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Human UDP-Glucuronosyltransferase 1A1 is the Primary Enzyme Responsible for the *N*-glucuronidation of *N*-hydroxy-PhIP *in vitro*

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Abstract

UDP-Glucuronosyltransferase 1A proteins (UGT1A) catalyze the glucuronidation of many endogenous and xenobiotic compounds including heterocyclic amines and their hydroxylated metabolites (the main topic of this study). Studies have shown that in humans UGT1A mediated glucuronidation is an important pathway in the detoxification of food-borne carcinogenic heterocyclic amines. The biotransformation of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), the most mass abundant heterocyclic amine found in cooked meats, is highly dependent on cytochrome P4501A2 hydroxylation followed by UGT catalyzed glucuronidation of the *N*-hydroxy-PhIP reactive intermediate. To determine which UGT1A proteins are involved in the glucuronidation of *N*-hydroxy-PhIP, microsomal preparations from baculovirus infected insect cells that express all of the known functional human UGT1A isozymes (UGT1A1, -1A3, -1A4, -1A6, -1A7, –1A8, -1A9, -1A10) were exposed to *N*-hydroxy-PhIP and the reaction products were isolated by HPLC. All UGT1A proteins except UGT1A6 showed some degree of activity towards *N*-hydroxy-PhIP. The formation of both *N*-hydroxy-PhIP-N^2-glucuronide and *N*-hydroxy-PhIP-*N3*-glucuronide was both time and substrate concentration dependent in all the microsomal incubations that showed appreciable activity. UGT1A1 was the most efficient in converting *N*-hydroxy-PhIP to both conjugates producing 5 times more of the N^2-conjugate than UGT1A4, the next active UGT, and 286 times more than UGT1A7, the least active UGT. With an apparent K_m of 52 μM and a K_{cat} of 114 min^{-1}, UGT1A1 was also the most catalytically efficient in forming *N*-hydroxy-PhIP-N^2-glucuronide. Catalytic constants for UGT1A4, UGT1A8 and UGT1A9 were 52 min^{-1}, 35 min^{-1} and 3.7 min^{-1}, respectively. The catalytic

efficiency for *N*-hydroxy-PhIP-*N3*-glucuronide formation was 8, 10, and 6 times lower for UGT1A1, -1A4, and -1A8, respectively, when compared to the k_{cat} values for *N*-hydroxy-PhIP-N^2-glucuronide formation. These results clearly show that UGT1A1 is mainly responsible for glucuronidating *N*-hydroxy-PhIP. Polymorphic expression resulting in decreased UGT1A1 activity in humans can cause reduced rates of glucuronidation which can change the metabolic ratio between bioactivation and detoxification to favor bioactivation. This change will increase the susceptibility to the deleterious effects from PhIP exposure because the capacity to form nontoxic *N*-hydroxy-PhIP glucuronide conjugates will be diminished.

Footnotes

Abbreviations

[1]UGT, UDP-glucuronosyltransferase; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; *N*-hydroxy-PhIP, 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; UDPGA, uridine diphosphoglucuronic acid.

Introduction.

The UDP-glucuronosyltransferases (UGTs)[1] are a superfamily of enzymes that catalyze the glucuronidation of many endogenous and xenobiotic compounds. UGT proteins are divided into 2 families, UGT1A and UGT2B, based on sequence homologies (*1*). The UGT1A family is located on chromosome 2, and is derived from a single locus that contains a unique exon 1 sequence that codes for each of eight functional UGT proteins (*2*). The carboxy terminus (exons 2-5) is identical in all UGT1A proteins. UGT1A isozymes have been shown to catalyze the glucuronidation of a wide variety of xenobiotics including heterocyclic amines and their hydroxylated metabolites, and are primarily responsible for the formation of many different *N*-glucuronides. (*3,4*). In contrast to the UGT1A family, the UGT2B family is located on chromosome 4 and consists of six individual structural genes (*5*). Gene products of the UGT2B family are primarily involved in steroid metabolism.

Most UGT1A proteins are found in the liver, however, appreciable levels have been detected in several extrahepatic tissues. In addition to a wide tissue distribution of UGT1As, different isoforms can be preferentially expressed in different tissue. The UGT1A gene locus in the liver codes for UGT1A1, UGT1A3, UGT1A4, UGT1A6 and UGT1A9. Expression of UGT1A7, UGT1A8 and UGT1A10 is found exclusively in extrahepatic tissue, primarily in gastric, colon and biliary tissue, respectively (*6*). The colon expresses all the known functional UGT1A isozymes except UGT1A7 (*7*). In addition to tissue specific patterns of expression, polymorphic expression of certain UGT1As has been reported (*8-10*). The differential expression of the UGT1A family of

enzymes demonstrates the importance of site-specific metabolism and substrate selectivity of the UGT1A proteins.

Studies have shown that the detoxification of food-borne carcinogenic heterocyclic amines is highly dependent on UGT-mediated glucuronidation (*11-14*). 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), the most mass abundant heterocyclic amine found in well-done cooked meats, is extensively glucuronidated by human UGT1A proteins (*13-18*). The most significant conjugation has been shown to be the *N*-glucuronidation of the reactive intermediate 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-b]pyridine (*N*-hydroxy-PhIP).

The bioactivation of PhIP in humans is dependent on the cytochrome P4501A2 catalyzed *N*-hydroxylation of PhIP to the corresponding *N*-hydroxy-PhIP (*19,20*). Subsequent esterification by sulfotransferase and/or acetyltransferase results in the formation of the highly electrophilic O-sulfonyl and O-acetyl esters, respectively (*21,22*). These esters most likely go on to form an aryl-nitrinium ion that can bind DNA and potentially cause mutations. *N*-Glucuronidation competes with these activation reactions and results in the formation of the less reactive *N*-hydroxy-PhIP-N^2-glucuronide and *N*-hydroxy-PhIP-*N3*-glucuronide (Figure 1) (*13,14*). These conjugates can be excreted though the urine or bile, or be transported to multiple tissues where further metabolism can occur. Differential expression of UGTs in specific tissues can change the metabolic ratio between bioactivation and detoxification. A change favoring bioactivation, due to decreased glucuronidation activity, likely leads to an increase in the susceptibility to potential tumor formation from PhIP exposure. Knowing the glucuronidation capacity of each UGT protein will allow for a better understanding of the bioactivation/detoxification

mechanisms of PhIP. This will help in evaluating the individual susceptibility to the potential cancer risks involved in exposure to PhIP. It is hypothesized that individuals with low levels of specific UGT proteins will have a diminished capacity to detoxify PhIP, making them more susceptible to the deleterious effects from PhIP exposure.

