Published in final edited form as:

Chem Res Toxicol. 2016 May 16; 29(5): 879–891. doi:10.1021/acs.chemrestox.6b00046.

Bioactivation of Heterocyclic Aromatic Amines by UDP Glucuronosyltransferases

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Abstract

2-Amino-9*H*-pyrido[2,3-*b*]indole (AαC) and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) are carcinogenic heterocyclic aromatic amines (HAA) that arise during the burning of tobacco and cooking of meats. UDP-glucuronosyltransferases (UGT) detoxicate many procarcinogens and their metabolites. The genotoxic *N*-hydroxylated metabolite of AαC, 2-hydroxyamino-9*H*-pyrido[2,3-*b*]indole (HONH-AαC), undergoes glucuronidation to form the isomeric glucuronide (Gluc) conjugates *N*²-(β-D-glucosiduronyl)-2-hydroxyamino-9*H*-pyrido[2,3-*b*]indole (AαC-HON²-Gluc) and *O*-(β-D-glucosiduronyl)-2-hydroxyamino-9*H*-pyrido[2,3-*b*]indole (AαC-HN²-*O*-Gluc). AαC-HON²-Gluc is a stable metabolite but AαC-HN²-*O*-Gluc is a biologically reactive intermediate, which covalently adducts to DNA at levels that are 20-fold higher than HONH-AαC. We measured the rates of formation of AαC-HON²-Gluc and AαC-HN²-*O*-Gluc in human organs: highest activity occurred with liver and kidney microsomes, and lesser activity was found with colon and rectum microsomes. AαC-HN²-*O*-Gluc formation was largely diminished in liver and kidney microsomes, by niflumic acid, a selective inhibitor UGT1A9. In contrast, AαC-HON²-Gluc formation was less affected and other UGT contribute to *N*²-glucuronidation of HONH-AαC. UGT were reported to catalyze the formation of isomeric Gluc conjugates at the *N*² and *N*³ atoms of 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (HONH-PhIP), the genotoxic metabolite of PhIP. However, we found that the *N*³-Gluc of HONH-PhIP also covalently bound to DNA at higher levels than HONH-PhIP. The product ion spectra of this Gluc conjugate acquired by ion trap mass spectrometry revealed that the Gluc moiety was linked to the oxygen atom of HONH-PhIP and not the *N*³ imidazole atom of the oxime tautomer of HONH-PhIP as was originally proposed. UGT1A9, an abundant UGT isoform expressed in human liver and kidney, preferentially forms the *O*-linked Gluc conjugates of HONH-AαC and HONH-PhIP as opposed to their detoxicated *N*²-Gluc isomers. The regioselective *O*-glucuronidation of HONH-AαC and HONH-PhIP, by UGT1A9, is a mechanism of bioactivation of these ubiquitous HAAs.

Graphical abstract

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Supporting Information

The steady-state enzyme kinetic parameters for glucuronidation of HONH-AαC by recombinant UGT isoforms; high resolution mass spectra of Gluc conjugates of HONH-AαC; stability of Gluc conjugates of HONH-PhIP; the EROD activities in human kidney and liver microsomes. The methodology and performance of the method to measure Gluc conjugates of HONH-AαC and UGT1A expression levels in human microsomes are provided. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Keywords

2-amino-9H-pyrido[2, 3-*b*]indole; 2-amino-1-methyl-6-phenylimidazo[4, 5-*b*]pyridine; bioactivation; heterocyclic aromatic amines; red meat; tobacco smoke; UDP-Glucuronosyltransferase

Introduction

Tobacco smoking is a major risk factor associated not only for lung cancer, but also liver, colorectal and bladder cancers.^{1,2} Tobacco smoke contains more than 60 carcinogens that include polycyclic aromatic hydrocarbons (PAH), N-nitrosamines, aromatic amines, and heterocyclic aromatic amines (HAA).³ PAH and N-nitrosamines are believed to contribute to lung cancer, and aromatic amines are involved in the pathogenesis of bladder cancer of smokers;^{1,2} however, the causative agents of liver and gastrointestinal cancers are uncertain. 2-Amino-9H-pyrido [2,3-*b*]indole (AαC) is a mutagenic HAA that was originally isolated from the pyrolysis product of soybean globulin,⁴ and then identified in mainstream tobacco smoke and charred meats.^{5,6} The amount of AαC present in main stream tobacco smoke is 25–100 fold greater than that of 4-aminobiphenyl, a known human bladder carcinogen.^{1,2} AαC induces liver and blood vessel tumors in mice,⁷ and induces *lacI* mutations and aberrant crypt foci, early biomarkers of neoplasia, in the colon of mice.^{8–10} 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) is the most mass-abundant HAA formed in well-done cooked meats¹¹ and also detected in main stream tobacco smoke.¹² PhIP induces lymphoma, pancreatic, colorectal, and prostate cancers in rodents.⁷ Therefore, high levels of exposure to AαC and PhIP through tobacco smoke and cooked meats may contribute to DNA damage in these organs of smokers and carnivores.

