution including the online sample clean up step and suitable for high throughput analysis. This method provides good separation of the substrate and the metabolite to selectively quantitate the *N*-hydroxy PhIP from the incubation system and the lower limit of quantification (LLOQ) is 20 pg. The lowest limit of detection of the assay was the lowest amount the calibration curve, with acceptable precision (20%) and accuracy ($100 \pm 10\%$). The quality control (QC) samples were prepared in the same fashion as the calibration samples at concentrations corresponding to 1 and 10 ng/ml. A blank sample (no standard) was prepared by mixing 50:50 ratios of the mobile phase. The stability and reliability of the assay were determined by replicate analyses of QC samples. The LC-MS system was routinely checked for carryover by injecting the blank after every 5 samples and also after the injection of high concentration *N*-OH-PhIP.

The peaks were identified based on their retention time in the HPLC-UV chromatogram and their UV spectrum, which was performed by the human recombinant CYP1A2 (Fig. 4a) and their molecular weight precursor ion in the LC/MS mode. To further confirm the identity of *N*-hydroxy PhIP (m/z 241), the peak obtained from the incubation system was subjected to fragmentation in MS/MS mode by 30% collision energy. The base peak in

the MS/MS spectrum for *N*-hydroxy PhIP (m/z 224) originated from the loss of $[M+H-OH]^+$ from the *N*-hydroxy PhIP molecular ion (m/z 241). A similar base peak fragment was observed in the MS/MS spectrum from the authentic standard *N*-hydroxy PhIP. Similarly the, UV, MS and MSMS fragments for *N*-hydroxy IQ, (Fig. 4b) $[M+H]$ m/z 215 and $[M+H-OH]^+$ m/z 198, *N*-hydroxy MeIQ (Fig. 4c) $[M+H]$ m/z 229 and $[M+H-OH]^+$ m/z 212 and *N*-hydroxy MeIQx (Fig. 4d) $[M+H]$ m/z 230 and $[M+H-OH]^+$ m/z 213 were obtained. The authentic standards of *N*-hydroxy IQ and *N*-hydroxy MeIQ were highly unstable and could not be used for quantitation. In case of 4-hydroxy PhIP m/z 241 formed from the incubation system subjected to fragmentation in MS/MS mode by 40% collision energy. The base peak in the MS/MS spectrum for 4-hydroxy PhIP (m/z 226) originated from the loss of $[M+H-CH_3]^+$. The formation of 4-hydroxy PhIP was qualitatively confirmed by the standard samples and the formation of 4-hydroxy PhIP was consistent as reported by Hammons *et. al*, (Hammons et al., 1997), the formation of 4-hydroxy PhIP by human CYP1A1 was shown in Fig. 8.

Data Processing and Review

After analysis of a sample set, each raw file was opened for review and data processing. Xcalibur software (San Jose, CA) was used to process the data and extract the peak area and height into a spreadsheet.

Results

We have modified our earlier LC/ESI-ITMS method (Prabhu et al., 2001) into a direct injection method, which avoids the "solid phase extraction" sample preparation step and includes an online sample clean up step. The assay was validated for reliable, repeatable quantitation of *N*²-hydroxy PhIP and the linearity of ion-trap response; calibration standards were prepared in triplicates (1 to 1000 ng/ml). Different amounts of standard *N*²-hydroxy PhIP were spiked into the incubation mixture containing human liver microsomes, PhIP and NADPH. The samples were analyzed using direct injection LC/ESI-ITMS method and confirmed the reproducibility and the linearity of detector response. The lowest limit of detection of the assay was the lowest amount (20 pg) on the calibration curve, with acceptable precision (20%) and accuracy ($100 \pm 10\%$). Under similar conditions, this LC/MS method has also been applied for the analysis of IQ, MeIQ, MeIQx and their respective *N*-hydroxy metabolites.

Substantial amount of *N*-hydroxylated metabolites of PhIP, IQ, MeIQ and MeIQx were detected in the reactions with 5 μ g (0.02 mg/ml) human liver microsomes, CYP1B1, CYP1A2 and CYP1A1 (Fig. 4) but not in the negative controls with the pre-inactivated microsomes or incubation without NADPH. This confirms that the formation of the *N*²-hydroxy PhIP and *N*-hydroxylated metabolites of other HCAs is purely cytochrome P450 dependent. In case of the liver microsomes, the rate of *N*-hydroxy PhIP formation was found to be 150 ± 29 pmol/min/mg protein. We further analyzed a total of 31 human prostate microsome samples. Very low activity in the formation of *N*-hydroxy PhIP was only detected in two of the samples, P7 and P22 (Figure 6, 7 and 10). In P7 prostate microsomes the rate of *N*-hydroxylation was found to be 0.28 ± 0.05 pmol/min/mg protein, about 800 times lower than the liver. The 4'-OH PhIP was also formed in this sample (Fig. 9a) and was not detected in negative control (Fig. 9b). The PhIP-metabolizing activity was not detectable in the rest 29 samples even using high protein concentration (1mg/ml) and longer incubation time (60 min). When the metabolic activity of IQ, MeIQ and MeIQx were assayed for prostate microsomes the *N*-hydroxyl metabolite was not detected in all the prostate microsomes.

Since the sample P7 and P22 were both obtained from normal prostate tissues and the remaining tissues were all obtained from benign prostatic hyperplasia (BPH) patients (Table 1). The results indicate that the normal prostate microsomes might have very low metabolic activity. However, this observation needs to be confirmed with a larger number of normal prostate tissue samples. In summary, the results of our metabolism *in vitro* study suggest that human prostate microsomes do not metabolize the carcinogenic HCAs to any significant extent.

Difficulties

We have encountered following difficulties in this part of our project. Even though we have identified and tried our best to solve these problems in a timely manner, they did affect our working schedule.

Procuring the surgical human prostate tissue in a workable quantity is difficult. We have contacted various human tissue-providing networks such as Cooperative Human Tissue Network (CHTN, <http://www-chn.ims.nci.nih.gov>), National Disease Research Interchange (NDRI, <http://www.ndriresource.org>) and Cooperative Prostate Cancer Tissue Resource (CPCTR, <http://cpctr.cancer.gov>) but were unable to collect ~100

prostate tissue samples as we planned. The quantity of our received human prostate tissues was small in most cases, which limited the amount of microsomes for the metabolism study.

We had difficulties in obtaining the stable reference standards of the metabolites *N*-hydroxy MeIQ, *N*-hydroxy IQ and *N*-hydroxy MeIQx. When we purchased *N*-hydroxy IQ and *N*-hydroxy MeIQ from Toronto Research Chemicals Inc., Canada, they were already decomposed. The manufacturer confirmed that the stock at their facility had also same problem. The could attribute largely to the structure and ionization efficiency differences between analytes. The metabolite with OH- moiety has much lower stability than the substrate. As a provision, while reference standards are not readily available, qualitative analysis of metabolite level was done based on the structurally related analog (substrate) with comparable ionization efficiency. The 4-hydroxy PhIP standard obtained from LLNL and NCTR was also not suitable for low-level metabolite quantitation and was used as qualitative reference.

The LC/MS instrument used for our study was in the Core Facility of EOHSI Analytical Center, which is shared by a large number of users. The time limit in using the instrument affected the efficiency of our analysis.

The start of our project was significantly delayed due to the lengthy procedures of hiring and security-checking after the September 11th Tragedy. Dr. K.Gajaraj, a chemist who was recruited to replace Dr. S. Prabbu for this project, had to wait for approximately 6 months (from Aug 2002 to March 2003) to start his work as a regular employee. The progress was also interrupted by moving our laboratory from the EOHSI building to the new School of Public Health Building.

The originally proposed Aim 2 was to examine if there exists a correlation between the individual variation in prostate HCA-metabolizing activity and the expression profiles of HCA-metabolizing CYP enzymes in the prostate. However, this aim could not be accomplished mainly due to the lack of detectable PhIP-metabolizing activity in most of prostate tissue samples (29 of total 31). As a result, we were unable to perform the correlation study. In addition, it is difficult to conduct the expression profile study due to the quantity limitation of most available tissue samples.

Aim 3 was to investigate the functional significance of the genetic variants of the human CYP enzymes which are responsible for the metabolism of PhIP and other HCAs in human prostate tissue. We have focused on CYP1B1, a major PhIP-metabolizing enzyme in extrahepatic tissues including prostate. Based on the reported naturally-occurring CYP1B1 missense variants, we first generated the variant cDNAs by site-directed mutagenesis then expressed the variant proteins in a baculovirus/insect cell system. The variant proteins were compared with the wild-type CYP1B1 protein in metabolizing PhIP. Because the expressed CYP1B1 proteins have much higher activities than the microsomes prepared from extrahepatic tissues, we used a LC-fluorescence method, instead of the highly sensitive LC/MS method we established in Aim 1, for the metabolism study. This change also allowed us to control our schedule for sample analysis instead of relying on the LC/MS instrument in the EOHSI Core Facility.

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). Additional rounds of mutagenesis were conducted to generate the variant cDNAs containing more than one nucleotide variations (Table 2). All the variant cDNAs were sequenced after mutagenesis to ensure there were no extra mutations produced in PCR process.

Heterologous Expression and Microsome Preparation

A B_{AC}-TO-B_{AC} baculovirus expression system was used for the expression of CYP1B1 proteins. Wide-type and variant cDNAs were individually subcloned into the expression vector pFastbac1. The reconstructed bacmid DNAs through 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 proteins. δ -Aminolevulinic acid and ferric citrate stock solutions (100 mM each) were prepared by dissolving in Grace insect medium and the stock solution was added to the culture medium for a final concentration of 100 μ M. The Sf9 cells were harvested 72 hr after infection and re-suspended in PBS buffer containing 5 mM imidazole, 20% glycerol, and 1 mM freshly added PMSF. The microsomes were prepared 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 Bio-Rad protein assay reagent.

Immunoblot Analysis and P450 Content Determination

Microsome proteins (1 µg) was 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 at a 1:3300 dilution with a CYP1B1-specific polyclonal antibody as the primary antibody for 1 h and at 1:5000 using a goat anti-rabbit horseradish peroxidase conjugate as the secondary antibody for 1 h. The immunoblot was visualized by ECL enhanced chemiluminescence reagents according to the manufacture's protocol (Amersham Biosciences Inc., Piscataway, NJ).

Microsomal P450 content was determined by reduced CO-difference spectrum using an UV/visible spectrophotometer as previously described (He et al., 2004).

HPLC analysis

PhIP metabolites were analyzed using an HPLC-fluorescence (FL) 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), guard cartridge (µ Bondapak, 125 Å, 10 µm) and software PC1000 for data collection and analysis. Metabolites were eluted with 55% methanol in water containing 0.1% diethylamine (pH 4.0 with acetic acid). The isocratic solvents were 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 emission wavelength was 370nm. This analytical protocol was able to clearly resolve N²-OH-PhIP, PhIP and 4'-OH-PhIP in less than 20 min. Concentrations of the PhIP metabolites were determined by calibration curves prepared using standard solutions of N²-OH-PhIP and 4'-OH-PhIP. Under our analytical conditions, the limits of detection were 0.2 pg for N²-OH-PhIP and 3.2 pg for 4'-OH-PhIP, respectively.

Metabolism of PhIP and Kinetic Analysis

For PhIP metabolism, the incubation mixture consisted of 100 mM sodium phosphate buffer (pH 7.4), 5 mM MgCl₂, an NADPH generating system (15 mM glucose-6-phosphate, 1 mM NADP⁺, 1 unit of glucose-6-phosphate dehydrogenase), 30 pmol of CYP1B1 enzymes, NADPH-P450 oxidoreductase (reductase:P450 = 1:1 in molar ratio), and PhIP as the substrate, in a total volume of 300 µl. Reaction was carried out at 37°C

for 30 min, the mixtures were pre-incubated for 5 min at 37°C prior to initiation with the NADPH generating system and terminated by adding one volume of ice-cold methanol. The reaction solution was centrifuged at 4°C and filtered. Samples were stored at -70 °C prior to analysis by HPLC within 48 h after collection. An aliquot of 200 µl was injected onto a reverse-phase HPLC system equipped with a Fluorescence Detector. For enzyme kinetics studies, eight different PhIP concentrations, ranging from 0.31 µM to 50 µM, were used for incubation.