This lab and others has implicated UGT1A1 as the primary UGT protein involved in the glucuronidation of PhIP (*17,23*). This current study re-enforces the results from the previous reports and expands on them to obtain a more comprehensive understanding into the metabolic mechanisms of PhIP conjugation. Microsomal preparations containing recombinant human UGT1A proteins where used to assess the ability of each protein to catalyze the glucuronidation of *N*-hydroxy-PhIP. Kinetic parameters were measured for each of the UGT1A isozymes tested. Differential selectivity for the *N*-hydroxy-PhIP substrate was observed between each of the UGT1A isozymes.

Materials and Methods

Chemicals and reagents. Caution: *N-hydroxy-PhIP is carcinogenic to rodents and should be handled with care*. *N*-Hydroxy-PhIP was obtained from SRI International, Palo Alto, CA. The purity was checked by HPLC (isocratic at 40% methanol) and was determined to be greater than 95% pure. UDP-glucuronic acid (UDPGA), alamethicin and all other reaction buffer components were obtained as a mix from BD Gentest^{tm}, (Bedford, MA). All immunoblotting reagents were obtained from BioRad, (Hercules, CA.). Antibodies for immunoblotting were obtained from BD Gentest^{tm}. All other reagents were of analytical grade or better.

Quantification UGT1A protein expression in microsomal preparations. Immunoblotting was performed according to manufacturers recommendations (BD Gentest^{tm}). Briefly, one microgram of microsomal protein from each UGT1A expressing preparation was heated at 95°C for 4 min in loading buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 25% glycerol, 0.01% Bromophenol Blue) containing 2% 2-mercaptoethanol, and then separated on a 10% SDS-polyacrylamide gel. The separated proteins were electrotransferred onto a nitrocellulose membrane. The membrane was blocked in 5% nonfat powdered milk in 25 mM Tris-HCl (pH 7.5), 150 mM NaCl (Solution A) for 1 h, and then washed 3 times with Solution A containing 0.1% Tween 20. Membranes were then incubated for 1 h in 0.5% nonfat powdered milk in Solution A containing an antibody (1:1000) prepared from a rabbit immunized with a peptide specific for the conserved carboxy-terminal region of all human UGT1A isoforms (WB-UGT1A; BD Gentest^{tm}). After 3 more washes the membrane was incubated for 1 h in 0.5% nonfat

powdered milk in Solution A containing an HRP-conjugated goat anti-rabbit IgG secondary antibody (1:1000) followed by washing. Visualization was performed using Immun-Star HP substrate detection kit (BioRad). UGT1A protein content was estimated based on comparison of chemiluminescence intensity to a known 50 kD protein standard of known concentration (Precision Plus Protein Standard, BioRad) using a Shimadzu CS-9000 Dual Wavelength Flying Spot Scanner (Shimadzu Scientific Instruments, Columbia, MD).

Time dependent glucuronidation of N-hydroxy-PhIP. Microsomal preparations from baculovirus infected insect cells (BTI-TN-5B1-4) separately expressing UGT1A1, -1A3, -1A4, -1A6, -1A7, -1A8, -1A9 or -1A10 from human cDNA, which represent all the functional UGT1A proteins, were purchased from BD Gentest^{tm}, (Bedford, MA). Incubations were prepared on ice in 1.5 ml conical plastic tubes and consisted of 0.5 mg/ml microsomal protein, 8.0 mM $MgCl_2$, 0.5 mM EDTA, 2.0 mM UDPGA, 25 μg/ml alamethicin, and 5 μM or 100 μM *N*-hydroxy-PhIP (dissolved in dimethylsulfoxide delivered in 10 μl) in 50 mM Tris-HCl buffer, pH 7.5, in a final volume of 1.0 ml. Samples were incubated for up to 6 h at 37°C with no agitation after initial mixing. At 0.5, 1, 3, and 6 h incubation times a 250 μl aliquot was removed from each sample and placed into 2 volumes of ice-cold methanol to precipitate the proteins and stop the reaction. The samples were then allowed to stand at -20° C for 30 min. The protein was then removed by centrifugation in a microcentrifuge at maximum speed for 5 minutes. The methanolic extracts containing the reaction products were placed in clean plastic tubes and stored at -80°C until HPLC analysis.

Kinetics. Kinetic parameters were determined using the various microsomes with *N*-hydroxy-PhIP concentrations ranging from 0.5-500 μM. Microsomal incubations were carried out in the same manner as described above except that the final volume of each sample was 200 μl and the incubation time was 2 h. After the incubation time 2 volumes of ice-cold methanol were added to each sample to precipitate the proteins, as before. Metabolic products were isolated by HPLC. Apparent K_m and V_{max} values for *N*-glucuronidation by each enzyme were derived by fitting the data to the Michaelis-Menton equation, and obtaining a best fit curve from the data using the spreadsheet program Excel (Microsoft Corp., Redmond, WA).