The evaluation of the human health risk of HAA requires an understanding of the enzymes involved in bioactivation and detoxication of these procarcinogens. While there is extensive data on the metabolism of PhIP in humans,¹³ the knowledge on the metabolism of AαC is limited.¹⁴ Human cytochrome P450 1A2 catalyzes the N-oxidation of the exocyclic amine groups of AαC and PhIP, to form the genotoxic metabolites 2-hydroxyamino-9H-pyrido[2,3-*b*]indole (HONH-AαC) and 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (HONH-PhIP). These HONH-HAA metabolites can serve as substrates for *N*-acetyltransferases or sulfotransferases to form highly unstable esters that covalently adduct to DNA.¹⁵ Furthermore, human hepatocytes efficiently bioactivate AαC and PhIP to reactive metabolites that form DNA adducts.^{16,17}

UDP-glucuronosyltransferases (UGT) are important conjugation enzymes and catalyze the glucuronidation of numerous drugs, endobiotics, and procarcinogens. UGT are present in the 1A, 2A, and 2B subfamilies, which are expressed in liver and extrahepatic tissues.^{18,19} HAA and structurally related aromatic amines undergo detoxication by UGT-mediated *N*-glucuronidation of the parent amines and their *N*-hydroxylated metabolites.^{13,20,21} HONH-PhIP was reported to undergo glucuronidation by several UGT isoforms.^{22–27} The major glucuronide (Gluc) conjugate was characterized as *N*²-(β-D-glucosiduronyl)-2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP-HON²-Gluc), on the basis of ¹H- and ¹³C-NMR.²² The structure of the minor Gluc conjugate was deemed to have formed at the *N*3 imidazole atom of HONH-PhIP (PhIP-HON³-Gluc).²³ Both Gluc conjugates of HONH-PhIP are formed in human hepatocytes²⁸ and present in urine of carnivores.^{29–31} We recently identified *N*²-(β-D-glucosiduronyl)-2-hydroxyamino-9*H*-pyrido[2,3-*b*]indole (AαC-HON²-Gluc), a detoxicated conjugate of HONH-AαC; however, we also discovered that UGT catalyzed an unusual pathway of bioactivation of HONH-AαC, through the conjugation of Gluc to the oxygen atom of HONH-AαC, to form *O*-(β-D-glucosiduronyl)-2-hydroxyamino-9*H*-pyrido[2,3-*b*]indole (AαC-HN²-*O*-Gluc).³² This intermediate covalently binds to DNA at much higher levels than HONH-AαC under physiological conditions (Scheme 1).³² Among the recombinant UGT isoforms assayed (UGT1A1, -1A3, -1A4, -1A6, -1A8, -1A9, and -1A10 and UGT2B7), UGT1A1 and UGT1A9 were identified as principal UGT isoforms involved in the *N* and *O*-glucuronidation of HONH-AαC.³² Both AαC-HON²-Gluc and AαC-HN²-*O*-Gluc were shown to form in human hepatocytes.³²

In this study, we examined the capacity of different human tissues to catalyze the glucuronidation of HONH-AαC to form AαC-HON²-Gluc and AαC-HN²-*O*-Gluc. The rates of formation of Gluc conjugates of HONH-AαC were correlated to the level of expression of UGT protein, measured by a quantitative mass spectrometry method,^{33,34} and by enzymatic activities of UGT1A1 and UGT1A9 employing selective probe substrates and inhibitors.^{35,36} During the course of our study, we reexamined the reactivity of PhIP-HON²-Gluc and PhIP-HON³-Gluc conjugates with DNA, and discovered that PhIP-HON³-Gluc also covalently bound to DNA at higher levels than HONH-PhIP. High resolution accurate mass spectral data showed that the proposed *N*3-Gluc of HONH-PhIP is actually a Gluc conjugate linked to the oxygen atom of HONH-PhIP and not the *N*3 imidazole atom of the oxime tautomer of HONH-PhIP as was originally proposed.²³ Thus, *O*-glucuronidation serves as a mechanism of bioactivation of the *N*-hydroxylated metabolites of two of the most prominent HAA formed in tobacco smoke and well-done cooked meats.

Experimental procedures

Caution—AαC, PhIP, and their derivatives are potential human carcinogens and should be handled with caution in a well ventilated fume hood with the appropriate protective clothing.

Chemicals and Reagents

AαC and PhIP were purchased from Toronto Research Chemicals (Toronto, Canada). Uridine-5'-diphosphoglucuronic acid (UDPGA), alamethicin, ascorbic acid, trypsin,

iodoacetamide (IAA), dithiothreitol (DTT), estradiol, estradiol-3-*O*-Gluc, niflumic acid, propofol, and propofol-*O*-Gluc, were from Sigma. All other reagents were ACS reagent grade unless specified. The targeted peptides monitored for UGT1A1 and UGT1A9 were, respectively, T⁷⁸YPVPFQR⁸⁵ and A⁹²FAHAQWK^{99,37}. These peptides and isotopically labeled peptides T⁷⁸YPVPFQR⁸⁵ [R(¹³C₆,¹⁵N₄)], A⁹²FAHAQWK⁹⁹ [K(¹³C₆,¹⁵N₂)] were purchased from Thermo Fisher (Waltham, MA). Human liver samples were obtained from Tennessee Donor Services, Nashville, TN.³⁸ Human colon and rectum samples (non-tumor adjacent) were purchased from the National Disease Research Interchange (NDRI), and non-tumorous human kidney cortex samples were from the Collaborative Human Tissue Network (CHTN). *N*-(2'-Deoxyguanosin-8-yl)-2-amino-9*H*-pyrido[2,3-*b*]indole (dG-C8-AαC) and [¹³C₁₀]-dG-C8-AαC were prepared as previously described.³⁹