Results

Heterologous Expression of the Wild-type and Variant CYP1B1 Proteins

The wild-type and variant CYP1B1 cDNAs were all successfully constructed and used for protein expression in the B_{AC}-TO-B_{AC} baculovirus/insect cells. For most of the variants, immunoblot analysis detected a single protein band with the expected molecular weight same as the wild-type CYP1B1 (Fig. 11). There were no detectable CYP1B1 proteins in the microsomes prepared from the cells transfected with only the vector (containing no CYP1B1 cDNA) or the Asn⁴⁵³Ser variant cDNA. The CYP1B1 protein levels for the Gly³⁶⁵Trp, Glu³⁸⁷Lys and Arg³⁹⁰His variants were reproducibly lower than the wild-type and most of the variants.

The average P450 content in the cells expressing CYP1B1 was 219 pmol/mg (range 118-324 pmol/mg) based on the CO-difference spectrum analysis (Table 2). Most of the microsomes containing the expressed recombinant CYP1B1 displayed the characteristic P450 absorption peak. But a significant decreased in P450 content was observed in the microsomes containing Gly³⁶⁵Trp, Pro³⁷⁹Leu, and Glu³⁸⁷Lys variant proteins (data not shown) whose P450 content averaged 39 pmol/mg (range 24-55 pmol/mg). The CYP1B1 protein level was extremely low or non-detectable for the Arg³⁹⁰His and Asn⁴⁵³Ser variants, which also did not show detectable P450 peaks (Fig. 11). The CYP1B1 protein expression level generally correlates with the P450 content, except for the Pro⁴³⁷Leu variant, which displayed a substantial amount of protein expression but lack of detectable P450 peaks. These results were confirmed by 3 to 5 independent expression in Sf9 cells. For the variants with significant lower level of P450 content, repeated expressions were started from reconstruction of the expression vector.

Optimization of the HPLC-FL Detection

Our pilot study tried a reported HPLC-UV method and experimental conditions (Crofts et al., 1997) but failed to detect the formation of N²-OH-PhIP or 4'-OH-PhIP in the metabolism of PhIP by our expressed CYP1B1 proteins. We then changed to an HPLC-FL method reported by the same group and found that the above two metabolites could be satisfactorily detected. To ensure that analysis of a large number of samples can be completed within 48 hr to reduce the possibility of degradation, we further optimized the analytical program by using isocratic elution with 55% methanol instead of the reported gradient protocol. This change resolves N²-OH-PhIP, PhIP and 4'-OH-PhIP in less than 20 min instead of 40 min. Using this new protocol, the detection limits was also greatly improved (400-fold more sensitive) compared to original report (Crofts et al., 1997) for these two PhIP metabolites.

As shown in Figure 12, authentic N²-OH-PhIP and 4'-OH-PhIP, and PhIP are eluted with a retention time at 4 min, 8 min, and 13 min, respectively. When PhIP was incubated with microsomes expressing wild-type CYP1B1, N-hydroxy PhIP and 4-hydroxy PhIP were detected as a function of time and microsomal protein concentration. The production of the metabolites was linear up to 60-min incubation time and up to 120 pmol/ml microsomal proteins.

Activity of CYP1B1 Variants and Enzyme Kinetics

The activity assay was conducted using a 30-min incubation time and 100 pmol/ml microsomal protein. For variants with no detectable activity, the assay was repeated with up to 20-fold amount of microsomal proteins compared to the wild-type CYP1B1 proteins. The activity assay was conducted at high (10 μ M) and low (1 μ M) substrate concentrations. As shown in Fig. 13, 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 variants Trp⁵⁷Cys, Gly⁶¹Glu, Arg⁴⁸Gly/Ala¹¹⁹Ser, Arg⁴⁸Gly/Ala¹¹⁹Ser/Leu⁴³²Val, Arg⁴⁸Gly/Ala¹¹⁹Ser/Leu⁴³²Val/Ala⁴⁴³ showed a lower activity than the wild-type CYP1B1. There was no detectable 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 has a total loss of PhIP-metabolizing activity. This activity profile was consistent with that assayed at 1 μ M substrate concentration (data not shown).

Five variants with over two-fold decrease in PhIP-metabolizing activity were selected for enzyme kinetics studies. 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 0.31 to 50 μ M, in duplicate experiments with less than 10% of assay variations (data not shown). When different amounts of PhIP were incubated with the microsomes containing the wide-type CYP1B1, N²-OH-PhIP and 4'-OH-PhIP were produced in a concentration-dependent manner. The rate of formation of N²-OH-PhIP and 4'-OH-PhIP appeared to reach saturation at concentrations of PhIP above 10 μ M. The values of apparent K_m , V_{max} and catalytic efficiency (V_{max}/K_m) derived from the kinetics curves are shown in Table 3. While the K_m for N-hydroxylation of PhIP was almost not changed 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 showed almost a 10-fold reduction because of the substantial increase of K_m value.

The effect of the missense changes on the formation of the N²-OH-PhIP and 4'-OH-PhIP appears to be different for different variants. For the Trp⁵⁷Cys variant, the K_m values for N²-OH-PhIP and 4'-OH-PhIP formation were both increased, but the V_{max} values were both decreased. For the Gly⁶¹Glu variant, the K_m value for N²-OH-PhIP formation was increased, but for 4'-OH-PhIP formation was unchanged, yet the V_{max} values were both decreased. The K_m values of the variants Arg⁴⁸Gly/Ala¹¹⁹Ser/Leu⁴³²Val and Arg⁴⁸Gly/Ala¹¹⁹Ser/Leu⁴³²Val/Ala⁴⁴³Gly for N²-OH-PhIP formation were increased, while there was opposite trend of K_m value 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. In summary, these results identify several CYP1B1 genetic variants with functional changes in their protein expression and catalytic activity for PhIP metabolism.

Key Research Accomplishments

- Our original LC/MS method has been further improved, validated and modified to a highly sensitive and selective LC-MS/MS method for the analysis of PhIP, IQ, MeIQ, MeIQx and their N-hydroxy metabolites. The new method also has the advantage to

avoid the “solid phase extraction” step in sample preparation step and to include an online sample clean up step.

- We have completed the study on the metabolism of PhIP in 31 human prostate tissues. This is the first report on HCA metabolism in human prostate microsome with relatively good sample size.
- There was no detectable PhIP metabolism in all 29 benign prostatic hyperplasia (BPH) samples and a very low metabolic activity in 2 samples obtained from normal prostate tissues. This result suggests that the expression profile of PhIP-metabolizing enzymes in the normal prostate tissues might be different from that in the BPH tissues. However, this observation needs to be confirmed with a larger number of normal prostate tissue samples. Nevertheless, the results of our metabolism *in vitro* study suggest that human prostate microsomes do not metabolize the carcinogenic HCAs to any significant extent.
- We have conducted an extensive functional characterization study of the genetic variants of CYP1B1, a major enzyme for PhIP metabolic activation in extrahepatic tissues, and identified several variants that have altered protein expression or catalytic activity. These results provide important information on the structure-activity relationship of this important carcinogen-metabolizing enzyme and for the selection of appropriate cancer susceptibility biomarkers for human population studies related to PhIP exposure.

Reportable Outcomes

Abstracts and Manuscripts

An abstract entitled “Determination of PhIP and its metabolite N2-OH-PhIP by LC/ESI-ITMS” was presented at the 5th Annual Research Day Conference in the UMDNJ-Robert Wood Johnson Medical School and School of Public Health, October 23, 2003, Piscataway, New Jersey

Another abstract entitled “Metabolism of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) by Human CYP1B1 Genetic Variants” will be presented at the 2007 Annual Retreat on Cancer Research In New Jersey, May 31, 2007, Piscataway, New Jersey

The corresponding manuscripts are in preparation. We will inform USAMRPCP once

they get published.

Degrees obtained through the Award Support

Jing-Fen Han, in the Graduate Program of Environmental Sciences jointly sponsored by Rutgers University and University of Medicine and Dentistry of New Jersey, will defense her Ph.D. thesis on April 16, 2007. The functional characterization of CYP1B1 genetic variants constitutes a major part of her thesis work.

In addition, two postdoctoral researchers and one visiting scientist were trained by this project.

Cell Lines, Tissue or Serum Repositories

We have constructed the expression vectors containing 15 missense CYP1B1 variant cDNAs and the corresponding proteins. We will be glad to share these with other investigators upon request.

Conclusions

We have established a highly sensitive LC/MS method for the analysis of PhIP and other carcinogenic HCAs as well as their metabolites. Using this method, we examined the PhIP-metabolizing activity in 31 human prostate tissue samples and found that there was no detectable formation of N²-hydroxy PhIP and 4'-hydroxy PhIP in the BPH samples and only a very low activity in two normal prostate tissues. This result suggests that the metabolic activation of PhIP and other carcinogenic HCAs in the prostate may play an insignificant role in prostate cancer related to HCA exposure. We also determined the functional significance of 15 CYP1B1 genetic variants in the metabolism of PhIP and identified several functional variants. This result is important for design and interpretation of molecular epidemiological studies on the human genetic susceptibility to PhIP-related cancers including prostate cancer.

Investigators Supported by This Award

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Saileta Prabhu, Postdoctoral Researcher

K. Gajaraj, Postdoctoral Researcher

Jing-Fen Han, Ph.D. student

Xiaoyang He, Research Teaching Specialist

References

- American Cancer Society, Atlanta, GA, Cancer facts and figures, 2004
- Archer, C. L., Morse, P., Jones, R. F., Shirai, T., Haas, G. P., and Wang, C. Y. , Carcinogenicity of the *N*-hydroxy derivative of 2-amino-1-methyl-6- phenylimidazo[4,5-b]pyridine, 2-amino-3, 8-dimethyl-imidazo[4,5- f]quinoxaline and 3, 2'-dimethyl-4-aminobiphenyl in the rat, *Cancer Lett*, 155: 55-60, 2000
- Bergman, K., Autoradiographic distribution of ¹⁴C-labeled 3H-imidazo[4,5-f]quinoline- 2- amines in mice, *Cancer Res*, 45:1351-6, 1985
- Crofts FG, Strickland PT, Hayes CL, and Sutter TR , Metabolism of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) by human cytochrome P4501B1. *Carcinogenesis* 18:1793-1798, 1997
- Crofts FG, Sutter TR, and Strickland PT, Metabolism of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine by human cytochrome P4501A1, P4501A2 and P4501B1. *Carcinogenesis* 19:1969-1973, 1998
- De Stefani, E., Boffetta, P., Mendilaharsu, M., Carzoglio, J., and Deneo-Pellegrini, H. , Dietary nitrosamines, heterocyclic amines, and risk of gastric cancer: a case-control study in Uruguay, *Nutr Cancer*, 30:158-62, 1998
- 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. , Contribution of CYP1A1 and CYP1A2 to the activation of heterocyclic amines in monkeys and human, *Carcinogenesis*, 15:829-36, 1994
- Gemzik B, Jacob S, Jennings S, Veltman J, Parkinson A., Species differences in 5 alpha-androstane-3 beta,17 beta-diol hydroxylation by rat, monkey, and human prostate microsomes Arch Biochem Biophys, 296:374-83, 1992.
- Guengerich, F. P. , Metabolism of chemical carcinogens, *Carcinogenesis*, 21:345-51, 2000
- Haaparanta T, Gustafsson JA, Glaumann H. Isolation of mitochondria, lysosomes, and microsomes from the rat ventral prostate with a note on inverted microsomal vesicles. Arch Biochem Biophys, 223:458-67,1983