HPLC analysis of glucuronide conjugates. The aqueous-methanol extracts from the microsomal incubations were evaporated to dryness under nitrogen, then reconstituted in 60 μl of HPLC starting mobile phase. The samples were centrifuged in a microcentrifuge for 1 min at maximum speed and 50 μl of supernatant was injected into an Alliance HPLC system (Waters Corp., Milford, MA) equipped with a 5 μm, 4.6 X 150 mm TSK-GEL ODS-80 TM column (Toso Bioscience, Montgomeryville, PA) and a Waters 990 photodiode array detector. Metabolites were eluted at 0.75 ml/min using a gradient starting at 30% methanol/0.1% triethylamine, pH 6.0, up to 55% methanol/0.1% triethylamine, pH 6.0, at 8 min. The methanol concentration was maintained at 55% from 8 to 20 min. The identities of the *N*-hydroxy-PhIP-glucuronide conjugates were confirmed by comparing the HPLC retention time and UV spectra to known metabolite standards, and quantified based on the molar extinction coefficient of PhIP.

Results

Quantification of UGT1A protein expression in microsomal preparations. Western Blot analysis was performed using an anti-human UGT1A peptide raised in a rabbit that is specific for the carboxy terminus of the protein, and whose sequence is conserved for all UGT1A isozymes. All microsomal extracts containing the baculovirus-expressed UGT1A isozymes revealed the expression of a protein with a molecular weight between 52-55 kDa (Figure 2). Expression of this protein was not detected in microsomes prepared using the wild type virus. The variability in the mobility of each protein band was due to the variability in the glycosylation (and thus mobility) of the UGT1A proteins. The chemiluminescence intensity of each band was compared to the intensity of a known molecular weight standard of known concentration. This comparison allowed for an estimate of the concentration of each UGT1A isozymes from the microsomal preparations. The estimated concentration of UGT protein present in the samples ranged from 0.57 ng/μl to 2.85 ng/μl. Microsomes containing the UGT1A10 protein had the highest expression level and microsomes containing UGT1A4 had the lowest. All subsequent experiments using the microsomal preparations were normalized to ng of UGT1A isozyme present in each preparation.

Stability of *N*-hydroxy-PhIP over time. The stability of the *N*-hydroxy-PhIP substrate was monitored to determine if any significant degradation of substrate occurred over the 6 h incubation time under the reaction conditions. Over the course of the experiment nonenzymatic degradation of *N*-hydroxy-PhIP resulted in a 2% loss in substrate over 3 h

and an additional 17% loss at 6 h (Figure 3). The 15% loss of substrate between 3 and 6 h could contribute to a significant reduction in the rate of glucuronide conjugate formation during that time.

Optimization of reaction pH. Each UGT1A isozyme was incubated with 25 μM *N*-hydroxy-PhIP at 4 different pH values (pH 6.5, 7.0, 7.5, 8.0) to determine the optimum pH for each UGT1A isozyme. All UGT activity was optimal at a pH of 7.5 except reactions containing UGT1A3 and 1A10, which were most efficient at pH 7.0 (data not shown). None of the differences in the amount of reaction product formed between the various pH values, however, were statistically significant.

Time dependent glucuronidation of *N*-hydroxy-PhIP. To determine which UGT1A proteins contribute the most to *N*-hydroxy-PhIP glucuronidation, each isozyme was incubated with either 5 μM or 100 μM of *N*-hydroxy-PhIP in a time dependent fashion and glucuronide metabolite formation was normalized to ng of UGT1A present in each sample. All UGT1As showed some degree of activity towards *N*-hydroxy-PhIP except UGT1A6. Each active UGT isozyme produced both *N*-hydroxy-PhIP-N^2-glucuronide and *N*-hydroxy-PhIP-*N3*-glucuronide in a time dependent manner at the 100 μM substrate concentration (Figure 4). However, the relative amount of each conjugate was very different (Figure 5). For incubations containing UGT1A3, 1A6 and 1A7 at 5 μM *N*-hydroxy-PhIP, there was no detectable glucuronide conjugate formation. In addition, incubations lacking the co-substrate UDP-glucuronic acid, produced no detectable *N*-hydroxy-PhIP glucuronide conjugates. Formation of both conjugates was linear with

time up to 3 h for both substrate concentrations in all UGT1A preparations that showed detectable activity. After 3 h the rate of formation began to level off, indicating a depletion of substrate and/or enzyme in the reaction (Figure 4). Based on the data, it was clearly evident that UGT1A1 was the most efficient in converting *N*-hydroxy-PhIP to both *N*-hydroxy-PhIP-N^2-glucuronide and *N*-hydroxy-PhIP-*N3*-glucuronide whereas UGT1A7 was the least efficient (Table 1). When 100 μM *N*-hydroxy-PhIP was used as substrate, at 3 h incubation time 220 pmol and 25 pmol of *N*-hydroxy-PhIP-N^2-glucuronide and *N*-hydroxy-PhIP-*N3*-glucuronide was formed per ng of UGT1A1 protein, respectively. UGT1A4, the next most active protein, formed 73 pmol and 6.1 pmol of *N*-hydroxy-PhIP-N^2-glucuronide and *N*-hydroxy-PhIP-*N3*-glucuronide per ng UGT1A4, respectively. The amount of *N*-hydroxy-PhIP-N^2-glucuronide formed from UGT1A7 was 286 times less than what was seen for UGT1A1. The ratio of *N*-hydroxy-PhIP-N^2-glucuronide to *N*-hydroxy-PhIP-*N3*-glucuronide was different between each of the UGT1A isozymes (Table 1). Incubations containing recombinant UGT1A1, 1A4, 1A8, and 1A10 produced *N*-hydroxy-PhIP-N^2-glucuronide at levels approximately 10 times higher than the levels of *N*-hydroxy-PhIP-*N3*-glucuronide, whereas, microsomes expressing UGT1A3, 1A7, and 1A9 showed the opposite relationship; as *N*-hydroxy-PhIP-*N3*-glucuronide was formed in 2 times excess of the N^2 conjugate. These ratios were consistent within each UGT1A isozyme over the course of the experiment.

UGT1A Kinetics. Enzyme kinetic analysis was performed using a subset of 4 of the 8 UGT1A isozymes with varying degree of activity (UGT1A1, UGT1A4, UGT1A8, UGT1A9) being found. All four isozymes produced a dose dependent increase in *N*-

hydroxy-PhIP-glucuronide conjugate formation. For UGT1A1 and 1A9 substrate inhibition occurred at 100 μM *N*-hydroxy-PhIP. Substrate inhibition was also evident for UGT1A4 and 1A8 but was not observed until 250 μM substrate concentration.