Synthesis of HONH-AαC, HONH-PhIP, and Biosynthesis of Their Gluc Metabolites

HONH-AαC and HONH-PhIP were prepared by the reduction of their nitro derivatives as previously described.^{15,40,41} The Gluc conjugates *N*²-(β-D-glucosiduronyl)-2-hydroxyamino-9*H*-pyrido[2,3-*b*]indole (AαC-HON²-Gluc) and *O*-(β-D-glucosiduronyl)-2-hydroxyamino-9*H*-pyrido[2,3-*b*]indole (AαC-HN²-*O*-Gluc), and *N*²-(β-D-glucosiduronyl)-2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP-HON²-Gluc), and *O*-(β-D-glucosiduronyl)-2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP-HN²-*O*-Gluc), were obtained by the incubation of HONH-AαC and HONH-PhIP with human liver and kidney microsomes as previously reported with modifications.^{23,32} In brief, the enzyme reactions were carried out in 100 mM Tris-HCl buffer (pH 7.5), which contained 10 mM MgCl₂ and 0.5 mM EDTA and microsomal protein (0.25 mg/mL). The microsomes were preincubated on ice for 30 min with alamethicin (50 μg/mg of protein) prior to adding other assay components in a final volume of 0.5 mL. Thereafter, the reaction was initiated by adding UDPGA (2 mM), followed by HONH-HAA (100 μM) and ascorbic acid (2 mM). The solution was gently purged with argon and incubated for 3 hours at 37 °C. The reaction was terminated by the addition of 1 volume of ice-cold CH₃OH. The microsomal protein was removed by centrifugation (22,000 g for 5 min), and the supernatant was retrieved and concentrated to dryness by vacuum centrifugation. The metabolites were reconstituted in water and separated by HPLC.³² The desired fractions were collected, dried under vacuum, and stored in liquid nitrogen.

DNA Adduct Formation with HONH-AαC, HONH-PhIP and Their Gluc conjugates

Calf thymus DNA (0.4 mg/mL) in 100 μL of 100 mM Tris buffer, pH 7.4 was incubated with HONH-AαC, HONH-PhIP or their Gluc conjugates (0.5 μM) for 30 min at 37 °C. Then, 0.1 volume of 5 M NaCl followed by 2 volumes of C₂H₅OH were added to precipitate DNA. The pelleted DNA was washed with C₂H₅OH:H₂O mixture (7:3). After drying, the DNA was reconstituted in 5 mM Bis-Tris buffer (pH 7.1) and digested in the presence of internal standards (5 adducts per 10⁷ DNA bases) as described.^{16,42} The samples were dried by vacuum centrifugation and reconstituted in 50 μL of DMSO: H₂O (1:1) for MS analysis.

Rates of Glucuronidation of Estradiol, Propofol and HONH-A α C by Human Liver, Kidney, Colon and Rectum Microsomes

The incubation consisted of human microsomal protein (0.25 mg/mL) in 100 mM Tris-HCl buffer (pH 7.5), containing 10 mM MgCl₂, 0.5 mM EDTA. The microsomes were preincubated on ice for 30 min with alamethicin (50 μ g/mg of protein) prior to adding other assay components. The assay was initiated by adding 2 mM UDPGA and substrates. The concentration of HONH-A α C was 1 μ M. The UGT isoform-specific substrates were selected based on their reported K_m values for UGT isoforms.^{35,43} Estradiol (25 μ M) was employed to assess UGT1A1 activity, and propofol (100 μ M) was employed as the substrate for measurement of UGT1A9 activity. The reactions were terminated with 1 vol of CH₃OH, followed by centrifugation to remove the protein as described above. The Gluc conjugates in the supernatant were monitored by HPLC-UV at wavelength of 215 nm for both propofol-*O*-Gluc and estradiol-3-*O*-Gluc.³⁵ The amounts of product formation were calculated employing calibration curves of the Gluc standards spiked into liver microsomes. The rates of glucuronidation were linear with the time and proportional to protein concentration.