- Hammons, G. J., Milton, D., Stepps, K., Guengerich, F. P., Tukey, R. H., and Kadlubar, F. F. , Metabolism of carcinogenic heterocyclic and aromatic amines by recombinant human cytochrome P450 enzymes, *Carcinogenesis*, 18: 851-4, 1997
- Han JF, Wang SL, He XY, Liu CY, and Hong JY, Effect of genetic variation on human cytochrome p450 reductase-mediated paraquat cytotoxicity. *Toxicol Sci* 91:42-48, 2006
- He XY, Shen J, Ding XX, Lu A-Y-H, and Hong JY (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
- Hong JY, Wang YY, Bondoc FY, Lee M, Yang CS, Hu WY, and Pan JM, 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, 1999
- King, R. S., Teitel, C. H., Shaddock, J. G., Casciano, D. A., and Kadlubar, F. F. , Detoxification of carcinogenic aromatic and heterocyclic amines by enzymatic reduction of the N-hydroxy derivative, *Cancer Lett*, 143:167-71, 1999
- Kolonel, L.N. Nutrition and prostate cancer, *Cancer Causes Control*, 7:83-94, 1996
- Layton, D. W., Bogen, K. T., Knize, M. G., Hatch, F. T., Johnson, V. M., and Felton, J. S. , Cancer risk of heterocyclic amines in cooked foods: an analysis and implications for research, *Carcinogenesis*, 16:39-52, 1995
- 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., The identification of [2-(14)C]2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine metabolites in humans, *Carcinogenesis*, 20:705-13, 1999
- Norrish, A. E., Ferguson, L. R., Knize, M. G., Felton, J. S., Sharpe, S. J., and Jackson, R. T., Heterocyclic amine content of cooked meat and risk of prostate cancer, *J Natl Cancer Inst*, 91:2038-44, 1999
- Peluso, M., Castegnaro, M., Malaveille, C., Friesen, M., Garren, L., Hautefeuille, A., Vineis, P., Kadlubar, F., and Bartsch, H. , 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*, 12:713-7, 1991

Philpot RM. Characterization of cytochrome P450 in extrahepatic tissues. *Methods Enzymol.* 206:623-31, 1991

Shirai T, Sano M, Tamano S, Takahashi S, Hirose M, Futakuchi M, Hasegawa R, Imaida K, Matsumoto K, Wakabayashi K, Sugimura T, Ito N. The prostate: a target for carcinogenicity of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) derived from cooked foods, *Cancer Res.*, 57:195-8, 1997

Shirai T., Asamoto M., Takahashi S., and Imaida K., Diet and prostate cancer, *Toxicology* 181-182:89-94, 2002

Sinha, R. and Rothman, N. , Role of well-done, grilled red meat, heterocyclic amines (HCAs) in the etiology of human cancer, *Cancer Lett*, 143:189-94, 1999

Sinha, R., Kulldorff, M., Swanson, C. A., Curtin, J., Brownson, R. C., and Alavanja, M. C. , Dietary heterocyclic amines and the risk of lung cancer among Missouri women, *Cancer Res*, 60:3753-6, 2000

Stuart, G. R., Holcroft, J., de Boer, J. G., and Glickman, B. W. , Prostate mutations in rats induced by the suspected human carcinogen 2- amino-1-methyl-6-phenylimidazo[4,5-b]pyridine, *Cancer Res*, 60:266-8, 2000

Sugimura, T., Overview of carcinogenic heterocyclic amines, *Mutat Res*, 376:211-9, 1997

Ushiyama, H., Wakabayashi, K., Hirose, M., Itoh, H., Sugimura, T., and Nagao, M. , Presence of carcinogenic heterocyclic amines in urine of healthy volunteers eating normal diet, but not of inpatients receiving parenteral alimentation, *Carcinogenesis*, 12:1417-22,1991

Williams, J. A., Martin, F. L., Muir, G. H., Hewer, A., Grover, P. L., and Phillips, D. H. , Metabolic activation of carcinogens and expression of various cytochromes P450 in human prostate tissue, *Carcinogenesis*, 21:1683-9, 2000

Zheng, W., Gustafson, D. R., Sinha, R., Cerhan, J. R., Moore, D., Hong, C. P., Anderson, K. E., Kushi, L. H., Sellers, T. A., and Folsom, A. R., Well-done meat intake and the risk of breast cancer, *J Natl Cancer Inst*, 90:1724-9, 1998

Appendices

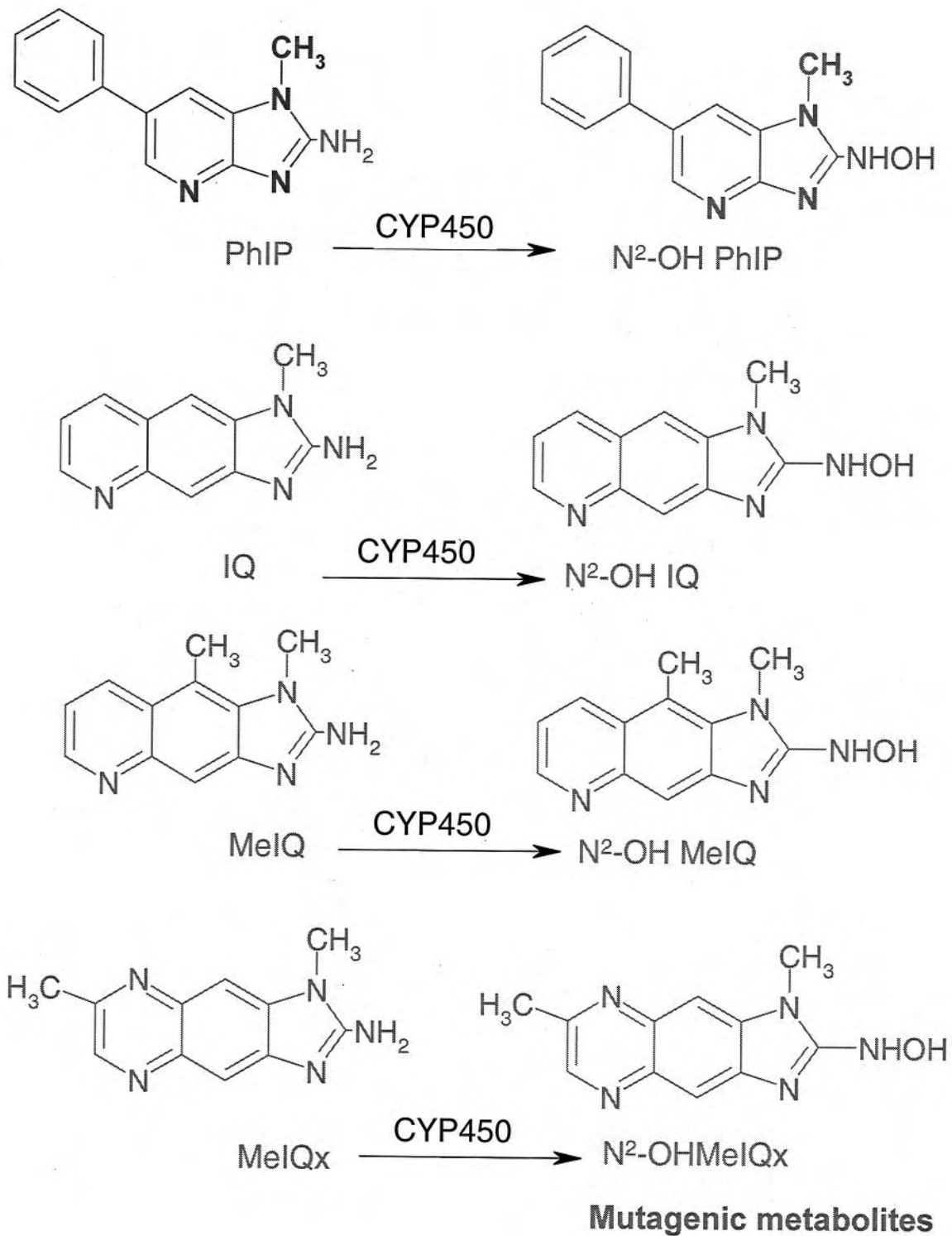


Figure 1. Chemical structure of the heterocyclic amines and its mutagenic metabolites

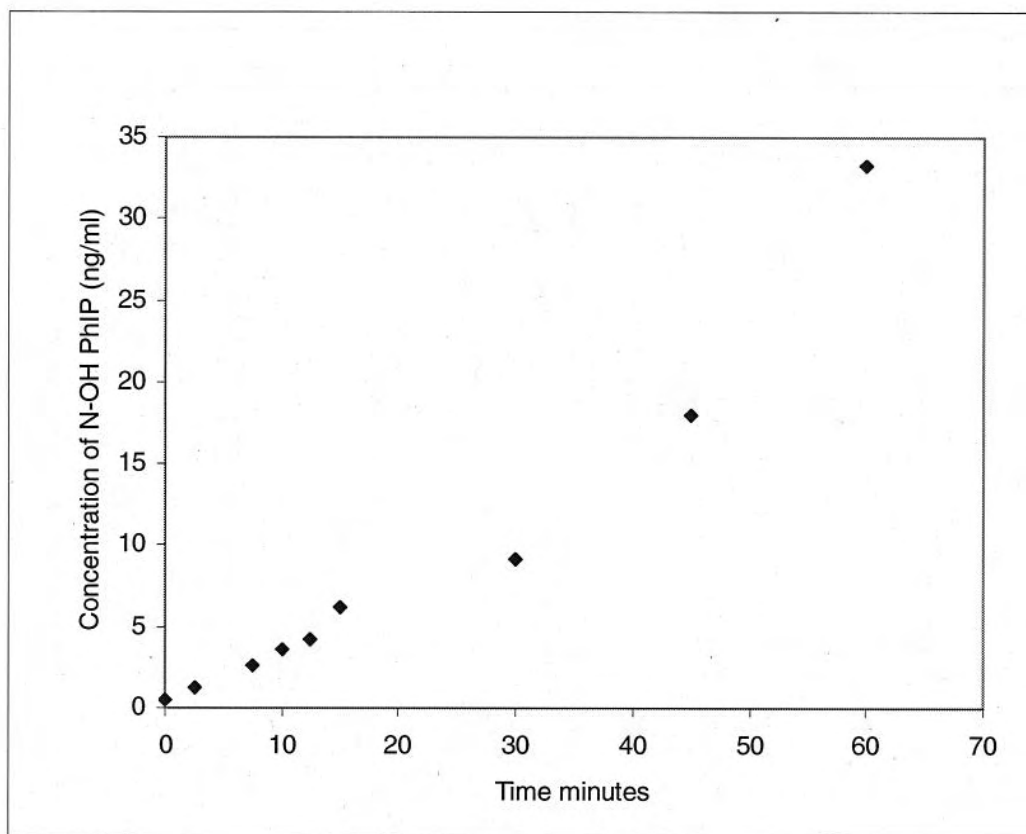


Figure 2. Formation of *N*-hydroxy PhIP vs incubation time, PhIP incubated with 5 μ g human liver microsome proteins for 2.5, 5, 7.5, 10, 12.5, 15, 30, 45 and 60 minutes. The *N*-hydroxy PhIP formation was linear up to 45 min and the incubations in prostate microsomes were done for 30 minutes.