Table 2 shows the kinetic parameters for the formation of *N*-hydroxy-PhIP-N^2-glucuronide and *N*-hydroxy-PhIP-*N3*-glucuronide from the four UGT1A isozymes. Since apparent substrate inhibition was observed at the higher substrate concentrations, the kinetic parameters were derived from the apparent activity values obtained at the lower substrate concentrations. UGT1A1 had the highest rate of catalysis for forming *N*-hydroxy-PhIP-N^2-glucuronide with a V_{max} of 2.07 pmol/min/ng UGT1A1, a K_m of 52 μM and a K_{cat} of 114 min^{-1}(Table 2). With a catalytic constant of 3.7 min^{-1}, and a maximum velocity of 0.07 pmol/min/ng UGT1A, UGT1A9 was the least efficient in forming *N*-hydroxy-PhIP-N^2-glucuronide. UGT1A4 and 1A8 had K_{cat} and V_{max} values of 51.9 min^{-1} and 35.5 min^{-1}, and 0.94 pmol/min/ng UGT1A and 0.64 pmol/min/ng UGT1A, respectively. The rate of catalysis for UGT1A1, 1A4, and 1A8 to form *N*-hydroxy-PhIP-*N3*-glucuronide was much lower than the catalysis rate to form *N*-hydroxy-PhIP-N^2-glucuronide. The maximal velocity and the catalytic constants for *N*-hydroxy-PhIP-*N3*-glucuronide formation were 8, 10, and 6 times lower for UGT1A1, 1A4, and 1A8, respectively, when compared to the values for *N*-hydroxy-PhIP-N^2-glucuronide formation. UGT1A9, however, was 2 times more efficient at producing the *N3*-conjugate over the N^2 conjugate. Although, the K_{cat} for UGT1A9 was still 2 times less that that of UGT1A1.

Discussion

Previous studies by this and other labs have investigated the glucuronidation of PhIP and *N*-hydroxy-PhIP. The results from this current study expand on the previous findings and are presented as a more comprehensive investigation of all the known functional UGT1A proteins for their capability to catalyze the formation of glucuronide conjugates of *N*-hydroxy-PhIP, and the significance of the findings. The results from these current experiments clearly show that microsomal preparations containing recombinant human UGT1A1 protein are most responsible for the glucuronidation of *N*-hydroxy-PhIP. All the other UGT1A proteins, except for UGT1A6, were capable of glucuronidating *N*-hydroxy-PhIP as well, but at significantly lower rates. These findings are in accordance with what has been reported in previous studies (*17,23*). Estimating the UGT concentration of each isozyme in the microsomal preparations by Western blot analysis allowed for the normalization of glucuronide conjugate formation to actual UGT1A content rather than total microsomal protein content. Although standardization of protein content was estimated based on comparison of chemiluminescence intensity to a non-UGT protein standard, it was assumed that the binding efficiency of the HRP-conjugate was similar for all proteins therefore, the chemiluminescence intensity should be linear across all samples. This estimation of protein content allowed for a more precise assessment of the roles each UGT1A isozyme played in the glucuronidation of *N*-hydroxy-PhIP. The difference in glucuronide formation between the isozymes differed significantly. The recombinant UGT1A1 catalyzed the metabolism of *N*-hydroxy-PhIP to the *N*-hydroxy-PhIP-N^2-glucuronide at rates that were 286 times greater than the recombinant UGT1A7 under the same reaction conditions. Based on total *N*-hydroxy-

PhIP-glucuronide formation, the results suggest the following relative ranking of UGT1A capacity to conjugate *N*-hydroxy-PhIP: UGT1A1 > UGT1A4 > UGT1A8, UGT1A3 > UGT1A9 > UGT1A10 > UGT1A7 >> UGT1A6. The ability of UGT1A4 to catalyze the glucuronidation of *N*-hydroxy-PhIP in this current study is in contrast to previous studies that reported no observable activity for UGT1A4 to catalyze the glucuronidation of *N*-hydroxy-PhIP (*18,23*). This discrepancy could be due to different enzyme sources and/or different reaction conditions. The difference in glucuronidation activity between the different UGT1A proteins demonstrates the wide substrate selectivity each isozyme has and emphasizes the implications differential tissue expression can have on glucuronidation capability. For example, tissues with low levels of UGT1A1 will not be able to form the *N*-hydroxy-PhIP-glucuronides very efficiently which could lead to increased bioactivation of *N*-hydroxy-PhIP.

Two patterns emerged with regards to the formation of the N^2 and *N3* conjugates between the different UGT1A isozymes. The ratio of *N*-hydroxy-PhIP-N^2-glucuronide to *N*-hydroxy-PhIP-*N3*-glucuronide from incubations containing recombinant human UGT1A1, 1A4, 1A8, or 1A10 was 10:1, whereas, the ratio from incubations containing UGT1A3, 1A7 and 1A9 was 0.5:1. The finding that *N*-hydroxy-PhIP-N^2-glucuronide was the most abundant conjugate in incubations containing UGT1A1, 1A4, 1A8, 1A10 concurs with the findings that *N*-hydroxy-PhIP-N^2-glucuronide was the most abundant conjugate detected in human urine after exposure to PhIP (*15,16*). These findings suggest that these isozymes may play a significant role in *N*-hydroxy-PhIP glucuronide conjugation *in vivo*.