Glucuronidation of HONH-A α C by Human Microsomes and UGT Inhibition Studies

The enzyme incubation system was the same as that described above except that the reaction was conducted in a final volume of 50 μ L containing HONH-A α C (1 μ M). The enzyme reactions were conducted for 20 min except for kidney microsomes, where the time of incubation time was decreased to 5 min because of non-linear product formation. The reactions were terminated by the addition of 1 volume CH₃OH, and the microsomal protein was removed by centrifugation. The supernatants were concentrated to dryness by vacuum centrifugation, and resuspended in 100 μ L of H₂O. The rates of HONH-A α C-Gluc formation were determined by UPLC/MS (*vide infra*). Calibration curves were performed daily by spiking known quantities of A α C-HON²-Gluc and A α C-HN²-*O*-Gluc (0.005, 0.01, 0.02, 0.05, 0.1 μ M) into incubation systems, following termination of the UGT assay with CH₃OH. For the UGT inhibition studies, niflumic acid (0, 0.1 μ M, 1 μ M, or 2.5 μ M) was incubated with human liver or kidney microsomes for 5 min before the addition of HONH-A α C (1 μ M). The amount of methanol in the incubations was less than 1% (v/v).

NanoUPLC-ESI-MS³ Analyses

The measurements of DNA adducts, metabolites, and peptides were performed with a nanoACQUITY UPLC system (Waters Corporation, Milford, MA) interfaced to an Advance CaptiveSpray source (Michrom Bioresource Inc., Auburn, CA), and the Velos Ion-Trap mass spectrometer (Thermo Fisher, San Jose, CA). A Waters UPLC 2G-V/V Symmetry C18 trap column (180 μ m x 20 mm, 5 μ m particle size) was used for online sample enrichment of DNA adducts. A Magic C18AQ columns (Michrom Bioresource, Inc.) were employed for chromatography. A Michrom Captive Source was used for all analyses.

Measurement of HAA-DNA adducts

DNA digests (5 μ L) were injected onto the trap column (Waters Symmetry trap column and washed with mobile phase A (0.01% HCO₂H) at a flow rate of 12 μ L/min for 3 min. Then, the DNA adducts were back-flushed onto a Magic C18 AQ column (0.3x150 mm, 3 μ m,

Michrom Bioresource, Inc.). The flow rate was 5 $\mu\text{L}/\text{min}$. The gradient was started at 5% of B (95% CH_3CN , 0.01% HCO_2H), and increased to 95% B at 10 min, holding at 95% B for 2 min. Adducts were measured at the MS^3 scan stage in the positive ionization mode (dG-C8-A α C: m/z 449.1 > 333.1 > [$^{13}\text{C}_{10}$]-dG-C8-A α C: m/z 459.1 > 338.1 > dG-C8-PhIP: m/z 490.1 > 374.1 > [$^{13}\text{C}_{10}$]-dG-C8-PhIP: m/z 500.1 > 379.1 >). The collision energy was set at 35 eV for MS^2 and 45 eV for MS^3 . The activation Q value was 0.35, and the activation times were 10 ms. The isolation width was set, respectively at m/z 4.0 and 1.0 for MS^2 and MS^3 scan stages. The spray voltage was set at 1.5 kV and the capillary temperature was 270 $^\circ\text{C}$.

Measurement of Rates of A α C-HON²-Gluc and A α C-HN²-O-Gluc Formation in Human Tissues

The microsomal extracts (5 μL) containing HONH-A α C-Gluc metabolites were trapped with the Waters Symmetry trap column and washed with mobile phase A (0.01% $\text{CH}_3\text{CO}_2\text{H}$) at a flow rate of 12 $\mu\text{L}/\text{min}$ for 5 min. Then, the metabolites were back-flushed onto the C18 AQ column (0.3 $\times$ 150 mm, 3 μm). The flow rate was 5 $\mu\text{L}/\text{min}$. The gradient of elution started from 5% of B (95% CH_3CN , 0.01% $\text{CH}_3\text{CO}_2\text{H}$), increased to 25% B at 1 min, reached to 50% B at 14 min, and then 99% at 15 min with holding for 1 minute. The collision energy was 25 eV for MS^2 and 35 eV for MS^3 (A α C-HN²-O-Gluc, m/z 376.1 > 184.1 > A α C-HON²-Gluc, m/z 376.1 > 212.1 >). The activation Q and activation time values were set at 0.35 and 10 ms. The isolation widths were set at, respectively, m/z 3.0 and 1.0 at the MS^2 and MS^3 scan stages. The spray voltage was set at 1.5 kV and the capillary temperature was 270 $^\circ\text{C}$.

Quantitation of UGT1A1 and UGT1A9

The targeted peptides monitored for UGT1A1 and UGT1A9 were, respectively, T⁷⁸YPVPFQR⁸⁵ and A⁹²FAHAQWK⁹⁹. These peptides are unique to these UGT isoforms.³⁷ Human microsomal protein samples (20 μg) in 100 μL of 50 mM ammonium bicarbonate buffer (pH 8.0) containing 1 mM CaCl_2 were denatured by adding 40 mM DTT (10 μL) and heating at 60 $^\circ\text{C}$ for 40 min. After cooling to room temperature, 120 mM IAA (10 μL) was added and the solution was incubated at room temperature in the dark for 30 min. Thereafter, another 10 μL of DTT was added to quench excess IAA. Isotopically labeled peptides (1 pmol) were added as internal standards, followed by trypsin (0.08 $\mu\text{g}/\mu\text{L}$, 10 μL in 50 mM ammonium bicarbonate, pH 8.0) to digest the protein (trypsin/nominal protein ratio of 1:25) for 15 h at 37 $^\circ\text{C}$. The digest was then diluted with water (1 mL) and peptides were purified by solid phase extraction (SPE). The SPE cartridges (C18) were preconditioned with methanol followed by water. The diluted samples were added to the SPE, washed with 5% CH_3OH in water, and eluted with 1 mL of CH_3OH . Then, the samples were dried under vacuum and reconstituted in 50 μL of DMSO: H_2O mixture (1:9, v/v).