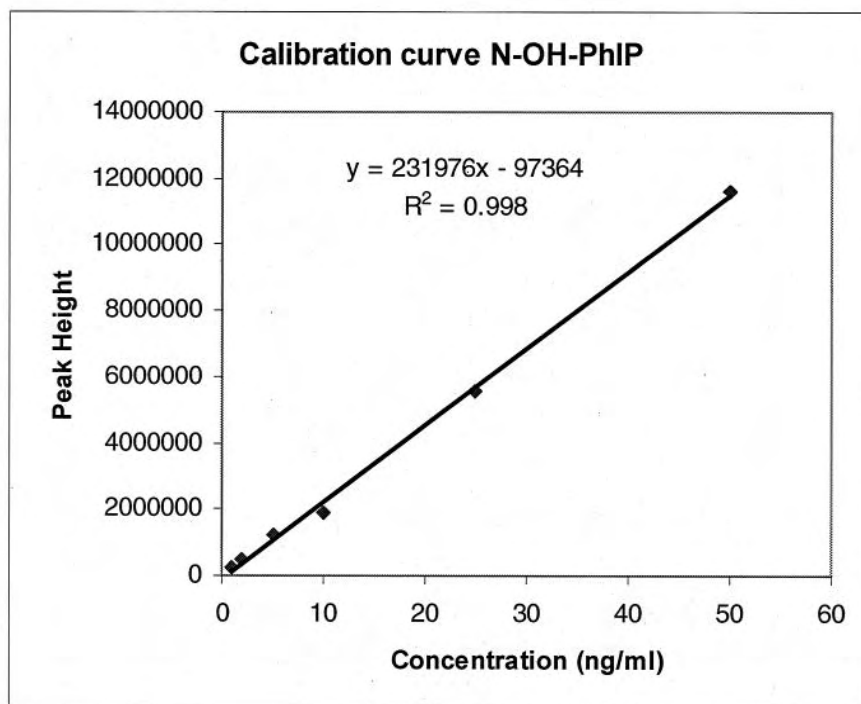


Figure 3. Calibration curve for *N*-hydroxy PhIP (1 ng/ml to 50 ng/ml standards were spiked into the incubation mixture containing inactivated human liver microsomes and the mixture was processed as described in sample preparation, constant volume 20 μ l was injected for the analysis).

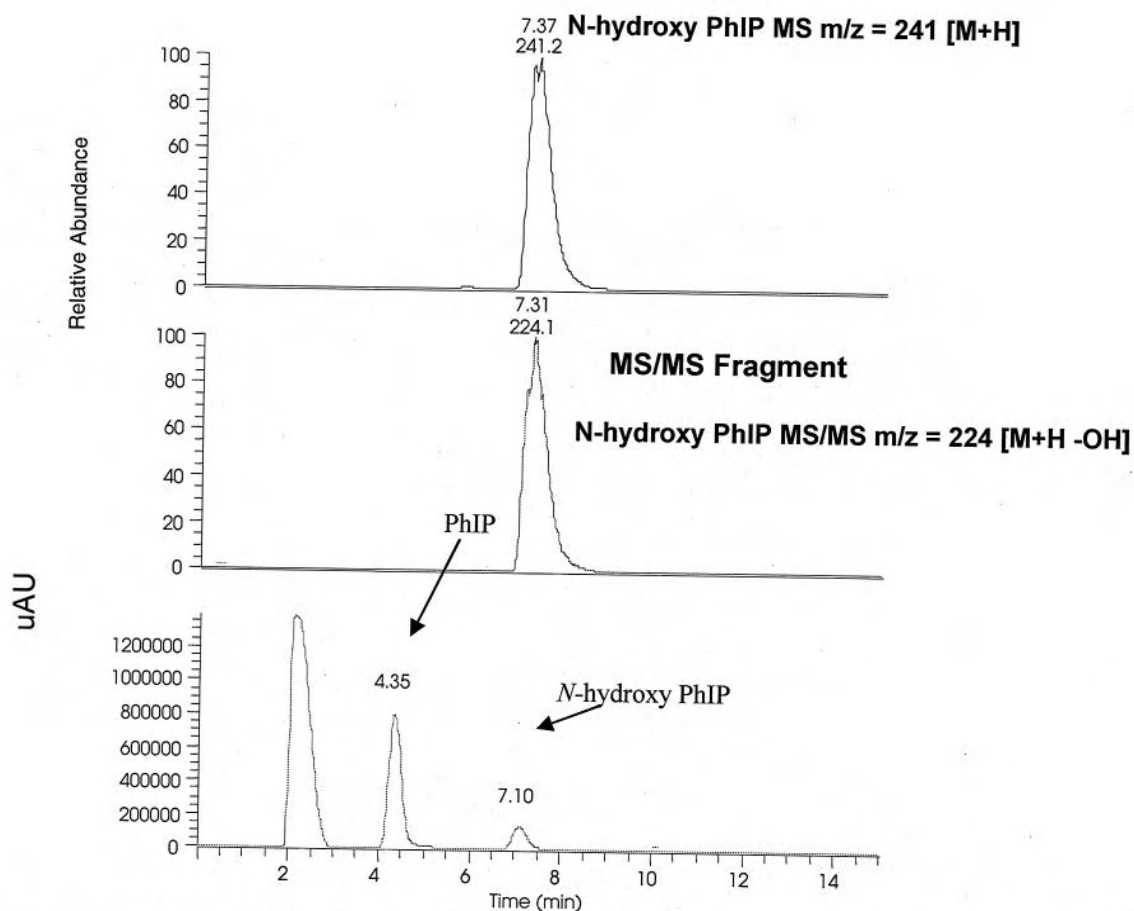


Figure 4a. HPLC-UV, MS and MS/MS analysis of PhIP and *N*-hydroxy PhIP, metabolism of PhIP by 5 pmol of human recombinant CYP1A2 incubated with 200 μ M PhIP for 30 min. Similarly the *N*-hydroxy PhIP formation was detected in human recombinant CYP1A1, CYP1B1 and human liver microsomes.

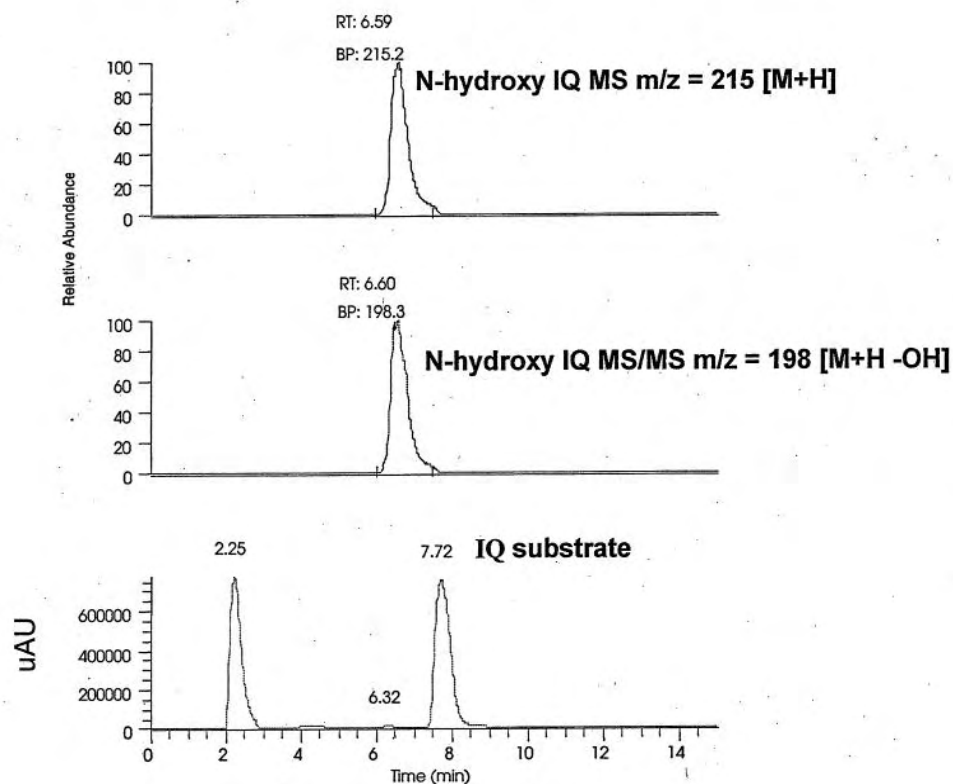


Figure 4b: HPLC-UV, MS and MS/MS analysis of IQ and *N*-hydroxy IQ, metabolism of IQ by 5 pmol of human recombinant CYP1A2 incubated with 200 μ M IQ for 30 min. Similarly the *N*-hydroxy IQ formation was detected in human recombinant CYP1A1, CYP1B1 and human liver microsomes at a lower level.

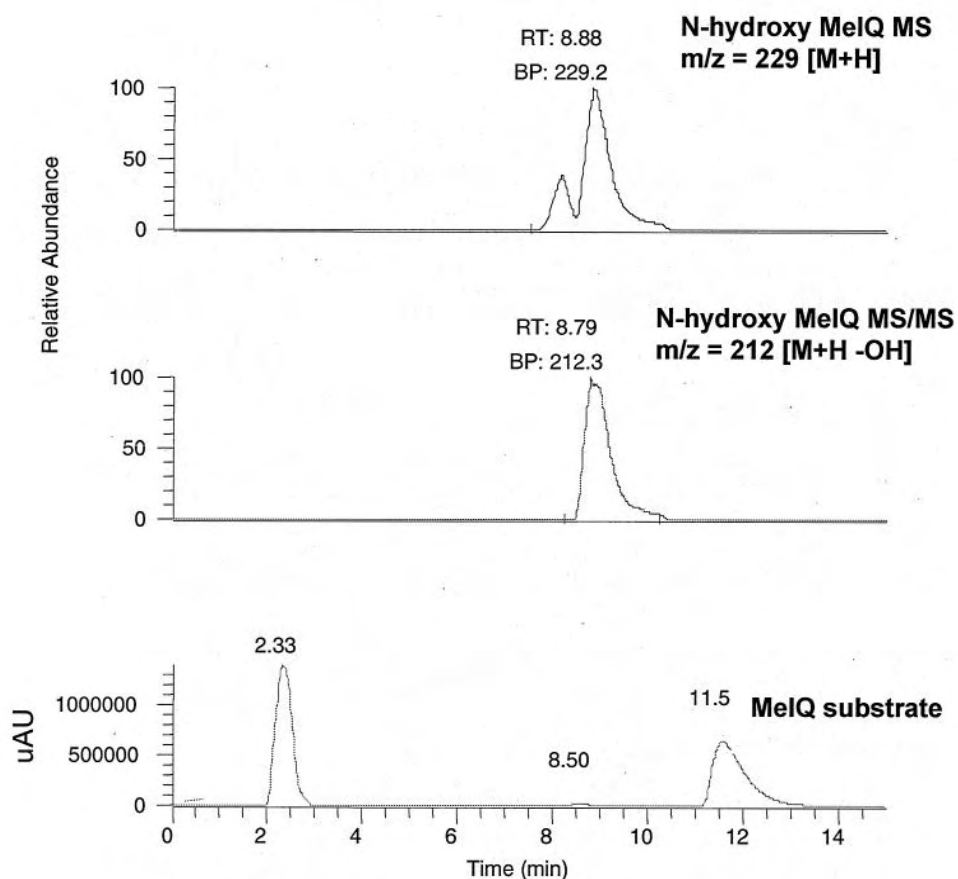


Figure 4c. HPLC-UV, MS and MS/MS analysis of MelQ and *N*-hydroxy MelQ, metabolism of MelQ by 5 pmol of human recombinant CYP1A2 incubated with 200 μ M MelQ for 30 min. Similarly the *N*-hydroxy MelQ formation was detected in human recombinant CYP1A1, CYP1B1 and human liver microsomes at a lower level.