The proposition of UGT1A1 being the primary UGT protein responsible for *N*-hydroxy-PhIP glucuronidation has several implications. UGT1A1 plays an important role in the metabolism and elimination of both endogenous and exogenous compounds. UGT1A1 is most prevalent in the liver, but appreciable levels have been detected in the stomach, intestine and colon (reviewed in (*24*)). This differential tissue expression could render tissues with little or no UGT1A1 activity more susceptible to PhIP bioactivation, and ultimately tumor formation, because their ability to detoxify *N*-hydroxy-PhIP will be diminished. Competition between other UGT1A1 substrates, such as bilirubin, can also affect the rate at which *N*-hydroxy-PhIP is detoxified. It is possible that the relatively high concentration of bilirubin could out compete *N*-hydroxy-PhIP for the UGT1A1 binding site. UGT1A1 activity can also be altered by genetic variability. Mutations in the UGT1A gene have been shown to alter the expression of the UGT1A1 protein causing reduced rates of glucuronide conjugate formation. Most notably are the clinical conditions Crigler-Najjar syndrome and Gilbert's syndrome which cause varying degrees of hyperbilirubinemia. Crigler-Najjar syndrome is characterized by a complete lack of UGT1A1 mediated bilirubin conjugation caused by mutant coding region UGT1A1 alleles (reviewed in (*24*)). Gilbert's syndrome is characterized by the allelic variant UGT1A1*28, that contains an additional (TA) dinucleotide repeat in the "TATAA" box of the UGT1A1 promoter region (*25,26*). This polymorphism results in hepatic bilirubin UGT conjugation being reduced to about 30% of normal (*27,28*). UGT1A1 is the only UGT isoform known to be involved in these inherited diseases involving bilirubin metabolism (*29,30*). It's been reported that Gilberts syndrome occurs in 7-10% of the general population. This would suggest that individuals with Gilbert's syndrome maybe

at greater risk from toxicants that are detoxified by UGT1A1 because their ability to detoxify these compounds would be diminished (*31*). For example, a recent study has reported a correlation between the UGT1A1 "TATAA" box polymorphism and a decreased ability to glucuronidate and detoxify benzo(a)pyrene-7,8-dihydrodiol(—) [BPD(—)] in human liver microsomes (*32*). Subjects possessing the UGT1A1*28 allelic variant showed significant decreases in UGT1A1 protein expression and BPD(—) glucuronidation activity in liver microsomes when compared to subjects having the wildtype UGT1A1(*1/*1) genotype. Since *N*-hydroxy-PhIP is also conjugated by UGT1A1 to a nontoxic excretable glucuronide, it is logical to infer that persons with the UGT1A1*28 variant will be more susceptible to the mutagenic and/or carcinogenic effects from PhIP exposure because their capacity to form nontoxic *N*-hydroxy-PhIP glucuronide conjugates will be compromised.

Figure 6 shows a proposed schematic representation of the fate of *N*-hydroxy-PhIP. Glucuronidation of *N*-hydroxy-PhIP can occur in the liver and extrahepatically, catalyzed by a multitude of UGT1A proteins. The formation of *N*-hydroxy-PhIP glucuronides predominately results in the elimination of the conjugates via urine or bile. However previous studies have shown that the *N*-hydroxy-PhIP-*N3*-glucuronide conjugate is susceptible to bacterial β-glucuronidase which results in cleavage of the glucuronide conjugate leaving *N*-hydroxy-PhIP for further metabolism (*13*). Further activation of *N*-hydroxy-PhIP also occurs in the liver and various extrahepatic tissues leading to the formation of highly reactive esters that can go on to form DNA adducts. As seen in Figure 6, competition between glucuronidation and activation depends on the differential expression of UGTs in specific tissues. A change in the metabolic ratio

favoring bioactivation, due to down regulation of specific UGTs, can likely lead to an increase in the susceptibility to potential tumor formation from PhIP exposure because the ability to detoxify PhIP will be diminished. Studies are ongoing in our laboratory to determine if reduced UGT1A activity contributes to a significant increase in the susceptibility to the mutagenic effects of PhIP.

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Table 1. Formation of *N*-hydroxy-PhIP-N^2- and *N*-hydroxy-PhIP-*N3*-glucuronides in microsomes expressing human UGT1A isozymes at two substrate concentrations[a]

	5 μM N-hydroxy-PhIP			100 μM N-hydroxy-PhIP		
Enzyme	N^2-gluc.	*N*3-gluc.	N^2-*gluc*/ *N3-gluc*	N^2-gluc.	*N*3-gluc.	N^2-*gluc*/ *N3-gluc*
UGT1A1	23.5 ± 0.2[b]	2.2 ± 0.3	10.7	220.4 ±1.9	24.7 ± 0.2	8.9
UGT1A3	nd	nd	na	8.8 ± 0.5	17.4 ± 0.5	0.51
UGT1A4	4.1 ± 0.3	nd	na	72.9 ± 0.6	6.1 ± 0.4	12
UGT1A6	nd	nd	na	nd	nd	na
UGT1A7	nd	nd	na	0.77 ± 0.2	2.0 ± 0.03	0.39
UGT1A8	2.9 ± 0.2	nd	na	40.7 ± 1.4	5.2 ± 0.4	7.8
UGT1A9	0.84 ± 0.1	4.4 ± 0.2	0.19	6.6 ± 0.1	13.6 ± 0.1	0.48
UGT1A10	0.93 ± 0.04	nd	na	10.6 ± 0.2	1.4 ± 0.02	7.6

[a]Incubation time was 3 hr

[b] Data is expressed as pmol metabolite/ng UGT of 3 incubations ± SD; nd = not detected; na = not applicable.