The peptide digests (2 μL) were resolved with a Magic C18 AQ column (0.1 m $\times$ 150 mm, 3 μm) at a flow rate of 1 $\mu\text{L}/\text{min}$. The gradient started from 2% B (95% CH_3CN , 0.01% HCO_2H), increased to 40% B at 15 min, and reached 99% B at 16 min. The peptides were measured in the positive ionization mode. The isolation widths were set, respectively, at m/z 2.0 and 1.0 at the MS^2 and MS^3 scan stages, and the activation time was 10 ms. For UGT1A9 peptides, the collision energies were set at 30 and 33 eV for the MS^2 and MS^3

scan stages, and the activation Q was set at 0.25 for both scan stages ($A^{92}FAHAQWK^{99}$: $[M+H]^+$, m/z 479.7 > 740.5>; $A^{92}FAHAQWK^{*99}$: $[M+H]^+$, m/z 483.75 > 748.5 >). For UGT1A1 peptides, the collision energies and activation Q at the MS² and MS³ scan stages were set, respectively, at 34 eV and 0.30, and 38 eV and 0.3 ($T^{78}YPVPFQR^{85}$: $[M+H]^+$, at m/z 504.5 > 743.4>; $T^{78}YPVPFQR^{85}$: $[M+H]^+$, m/z 509.5 > 753.5 >). The quantitative analysis of peptides was based on the following extracted ions at the MS³ scan stage: $A^{92}FAHAQWK^{99}$ (m/z 408.2, 532.3, 566.3, 594.4); $A^{92}FAHAQWK^{*99}$ (m/z 408.2, 540.3, 566.3, 594.4); $T^{78}YPVPFQR^{85}$ (m/z 530.3, 547.4, 726.5); and $T^{78}YPVPFQR^{85}$ (m/z 539.3, 557.4, 735.5).

High Resolution MS Characterization of HONH-A α C and HONH-PhIP Gluc Conjugates

The characterization of A α C- and PhIP-Gluc conjugates were carried out with a Dionex Ultimate 3000 LC instrument (Thermo Scientific, Waltham, MA) coupled with an Orbitrap EliteTM Hybrid Ion Trap Orbitrap mass spectrometer (Thermo Scientific, Waltham, MA). The mass accuracy and MS performance were calibrated with Pierce ESI Ion Calibration Solution (Pierce Biotechnology, Grand Island, NY). The purified PhIP and Gluc metabolites were analyzed with a Magic C18AQ column (0.3 mm $\times$ 150 mm, 3 μ m.) as described above. The capillary temperature was 270 °C and ionization voltage was 2 kV for negative and positive ion modes. The resolution was set as 30,000 for all transitions. The isolation width was set at m/z 1 for both MS² and MS³ scan modes for all Gluc conjugates except for the PhIP-HN²-O-Gluc conjugate in the negative ion mode where an isolation width of m/z 3 was employed. The activation Q was set at 0.25 for MS² and 0.35 for MS³ for both negative and positive modes. The collision energy was 25 eV for MS² and 35 eV for MS³. The A α C-HON²-Gluc was monitored at the MS² scan stage in negative ion mode ($[M-H]^-$ at m/z 374.0994 >) and positive ion mode ($[M+H]^+$ at m/z 376.1139 >); A α C-HN²-O-Gluc was monitored at the MS³ scan stage in negative ion mode ($[M-H]^-$ at m/z 374.0994 > 193.0354 >) and in positive ion mode ($[M+H]^+$ at m/z 376.1139 > 184.0869 >). The PhIP-HN²-O-Gluc transitions in negative mode ($[M-H]^-$ at m/z 415.1259 > 191.0197 >, or m/z 415.1259 > 223.0989 >). The same collision energies were employed in positive ion mode ($[M+H]^+$ at m/z 417.1405 > 225.1135 >). The PhIP-HON²-Gluc conjugate was monitored in negative ion mode ($[M-H]^-$ at m/z 415.1259 > 239.0938 >) and in positive ion mode ($[M+H]^+$ at m/z 417.1405 > 241.1084 >).

Quantum chemical calculations

Quantum chemical calculations based on density functional theory (DFT) was performed using Gaussian 09 A.02 (Gaussian Inc.) (Insert the reference) and GaussView (Gaussian Inc.).^{44,45} Geometry optimizations and energy calculations on the O-Gluc conjugates of HONH-A α C, HONH-PhIP and 2-hydroxyaminofluorene (HONH-AF) were conducted using the B3LYP method and 6-31 g (d, p) basis set. All calculations were done using water as the solvent at 298.15 K and atmospheric pressure. The energy was calculated by the equation: $H^{298.15} = E + ZPE + 2.5 \cdot R \cdot T$.