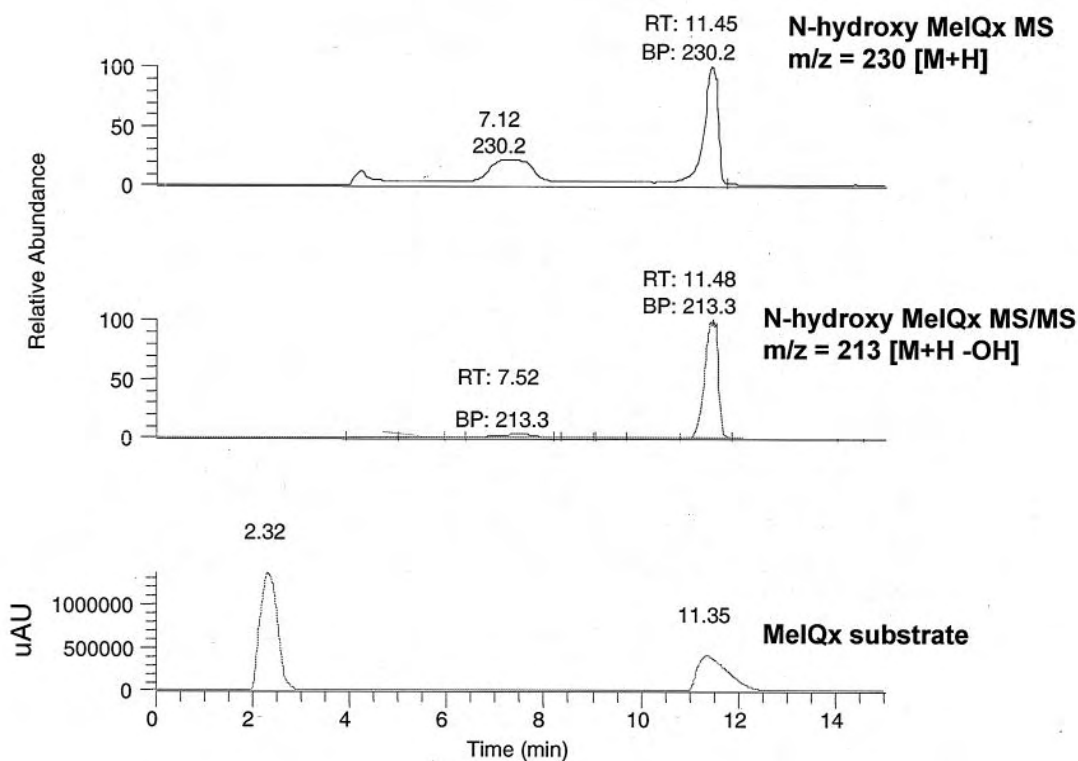
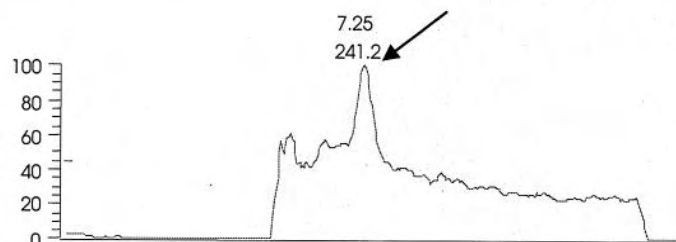


Figure 4d. HPLC-UV, MS and MS/MS analysis of MelQx and *N*-hydroxy MelQx, metabolism of MelQx by 5 pmol of human recombinant CYP1A2 incubated with 200 μ M MelQx for 30 min. Similarly the *N*-hydroxy MelQx formation was detected in human recombinant CYP1A1, CYP1B1 at very low level as compared to IQ and MelQ. The *N*-hydroxy MelQx formation was not detectable at 5 μ g human liver microsomes and comfortably detected at 50 μ g human liver microsomes.

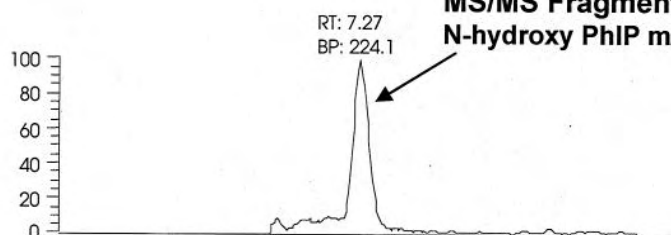
Relative Abundance

N-hydroxy PhIP MS $m/z = 241$ [M+H]



MS/MS Fragment

N-hydroxy PhIP $m/z = 224$ [M+H -OH]



PhIP Substrate

uAU

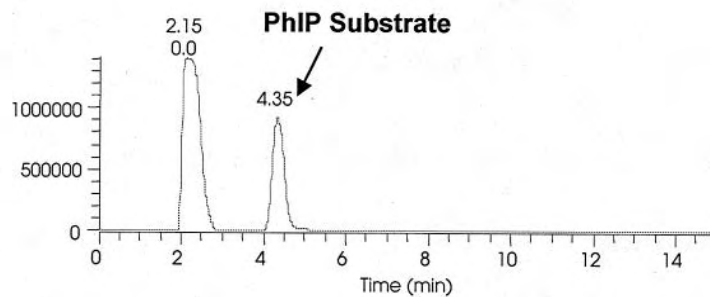


Figure 5. HPLC-UV, MS and MS/MS analysis of PhIP and N-hydroxy PhIP, metabolism of PhIP by human liver microsomes (1 μ g protein) incubated with 200 μ M of PhIP for 30 min.

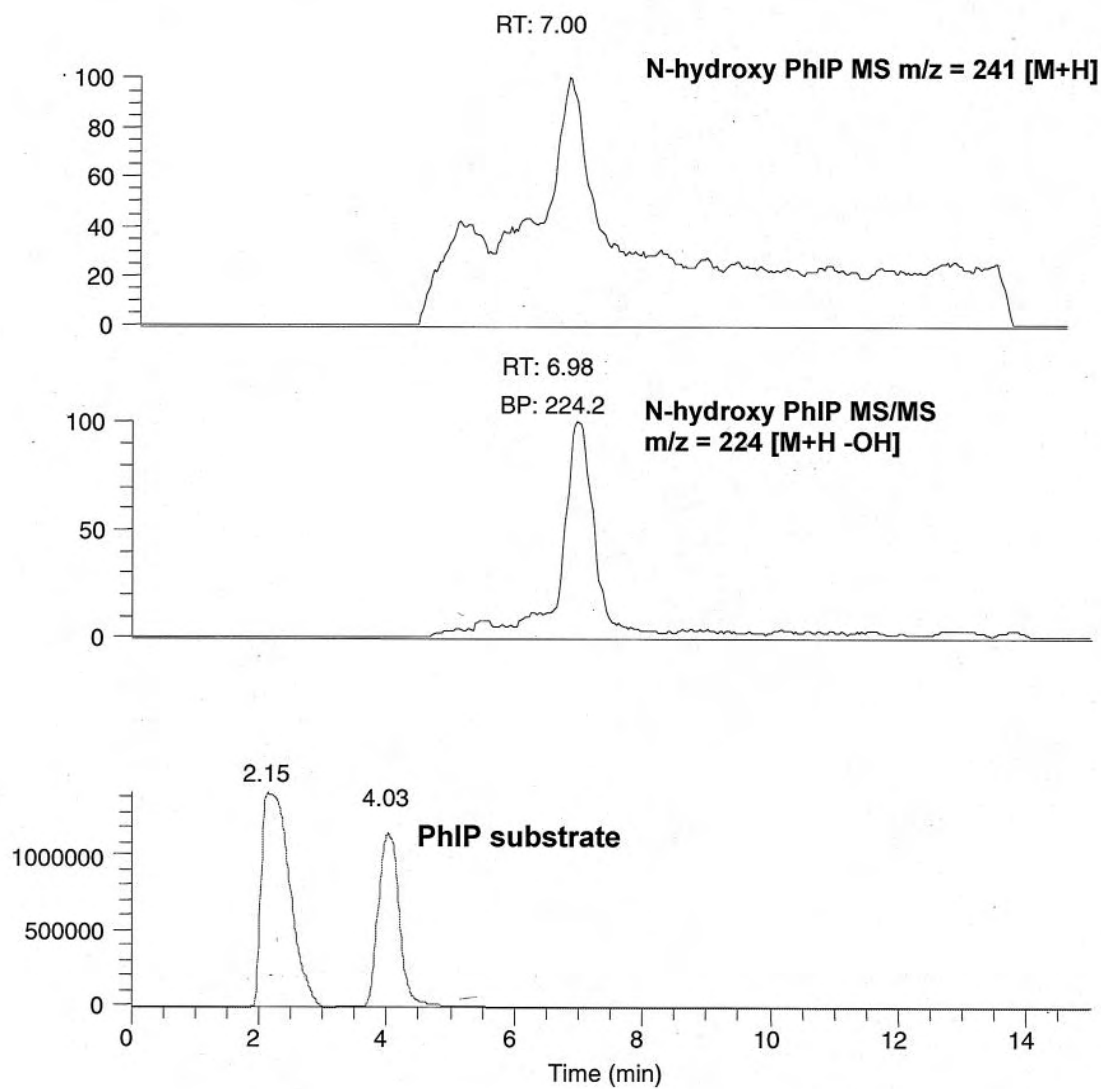


Figure 6. HPLC-UV, MS and MS/MS analysis of PhIP and *N*-hydroxy PhIP formation from the metabolism of PhIP by human prostate microsome P7 (140 μ g) incubated with 200 μ M of PhIP for 30 min.

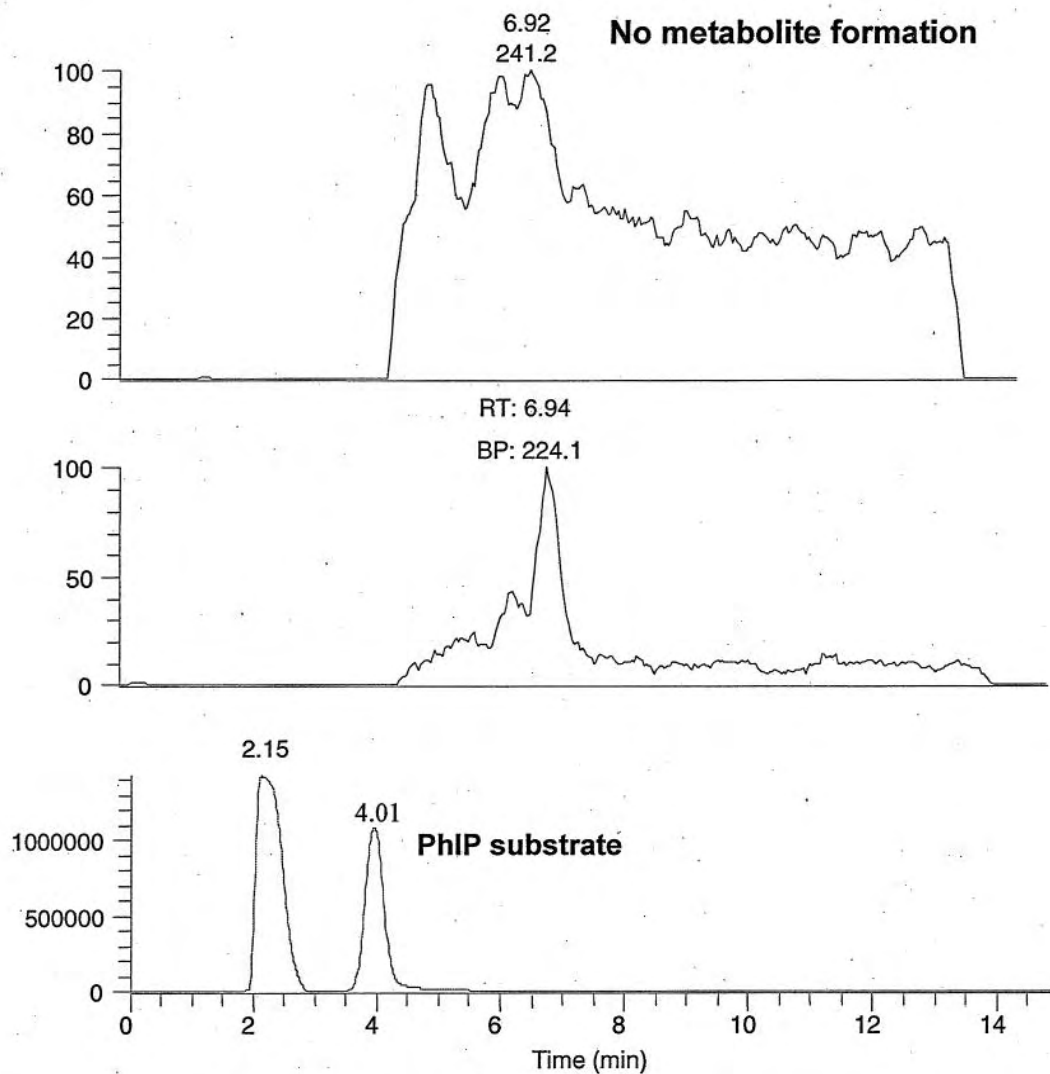


Figure 7: HPLC-UV, MS and MS/MS analysis of PhIP and *N*-hydroxy PhIP in the negative control. human prostate microsome 140 μ g denatured by ice cold methanol prior to incubation and incubated with 200 μ M of PhIP for 30 min. (Due to the very high sensitivity, the blank and negative control readings obtained in the MS/MS mode were subtracted for base line correction.)