Table 2. Kinetic parameters of N-hydroxy-PhIP glucuronidation in microsomes expressing human UGT1A1, 1A4 , 1A8 and 1A9

	N-OH-PhIP-N^2-glucuronide			N-OH-PhIP-N3-glucuronide		
Enzyme	K_m	V_{max} [a]	K_{cat} [b]	K_m	V_{max} [a]	K_{cat}
UGT1A1	52 μM	2.07	114 min^{-1}	59 μM	0.26	14.6 min^{-1}
UGT1A4	148 μM	0.94	51.9 min^{-1}	177 μM	0.09	5.1 min^{-1}
UGT1A8	162 μM	0.64	35.5 min^{-1}	197 μM	0.10	5.6 min^{-1}
UGT1A9	65 μM	0.07	3.7 min^{-1}	25 μM	0.13	6.9 min^{-1}

[a] V_{max} is expressed as pmol/min/ng UGT
[b] K_{cat} is based on the estimated protein concentration from the Western blot analysis

Figure Legends

Figure 1. Structure of *N*-hydroxy-PhIP glucuronides

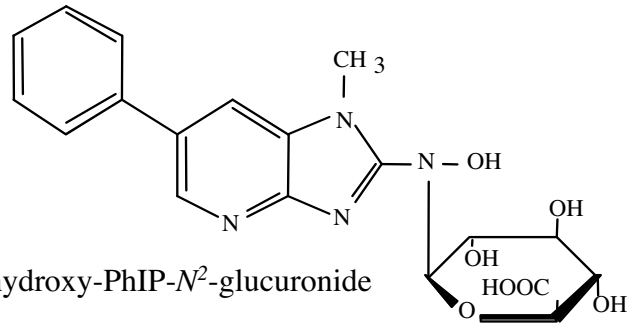
Figure 2. Western Blot of recombinant human UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9 or 1A10 from baculovirus infected insect cell microsomes using a rabbit anti-human UGT1A antibody. The antibody is specific for the carboxy-terminal region of the proteins, which is conserved for all UGT1A isoforms. The observed shift in mobility of each protein is due to variability in glycosylation of each UGT1A protein.

Figure 3. Nonenzymatic degradation of *N*-hydroxy-PhIP over time in non-UGT expressing microsomes exposed to *N*-hydroxy-PhIP. Data are the mean ± SD from 3 incubations.

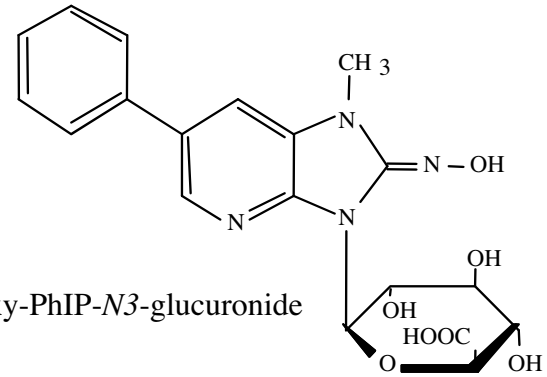
Figure 4. Time dependent formation of *N*-hydroxy-PhIP-glucuronides from recombinant human UDP-glucuronosyltransferase 1A proteins exposed 100 μM *N*-hydroxy-PhIP; **O** = *N*-hydroxy-PhIP-N^2-glucuronide; ■ = *N*-hydroxy-PhIP-*N3*-glucuronide. Data are the mean ± SD from 3 incubations.

Figure 5. Formation of *N*-hydroxy-PhIP-N^2-glucuronide and *N*-hydroxy-PhIP-*N3*-glucuronide from incubations containing recombinant human UDP-glucuronosyltransferase 1A proteins containing 100 μM *N*-hydroxy-PhIP. Incubations were performed at 37° C for 3 h. Data are the mean ± SD from 3 incubations.

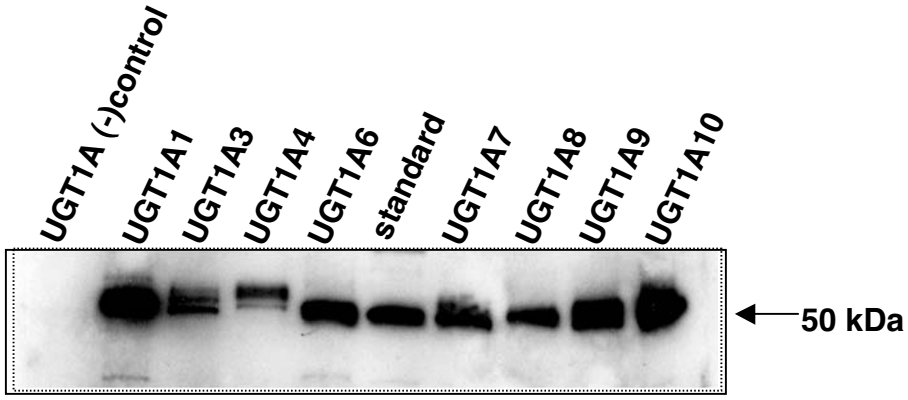
Figure 6. Proposed schematic representation of *N*-hydroxy-PhIP glucuronidation. Glucuronide conjugation can occur both in the liver and extrahepatically. The UGT isoforms are presented in their order of catalytic efficiency to form the *N*-hydroxy-PhIP glucuronides. *N*-hydroxy-PhIP-*N3*-glucuornide is susceptible to bacterial β-glucuronidase which results in cleavage of the glucuronide conjugate leaving *N*-hydroxy-PhIP for further metabolism.