Results

A α C-HON²-Gluc and A α C-HN²-O-Gluc Conjugate Formation Catalyzed by Human Liver, Colon, Rectum and Kidney Microsomes

We previously characterized the steady-state enzyme kinetic parameters for the glucuronidation of HONH-A α C with various recombinant UGT isoforms.³² The data are summarized in Table S1. UGT1A9 was the most catalytically efficient isoform at producing A α C-HN²-O-Gluc whereas UGT1A1 was most efficient at forming A α C-HON²-Gluc. UGT1A9 and UGT1A1 also displayed the lowest apparent K_m values for A α C-HN²-O-Gluc (0.7 μ M and 21.4 μ M) and A α C-HON²-Gluc (6.3 μ M and 49.8 μ M) formation. Therefore, we examined the relative contributions of UGT1A1 and UGT1A9 expressed in different human organs to catalyze the glucuronidation of HONH-A α C under a low substrate concentration (1 μ M) that approaches the level of human exposure to A α C. The A α C-HON²-Gluc and A α C-HN²-O-Gluc conjugates formed in liver were characterized by high resolution accurate mass measurement by Orbitrap at the MS² scan stage (Figure S1). The mass spectra support the previous assignments obtained by NMR spectrometry and ion trap multistage MS.³²

The rates of A α C-HON²-Gluc and A α C-HN²-O-Gluc formation were correlated to the level of expression of UGT1A1 and UGT1A9 protein, measured by mass spectrometry,^{33,34} and by enzymatic activities of UGT1A1 and UGT1A9 employing selective probe substrates and inhibitors.^{35,36} The results are reported below. The rates of formation HONH-A α C-Gluc conjugates from microsomal samples of different organs are shown in Figure 1. The amounts of A α C-HON²-Gluc and A α C-HN²-O-Gluc formed by human microsomes were calculated based on external calibration curves of Gluc standards spiked into extracts of human liver microsomes (Figure S2). Human liver and kidney microsomes were more active at forming HONH-A α C-Gluc conjugates than microsomes of colon and rectum. At the low substrate concentration of HONH-A α C (1 μ M) employed for these UGT assays, the A α C-HN²-O-Gluc was principal Gluc conjugate formed in liver and kidney, whereas A α C-HON²-Gluc was the predominant conjugate formed in colon and rectum microsomes. Kidney microsomes, in particular, preferentially formed A α C-HN²-O-Gluc over its isomeric conjugate A α C-HON²-Gluc. However, product formation was not linear over time with kidney microsomes, and therefore, the incubation time was decreased to 5 min because of non-linear product formation (Figure S3).

Specific Activities of UGT1A1 and UGT1A9 Isoforms

The activity of UGT1A1 was assessed by the glucuronidation of the 3-OH group of estradiol, and the glucuronidation of propofol was employed as a selective substrate for UGT1A9.^{35,46} The range of the rates of Gluc conjugate formation of estradiol and propofol with liver, kidney, colon, and rectum microsomes are depicted in Figure 2. The estradiol-3-O-Gluc conjugation activities are comparable for liver, colon and rectum microsomes, whereas kidney microsomes harbored the lowest activities (Figure 2A). In the liver, UGT1A1 is the major UGT isoform involved in formation of estradiol-3-O-Gluc, followed by UGT1A3 and UGT1A10, while UGT2B isoforms do not appreciably contribute to Gluc conjugation of the 3-OH group of estradiol.^{35,46} UGT1A8 is the most catalytically active

isoform involved in estradiol-3-*O*-Gluc conjugation;⁴⁶ however, UGT1A8 is not expressed in liver, but its prominent expression in the colon explains the high rate of estradiol-3-*O*-Gluc produced by colon microsomes.^{47,48} The relatively lower activity of estradiol-3-*O*-Gluc conjugation in kidney is attributed to the low levels UGT1A1 and UGT1A8 protein expression reported in this organ.³⁷ Conversely, the rate glucuronidation of propofol was highest in human kidney microsomes, where there is a higher level of UGT1A9 expression than liver, colon or rectum microsomes (Figure 2B).³⁷ These activities for the glucuronidation of estradiol 3-OH and propofol are consistent with the relative amounts of UGT1A1 and UGT1A9 expressed in these organs (*vide infra*).³⁷