RT: 0.00-2198 SM: 7B

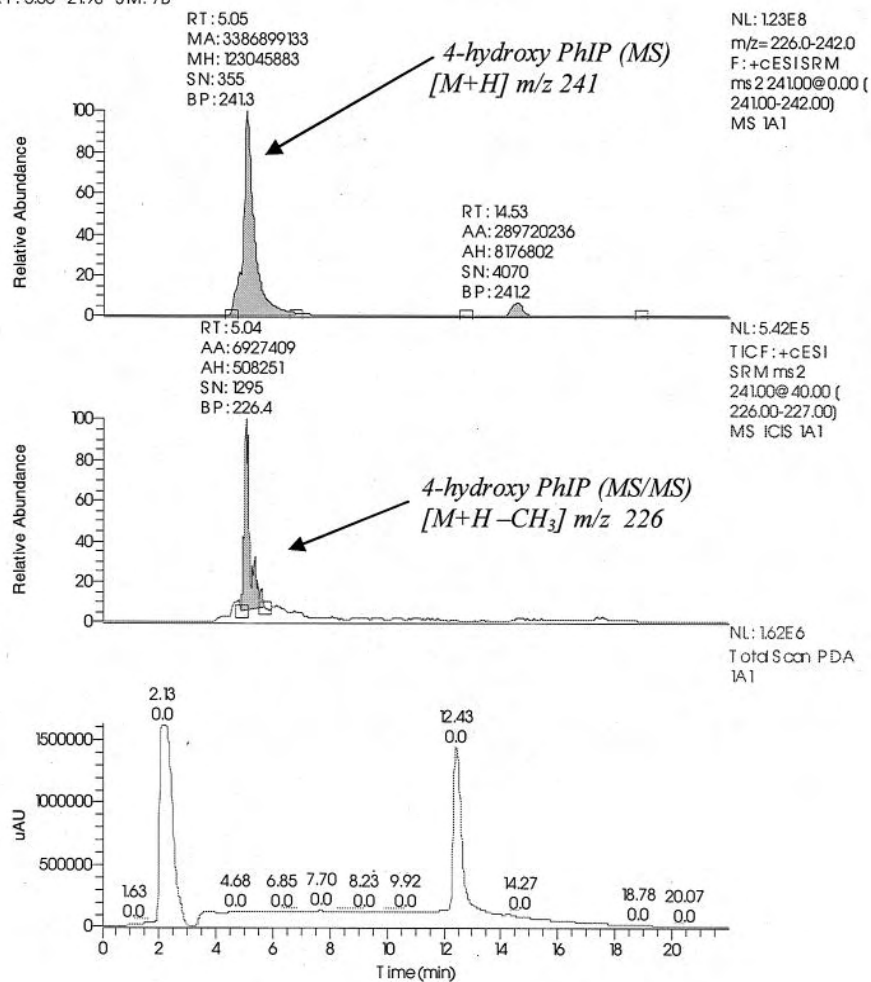


Figure 8. HPLC-UV, MS and MS/MS analysis of PhIP and 4-hydroxy PhIP, metabolism of PhIP by 5 pmol of human recombinant CYP1A1 incubated with 200 μ M PhIP for 30 min.

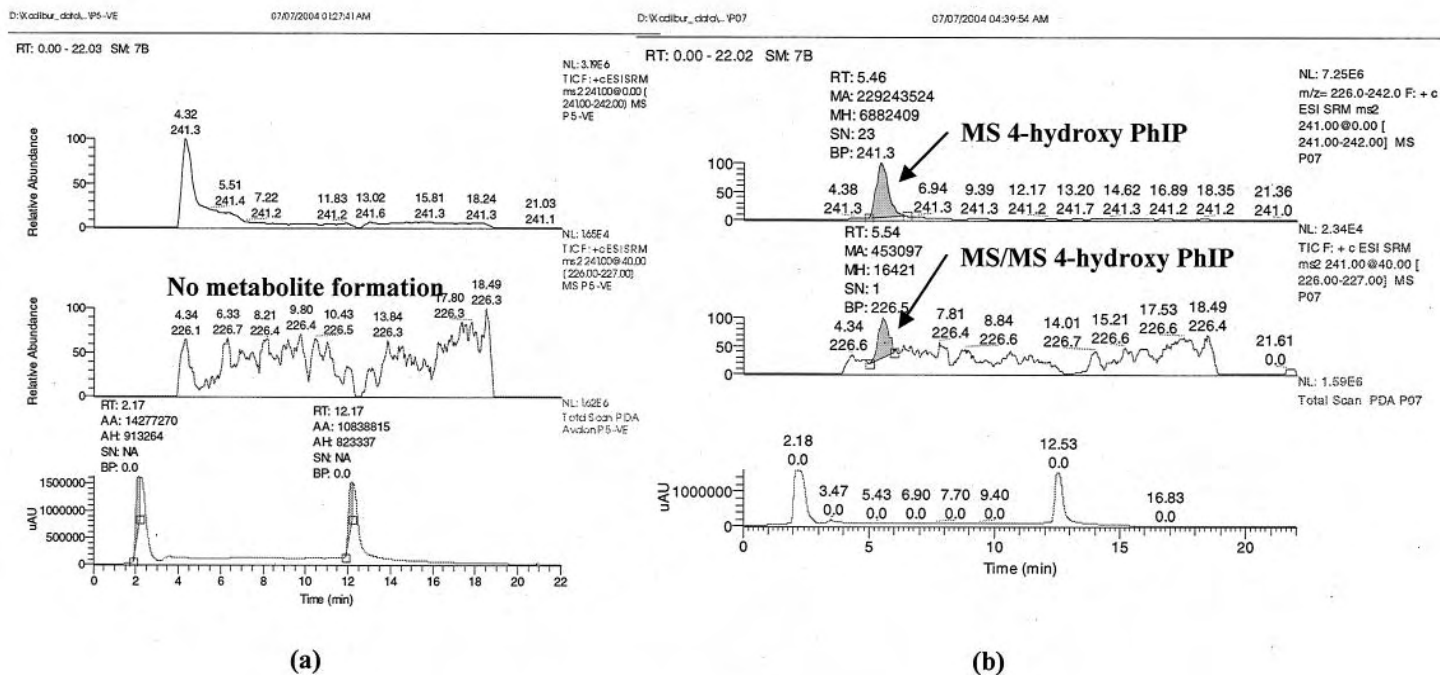


Figure 9. HPLC-UV, MS and MS/MS analysis of PhIP and 4-hydroxy PhIP, metabolism of PhIP by human prostate microsome incubated with 200 μ M PhIP for 30 min. (a) negative control where the microsomes were denatured prior to incubation and (b) is incubation with prostate microsome P7.

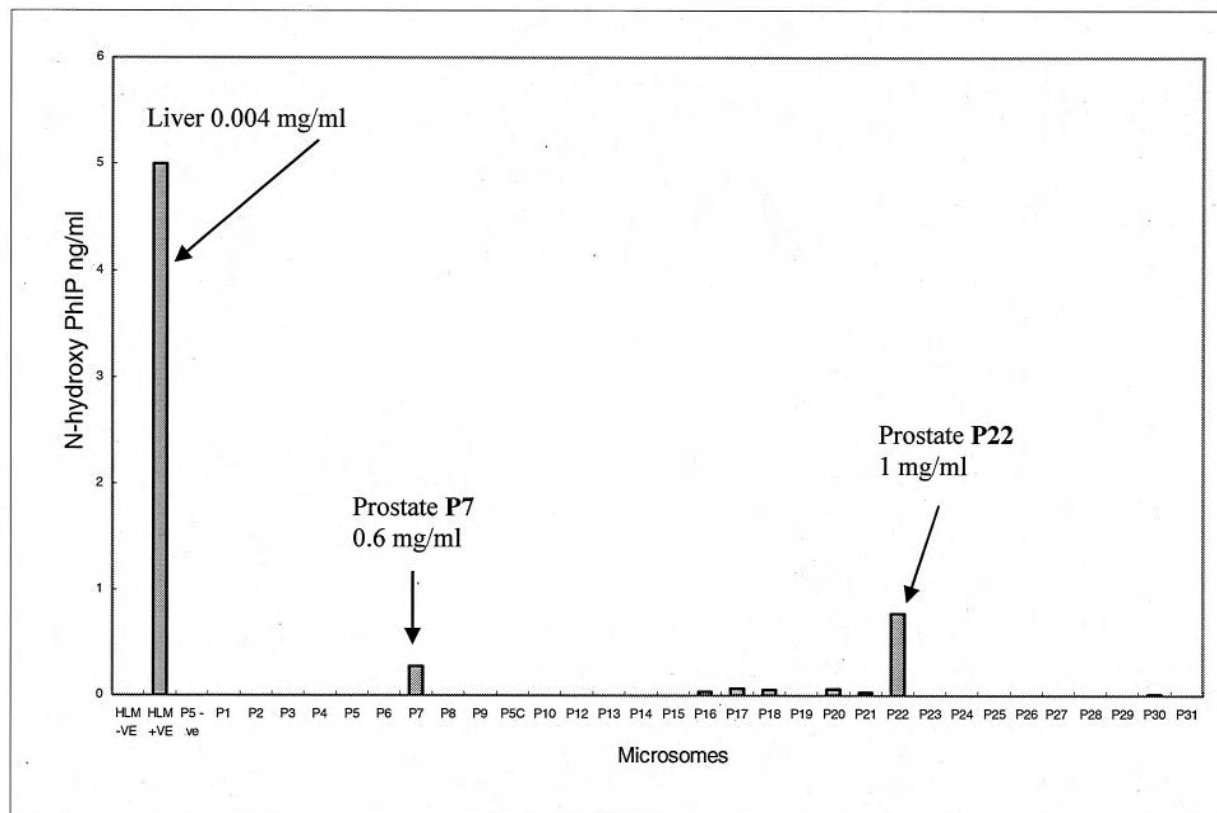


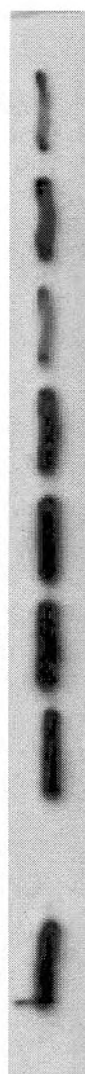
Figure 10. Comparison of metabolic activation of PhIP in human liver and prostate microsomes, final protein concentration was 0.004 mg/ml in human liver microsomes and in case of prostate P7 and P22 the protein concentration was 0.6 mg/ml and 1 mg/ml respectively.