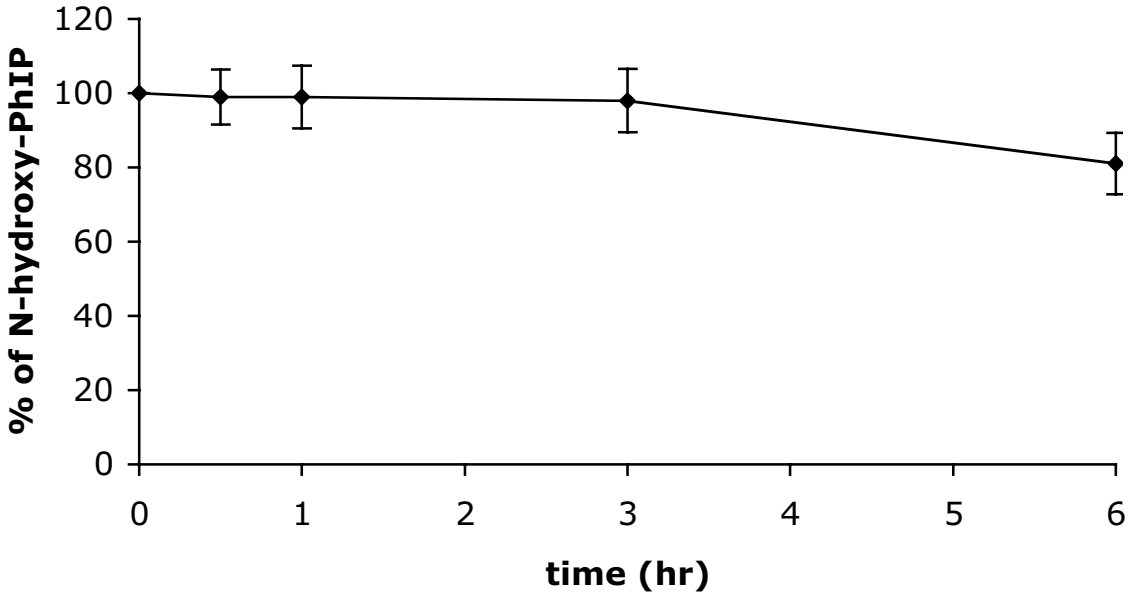


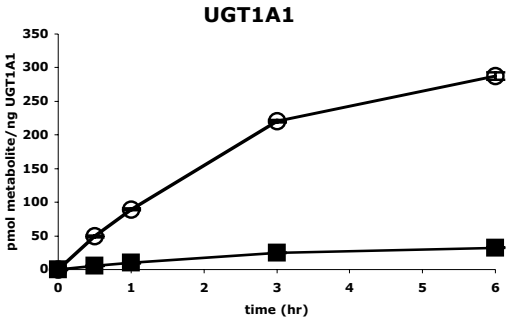
N-hydroxy-PhIP-N^2-glucuronide

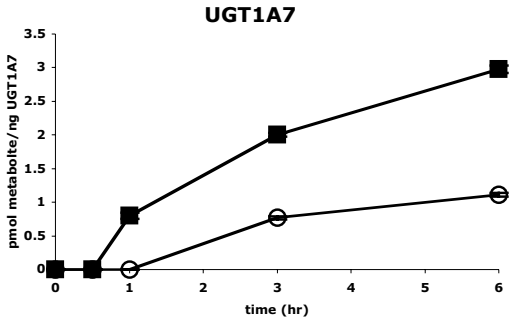


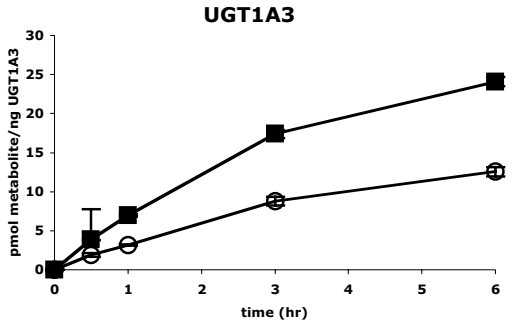
N-hydroxy-PhIP-*N3*-glucuronide

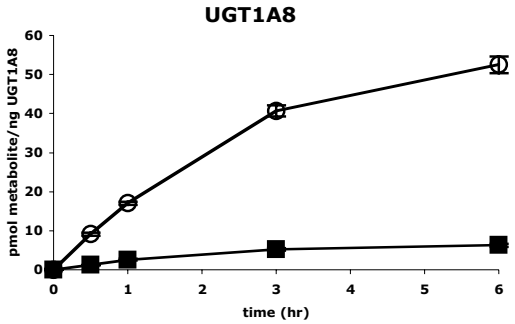

UGT1A (-)control
UGT1A1
UGT1A3
UGT1A4
UGT1A6
standard
UGT1A7
UGT1A8
UGT1A9
UGT1A10
50 kDa

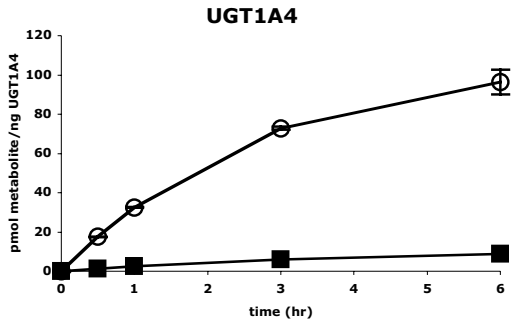

% of N-hydroxy-PhIP
120
100
80
60
40
20
0
0
1
2
3
4
5
6
time (hr)

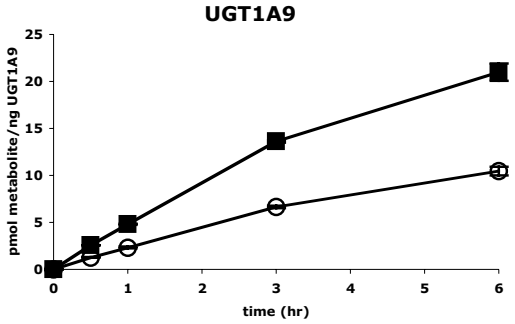

UGT1A1
pmol metabolite/ng UGT1A1
time (hr)

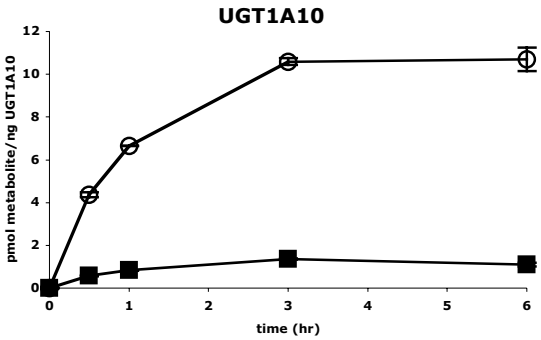

UGT1A7
pmol metabolte/ng UGT1A7
time (hr)