Quantification of UGT1A1 and UGT1A9 Isoforms

The UGT1A1 and UGT1A9 isoforms expressed in human liver, kidney, colon and rectum microsomes were measured by MS, employing the peptides T⁷⁸YPVPFQR⁸⁵ and A⁹²FAHAQWK⁹⁹, which are unique to UGT1A1 and UGT1A9, respectively.³⁷ We adapted the MS-based method established by Smith and co-workers,^{33,37} except that we employed ion trap MS. The calibration curves were constructed from spiked peptide standards in mouse liver microsomes, and the method of validation is provided in Figure S4, S5, and Table S3. The optimal proteolytic digestion conditions required 15 h, and a ratio of 1:25 of trypsin to microsomal protein. The levels of UGT1A1 and UGT1A9 in human liver, colon, rectum and kidney microsomes are reported in Figure 3. UGT1A1 and UGT1A9 were expressed at higher levels in human liver than colon and rectum, and kidney contained the highest amount of UGT1A9. The relative levels and amounts of these UGT1A isoforms expressed in liver and extrahepatic tissues are in agreement with previously reported data.^{33,37}

The Correlation among HONH-A α C-Gluc Conjugates, UGT1A1 and UGT1A9 Enzyme Activities and Protein Levels

The rates of formation of HONH-A α C-Gluc conjugates catalyzed by liver microsomes from 13 donors were plotted against the levels of expression and specific activities of UGT1A1 and UGT1A9. The rates of A α C-HON²-Gluc formation showed weak correlations with UGT1A1 and UGT1A9 enzyme activities and levels of protein expression, indicating that other UGTs also contribute to A α C-HON²-Gluc formation in liver.³² In contrast, the linear regression analyses showed that the rate of formation of A α C-HN²-*O*-Gluc correlated well to both enzyme activity and the protein level of UGT1A9 and also for UGT1A1 (Figure 4). The levels of expression of UGT1A1 and UGT1A9 proteins in human liver microsomes were also well correlated ($R^2 = 0.40$, $P = 0.02$) (Figure S-6.)

The Role of UGT1A9 in Catalyzing A α C-HN²-*O*-Gluc Formation

The role of UGT1A9 in the *O*-glucuronidation of HONH-A α C in liver and kidney microsomes was assessed by an inhibition study with niflumic acid, which is a substrate and a highly selective inhibitor of UGT1A9.³⁶ Niflumic acid was reported to inhibit recombinant and human liver microsomal UGT1A9 activities for various substrates with K_i values ranging from 0.03 to 0.40 μ M,^{36,49} but niflumic acid had little effect on the activities of other UGT1A and UGT2B isoforms^{36,50} Niflumic acid was effective in inhibiting the glucuronidation of propofol, a UGT1A9 substrate (Figure 5A); however, the formation of

estradiol-3-*O*-Gluc, which is catalyzed primarily by UGT1A1 in liver, was unaffected (Figure 5B).³⁵ Niflumic acid also resulted in a concentration-dependent inhibition in AαC-HN²-*O*-Gluc formation in liver (Figure 5C), whereas the amount of AαC-HON²-Gluc formed was unchanged, indicating that UGT1A1³² or other UGT isoforms catalyze AαC-HON²-Gluc formation (Figure 5D). Conversely, the formation of both AαC-HN²-*O*-Gluc and AαC-HON²-Gluc conjugates were greatly decreased in kidney microsomes co-incubated with niflumic acid (Figure 5C, 5D). The strong inhibition of HONH-AαC-Gluc formation, by niflumic acid, in kidney is attributed to the dominant expression and conjugating activity of UGT1A9 (Figure 3), whereas UGT1A1 and UGT2B10, another isoform involved in conjugation of basic amines, including nicotine and HONH-PhIP,^{27,51} are poorly expressed in kidney.^{37,52,53} Thus, the formation of AαC-HN²-*O*-Gluc in kidney appears to be largely carried out by UGT1A9.

Glucuronidation of HONH-PhIP

UGT also catalyze the glucuronidation of other HAA carcinogens and their *N*-hydroxylated metabolites, including PhIP.^{13,22,23} Two isomeric *N*-Gluc conjugates of HONH-PhIP are formed with human and rodent liver microsomes, and also produced in human hepatocytes.^{22,23,54} The major Gluc conjugate of HONH-PhIP, PhIP-HON²-Gluc, was characterized by ¹H- and ¹³C-NMR, and mass spectrometry.²² However, there was insufficient product to characterize the minor Gluc conjugate of HONH-PhIP by NMR. The linkage of this minor Gluc conjugate was proposed to have occurred at the *N*3 imidazole atom of the oxime tautomer of HONH-PhIP (PhIP-HON³-Gluc), based on comparison of UV spectral, chemical and enzymatic properties to those of the *N*²- and *N*3-Gluc conjugates of PhIP.⁵⁵ We re-examined the Gluc conjugates of HONH-PhIP by HPLC with UV and high resolution Orbitrap mass spectrometry. The HPLC-UV profile of Gluc product formation of HONH-PhIP, by human liver and kidney microsomes, is shown in Figure 6. The UGT in liver and kidney produced different proportions of two Gluc conjugates (M1 and M2) of HONH-PhIP. The UV spectral data of these isomeric HONH-PhIP-Gluc conjugates are in excellent agreement to those previously reported.^{22,23} The kidney showed selectivity and predominantly formed M2, possibly due to the abundant expression of UGT1A9, whereas M1 formation was the predominant conjugate formed with liver microsomes.