Fig 11

A. Immunoblot analysis:

CYP1B1

wt (-) a Cys⁵⁷ Glu⁶¹ Ser¹¹⁹ Trp³⁶⁵ Leu³⁷⁹ Lys³⁸⁷



b c d Trp⁴⁶⁹ e Gly⁴⁴³ His³⁹⁰ Ser⁴⁵³ Leu⁴³⁷



a -- Gly⁴³²/Val¹⁴³², b -- Gly⁴³²/Ser¹¹⁹/Val¹⁴³²/Gly¹³²⁸, c -- Gly⁴³²/Ser¹¹⁹/Val¹⁴³², d -- Gly⁴⁸/Ser¹¹⁹, e -- 60.8 kD Marker

B. CO-difference spectra

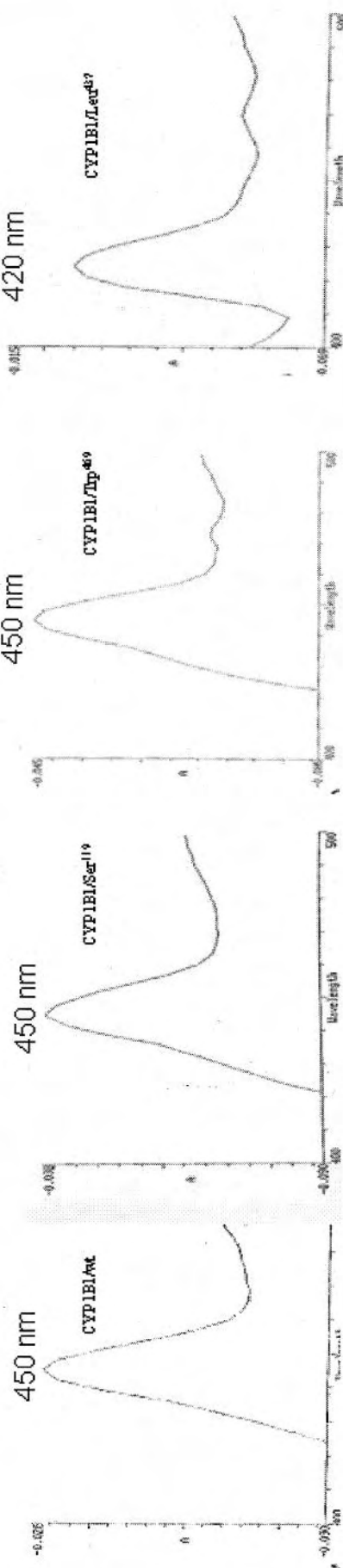
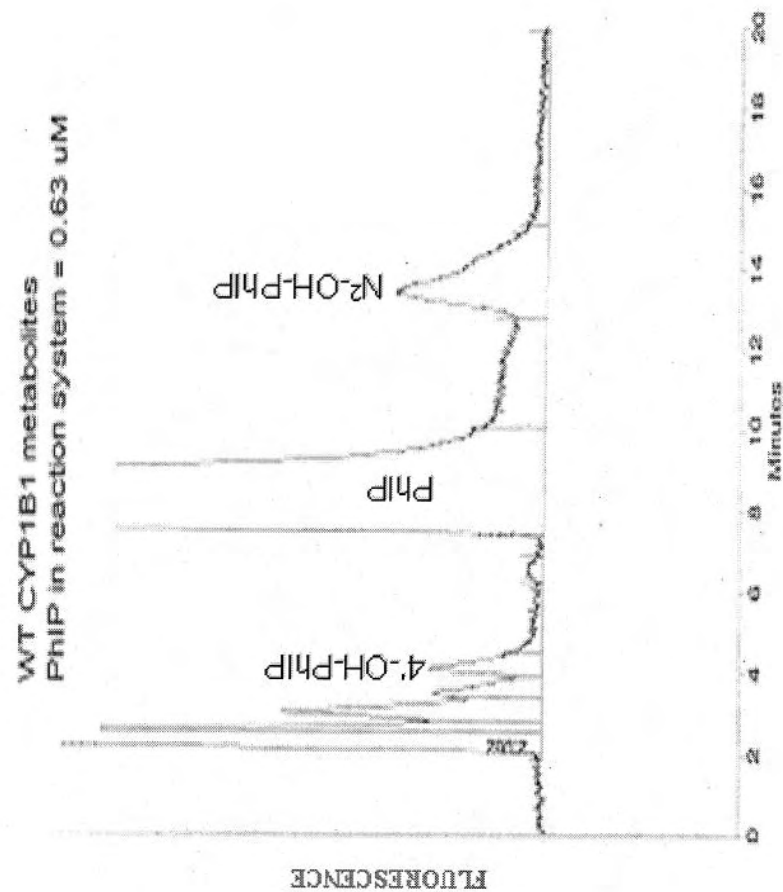


Fig 12

B.



A.

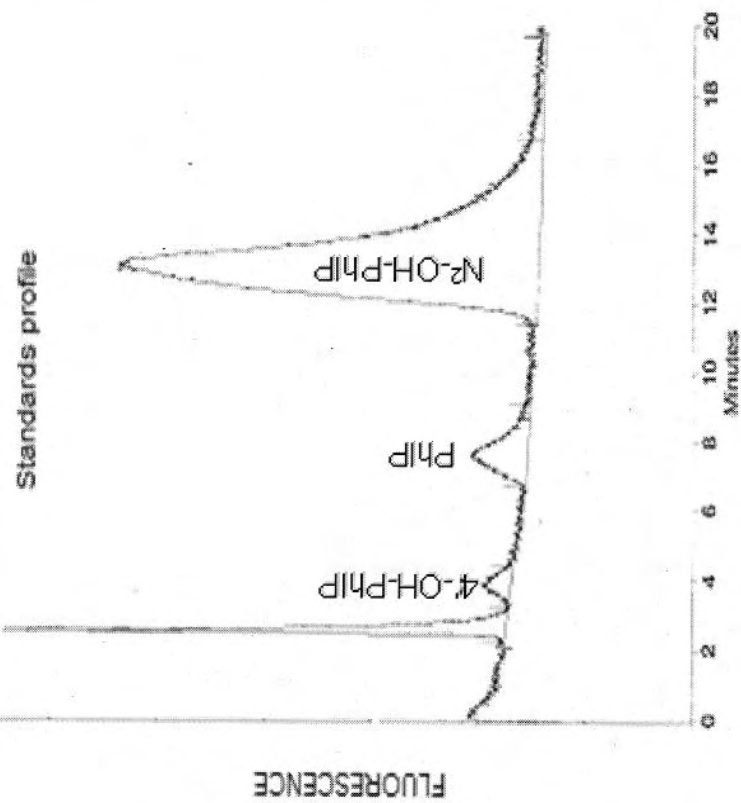
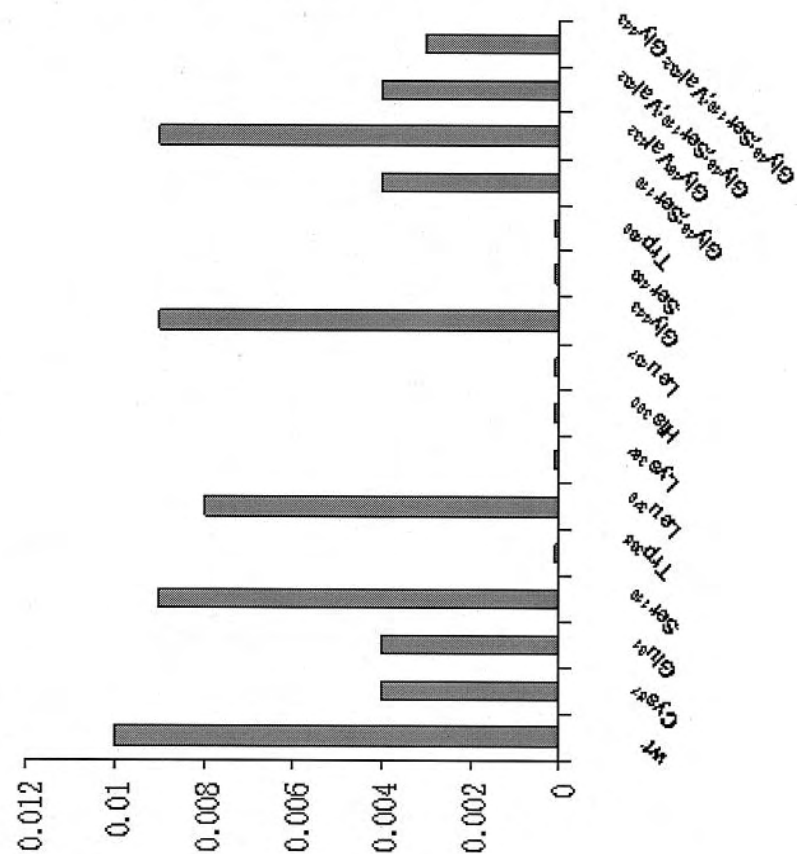


Fig 13

A. N²-OH-PhIP formation



B. 4'-OH-PhIP formation

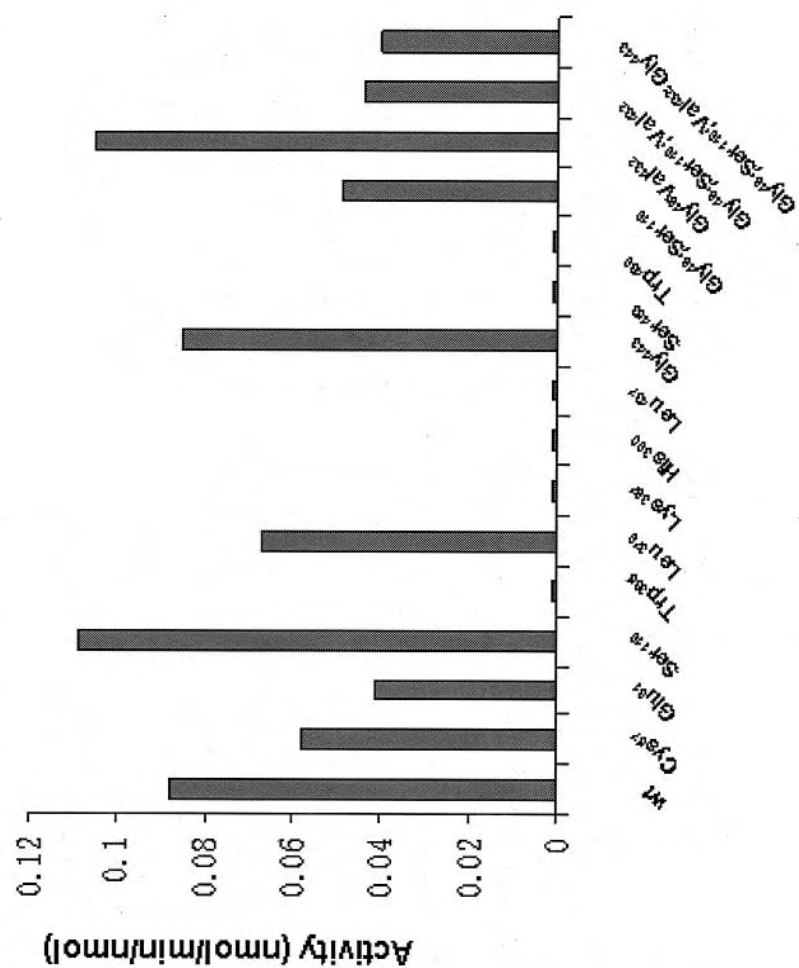


Table 1. Demographic data of subjects

ID	Age	Ethnic Status	Cause of surgery
P0	85	Caucasian	BPH
P1	73	Caucasian	BPH
P2	61	Caucasian	BPH & Bladder cancer
P3	76	Caucasian	BPH & prostate cancer
P4	77	Caucasian	BPH & Bladder cancer
P5	67	Caucasian	Chronic Prostatitis & BPH
P5c	67	Caucasian	Chronic Prostatitis & BPH
P6	54	Not available	Normal / unknown
P7	54	Not available	Normal prostate/ Bladder cancer
P8	81	Not available	BPH
P9	64	Not available	BPH
P10	75	Caucasian	BPH
P12	69	Caucasian	BPH
P13	68	Black	Chronic Prostatitis & BPH
P14	63	Caucasian	BPH
P15	72	Black	BPH
P16	66	Caucasian	BPH & prostate cancer
P17	75	Caucasian	BPH & prostate cancer
P18	68	Caucasian	BPH & prostate cancer
P19	71	Caucasian	BPH & prostate cancer
P20	75	Caucasian	Chronic Prostatitis & BPH
P21	77	Caucasian	BPH
P22	60	Caucasian	Normal prostate/ Bladder cancer
P23	66	Hispanic	BPH & prostate cancer
P24	77	Caucasian	Chronic Prostatitis & BPH
P25	62	Black	BPH
P26	60	Caucasian	Chronic Prostatitis & BPH
P27	75	Caucasian	BPH
P28	59	Black	BPH
P29	57	Caucasian	BPH
P30	58	Caucasian	BPH
P31	76	Caucasian	BPH