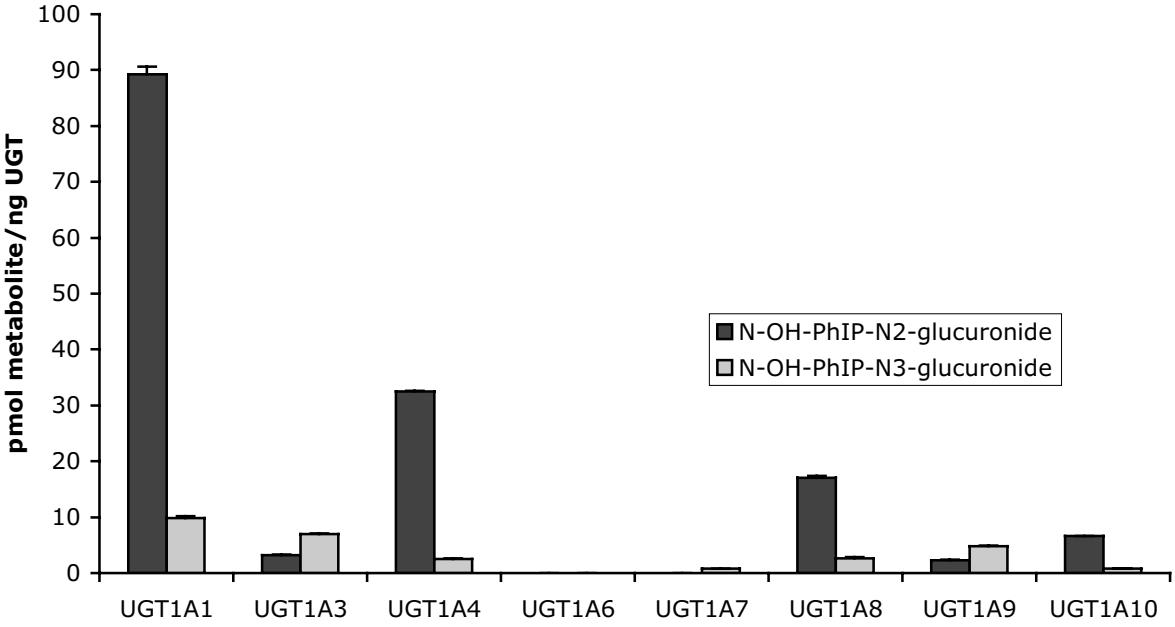

UGT1A3
pmol metabolite/ng UGT1A3
time (hr)

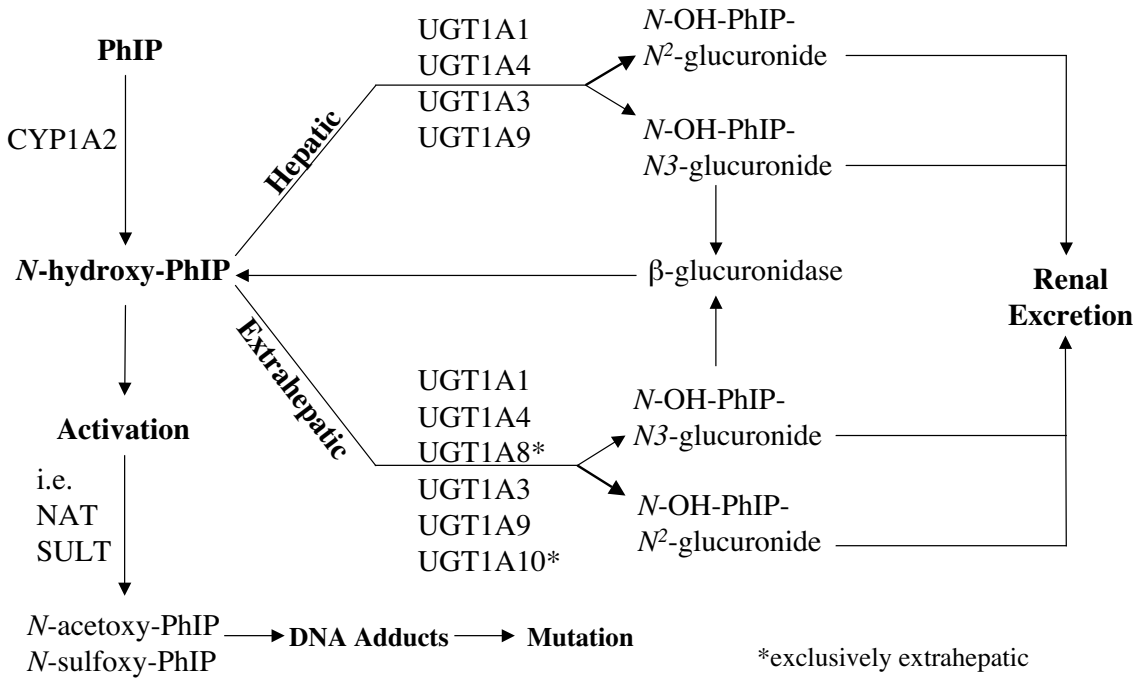

UGT1A8
pmol metabolite/ng UGT1A8
time (hr)


UGT1A4
pmol metabolite/ng UGT1A4
time (hr)


UGT1A9
pmol metabolite/ng UGT1A9
time (hr)


UGT1A10
pmol metabolite/ng UGT1A10
time (hr)


100
90
80
70
60
50
40
30
20
10
0
pmol metabolite/ng UGT
N-OH-PhIP-N2-glucuronide
N-OH-PhIP-N3-glucuronide
UGT1A1
UGT1A3
UGT1A4
UGT1A6
UGT1A7
UGT1A8
UGT1A9
UGT1A10


PhIP
CYP1A2
N-hydroxy-PhIP
Activation
i.e.
NAT
SULT
N-acetoxy-PhIP
N-sulfoxy-PhIP
DNA Adducts
Mutation
Hepatic
Extrahepatic
UGT1A1
UGT1A4
UGT1A3
UGT1A9
UGT1A1
UGT1A4
UGT1A8*
UGT1A3
UGT1A9
UGT1A10*
N-OH-PhIP-
N2-glucuronide
N-OH-PhIP-
N3-glucuronide
β-glucuronidase
N-OH-PhIP-
N3-glucuronide
N-OH-PhIP-
N2-glucuronide
Renal
Excretion
*exclusively extrahepatic