(BPH, Benign Prostatic Hyperplasia)

Table 2

CYP1B1 variant proteins heterogously expressed, their designed site-directed mutagenesis primers, and reported allele frequency

Allele	Nucleotide changes	Effect	Expression Level (pmol/μg)	Alle. Freq.	Primers Sequences	References
5	142C>G, 1294C>G	R48G; L432V	0.118	0.70%	5'-GGCGGCAGCTCGGGTCCGCGCCCC-3'	Akililu <i>et al.</i> , 2002
11	171G>C	W57C	0.105	(Ethiopian) rare	5'-GGGGCGCGGACCCGAGCTGCCGCGCC-3'	Stoilov <i>et al.</i> , 1998
12	182G>A	G61E	0.295	rare	5'-CCCCGTTTGCTGCGCCACTGATCGGAAAC-3'	Stoilov <i>et al.</i> , 1998
-	355G>T	A119S	0.157	50% (Seveso)	5'-GTTTCCGATCAGTGGGCACGCAAAACGGG-3'	Tanaka <i>et al.</i> , 2002
18	1093G>T	G365W	0.038	rare	5'-GGCCACTGATCGAAACGCGCGGCGG-3'	Stoilov <i>et al.</i> , 1998
19	1136C>T	P379L	0.055	rare	5'-CGCCGCGCGTTTTCGATCAGTGGCC-3'	Stoilov <i>et al.</i> , 1998
20	1159G>A	E387K	0.024	rare	5'-CCGACCGGCGGTCCTTTCGCTCCT-3'	Stoilov <i>et al.</i> , 1998
21	1169G>A	R390H	-	rare	5'-AGGAGGCGAAGGACGGCCGGTCGG-3'	Stoilov <i>et al.</i> , 1998
23	1310C>T	P437L	-	rare	5'-GGATCAGGTGCTGAGGACCGTCTG-3'	Stoilov <i>et al.</i> , 1998
-	1328C>G	A443G	0.263	-	5'-CAGACGGTCCCTCCACGACCTGATCC-3'	Stoilov <i>et al.</i> , 1998
4	1358A>G	N453S	-	2%	5'-CAGCCCAACCTGCTCTATGCTGGCCT-3'	Akililu <i>et al.</i> , 2002
25	1405C>T	R469W	0.19	(Ethiopian) rare	5'-AGCCAGGACATAGAGCAGGTGGGCTG-3'	Stoilov <i>et al.</i> , 1998
6	142C>G; 355G>T; 1294C>G	R48G; A119S; L432V	0.281	6.3% (Ethiopian)	5'-CCTGGCCTTCCCTTATAAGCCATGCGCTTCTCC-3'	Stoilov <i>et al.</i> , 1998
2	142C>G; 355G>T	R48G; A119S	0.261	36.7% (Ethiopian)	5'-GGAGAAGCGCATGGCTTATAAGGAAGGCCAGG-3'	Stoilov <i>et al.</i> , 1998
7	142C>G; 355G>T; 1294C>G; 1328C>G	R48G; A119S; L432V; A443G	0.324	7% (Ethiopian)	5'-CTTTATGAAGCCATGCACTTCTCCAGCTTGTGC-3'	Stoilov <i>et al.</i> , 1998
					5'-GCACAAAGCTGGAGAAAGTGCATGGCTTCATAAAG-3'	Stoilov <i>et al.</i> , 1998
					5'-GTGAAGTGGCCTAACCTGGAGAACTTTGATCCAG-3'	Stoilov <i>et al.</i> , 1998
					5'-CTGGATCAAAGTCTCCAGGTTAGGCCACTTCAC-3'	Stoilov <i>et al.</i> , 1998
					5'-GAGAACTTTGATCCAGGTCGATTCTTGGACAAGG-3'	Stoilov <i>et al.</i> , 1998
					5'-CCTTGTCCAAAGAATCGACCTGGATCAAAGTTCTC-3'	Stoilov <i>et al.</i> , 1998
					5'-GGATGGCCTCATCAGCAAGGACCTGACCCAG-3'	Stoilov <i>et al.</i> , 1998
					5'-CTGGTCAGGTCCTTGTGATGAGGCCATCC-3'	Stoilov <i>et al.</i> , 1998
					5'-CAGTGGGCAAAAGGTTGGTGCATTGGCGAAG-3'	Stoilov <i>et al.</i> , 1998
					5'-CTTCGCCAATGCACCACTCTTTTGCCTACTG-3'	Stoilov <i>et al.</i> , 1998
					5'-GTGAATCATGACCCAGTGAAGTGGCCTAAC-3'	Stoilov <i>et al.</i> , 1998
					5'-GTTAGGCCACTTCACTGGGTGATGATTTCAC-3'	Stoilov <i>et al.</i> , 1998

***, primers for 1294C>G nucleotide change.

Table 3

Kinetics parameters for heterogeneously expressed human PhIP hydroxylation by human CYP1B1 in PhIP N-hydroxylation and 4-hydroxylation.

CYP1B1 variant	Amino acid substitutions	N ² -OH-PhIP			4'-OH-PhIP		
		K_m	V_{max}	$K_{cat} (V_{max}/K_m)$	K_m	V_{max}	$K_{cat} (V_{max}/K_m)$
CYP1B1.1	None	μM	nmol/min/nmol		μM	nmol/min/nmol	
CYP1B1.11	Trp57Cys	3.6	0.012	0.003	4.5	0.120	0.026
CYP1B1.12	Gly61Glu	8.5	0.005	0.001	10.4	0.085	0.008
CYP1B1.2	Arg48Gly/Ala119Ser	7.5	0.009	0.001	3.9	0.056	0.014
CYP1B1.6	Arg48Gly/Ala119Ser/Val432Leu	3.2	0.006	0.002	4.1	0.049	0.012
CYP1B1.7	Arg48Gly/Ala119Ser/Val432Leu/Ala443Gly	14.8	0.016	0.001	6.0	0.100	0.017
		30.1	0.022	0.0007	2.7	0.057	0.021

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

DETERMINATION OF PHIP AND ITS METABOLITE N2-OH-PHIP BY LC/ESI-ITMS

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http://www2.umdj.edu/rdayweb/abstracts/abstracts_public_2.htm

Epidemiological studies suggests that exposure to heterocyclic amines, such as 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), formed in high-temperature cooked meat and cigarette smoke may play an important role in the causation of human prostate cancer. The formation of mutagenic PhIP metabolite N2-OH-PhIP by cytochrome P450 (CYP)-mediated N-hydroxylation is well documented. The capability of human prostate tissue in metabolizing PhIP, however has not been well studied. In this study, we have established a direct injection method based the modification to our previously reported LCMS/MS protocol for the determination of PhIP and its metabolites. This method is rapid, sensitive, selective and reliable for quantitation of N2-OH-PhIP. The method has been validated using human liver microsomes and human CYP1A1 and CYP1A2, the enzymes are known to be active in PhIP metabolism. In order to identify and confirm the N-hydroxylation fragmentation of N2-OH-PhIP in an ion trap was studied from liquid chromatography of the incubated samples containing the metabolites under assay in LCMS/MS and electrospray ionization as ion source. The MS2 spectrum obtained for N2-OH-PhIP was compared with other hydroxy PhIP metabolites. The base peak in the MS2 spectrum for N2-OH-PhIP (m/z 224, RT: 7.1) originated from loss of $[M+H-OH]^+$ from the N2-OH-PhIP molecular ion (m/z 241). A similar base peak fragment was also observed in the MS2 spectrum from the standard N2-OH-PhIP. With the same amount collision energy 4-hydroxy and 5-hydroxy PhIP (m/z 241, RT: 4.6 and 25.9) did not form the base peak 224 by MS2, which eventually confirms the N-hydroxylation. Preliminary study with the human prostate microsomes showed the formation of N2-OH PhIP. In addition, we examined PhIP metabolism by human CYP2S1 and found the activity is negligible as compared to the activity of CYP1A1 and CYP1A2 [Supported by US Army Medical Research Prostate Cancer Program, DAMD-17-02-1-0250].

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Metabolism of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) by Human CYP1B1 Genetic Variants

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Human cytochrome P4501B1 (CYP1B1) plays an important role in the metabolic activation of a variety of pro-carcinogens, including 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), a heterocyclic amine formed in fried, grilled or broiled meat and fish as a result of the pyrolysis of amino acids and proteins. PhIP is a suspected human carcinogen and induces cancers in the colon, breast, and prostate in rodents. The existence of human CYP1B1 missense genetic variants has been demonstrated but their activities in metabolizing PhIP are unknown. In this study, we used a baculovirus/insect cell system to express a total of 15 naturally-occurring CYP1B1 variants (with either a single or multiple amino acid substitution) 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 activities of 4 variants (Ala¹¹⁹Ser, Pro³⁷⁹Leu, Ala⁴⁴³Gly, Arg⁴⁸Gly/Leu⁴³²Val) were comparable to that of the expressed wild-type CYP1B1, 5 variants (Trp⁵⁷Cys, Gly⁶¹Glu, Arg⁴⁸Gly/Ala¹¹⁹Ser, Arg⁴⁸Gly/Ala¹¹⁹Ser/Leu⁴³²Val, Arg⁴⁸Gly/Ala¹¹⁹Ser/Leu⁴³²Val/Ala⁴⁴³Gly) exhibited a more than 2-fold decrease in the PhIP metabolizing activity which is consistent with the kinetic analysis data that the catalytic efficiency (V_{max}/K_m) for both N- and 4-hydroxylation was decreased in these five variants. 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 variants Trp³⁶⁵, Lys³⁸⁷, and His³⁹⁰ and was not detectable for the Ser⁴⁵³ variant, which correlates well with their low P450 content. 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 protein also had a substantial amount of expression in the microsomes but lack of detectable P450 peak and activity. Results from our study provide important information on the functionality of CYP1B1 genetic variations and should be useful in selection of appropriate cancer susceptibility biomarkers for future human population studies related to PhIP exposure [Supported by US Army Medical Research Prostate Cancer Program, DAMD-17-02-1-0250].

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