High resolution MS was employed to characterize the structures of M1 and M2. The product ion spectra acquired at the MS² and MS³ scan stages in positive and negative ion mode, and detailed fragmentation of the Gluc conjugates are depicted in Figures 6B and 6C. In the positive ion mode, M1 ([M+H]⁺ at *m/z* 417.1405) undergoes collision-induced dissociation (CID) to form the ion at *m/z* 241.1082 as the base peak at the MS² scan stage. This ion is assigned as protonated HONH-PhIP (*m/z* 241.1084). Consecutive reaction monitoring of the *m/z* 241.1084 at the MS³ scan stage, leads to the formation of ions at *m/z* 223.0978 and 224.1055, attributed to loss of OH and H₂O, respectively, from HONH-PhIP.⁵⁶ In the negative ion mode ([M-H]⁻ at *m/z* 415.1259), two prominent ions are observed in the product ion spectrum at *m/z* 239.0945 and 223.0996. These fragment ions are attributed to the oxide anion of HONH-PhIP (*m/z* 239.0938) and the nitrane of PhIP (*m/z* 223.0989). The pattern of fragmentation of M1 support the structure as an *N*-linked Gluc conjugate occurring through the *N*² atom of HONH-PhIP, and the UV and mass spectral data are

consistent with the assignment of this Gluc conjugate as PhIP-HON²-Gluc.^{22,23} In positive ion mode, the isomeric M2 Gluc conjugate underwent CID to produce prominent fragment ions at m/z 240.1005 and 225.1134; these ions are consistent with the proposed radical cation of HONH-PhIP (m/z 240.1006) and protonated PhIP (m/z 225.1135) (Figure 6B). The formation of protonated PhIP suggests the Gluc conjugate is linked through the oxygen atom of HONH-PhIP and not the *N*3 imidazole atom of HONH-PhIP.²³ Direct evidence for the proposed *O*-linked Gluc conjugate of HONH-PhIP was obtained by the product ion spectra of M2 in the negative ion mode. The CID of M2 shows two prominent fragment ions at m/z 223.0988 and 191.0197. These fragment ions are attributed to the nitrone of PhIP (m/z 223.0989) and the lactone of the glucuronate (m/z 191.0197). Consecutive reaction monitoring of m/z 223.0989 at the MS³ scan stage results in the loss of CH₃ to form the demethylated radical anion of PhIP at m/z 208.0755. The fragmentation of the m/z 191.0197 at the MS³ scan stage shows the typical CID fragmentation pattern previously reported for glucuronic acid.^{32,57} These findings show that the glucuronic acid is linked through the oxygen atom of HONH-PhIP and not through the *N*3-imidazole atom of the oxime of HONH-PhIP as was originally proposed for the structure of this Gluc metabolite, which was previously cited in the literature as *N*-OH-PhIP-*N*3-Gluc (Figure 7).^{23,24,26,27}

Reactivity of HONH-PhIP, HONH-AαC and Their Glucuronide Conjugates to Form DNA Adducts

The reactivity of Gluc conjugates of HONH-PhIP and HONH-AαC to covalently bind to calf thymus DNA was investigated, by measurement of dG-C8-PhIP and dG-C8-AαC, the two major adducts formed with these HAAs in human hepatocytes.^{16,17} Both HONH-AαC and HONH-PhIP bound to DNA at comparable levels. The level of DNA adduct formation with AαC-HN²-*O*-Gluc was about 20-fold greater than that formed with HONH-AαC (Figure 8). However, the binding of AαC-HON²-Gluc to DNA to form dG-C8-AαC adducts was considerably lower than HONH-AαC, signifying that the UGT-mediated pathway of *N*²-glucuronidation is a mechanism of detoxication of HONH-AαC. Similarly, PhIP-HN²-*O*-Gluc formed higher amounts of dG-C8-PhIP than HONH-PhIP, albeit at lower levels than AαC-HN²-*O*-Gluc. The chemical structures of the aryl groups for *N*-acetoxy-*N*-arylamides are also known to markedly influence the extent of reactivities of their *O*-Gluc conjugates with DNA.⁵⁸ In contrast, DNA adduct formation was not detected with PhIP-HON²-Gluc. T

Discussion

The UGT-mediated Gluc conjugation of chemicals is generally considered as a mechanism of detoxication, and the Gluc conjugates are less biologically or chemically reactive than their corresponding aglycones. The polarity of Gluc and the ionized carboxylic acid moiety facilitate the elimination of these conjugated metabolites from the body. However, there are two classes of chemicals where UGT pathways result in bioactivation. These chemicals include the acyl glucuronides of carboxylic acids of NSAIDs in particular,⁵⁹ and also the *N*-*O*-glucuronides of arylhydroxamic acids (*N*-hydroxy-*N*-acetylarylamines),^{58,60,61} which are electrophilic species capable of reacting to protein or DNA.

Apart from the endocyclic nitrogen atoms, AαC has the same chemical structure as 2-aminofluorene (2-AF), the carcinogenic aromatic amine most extensively studied during the past five decades.^{60,62} The *O*-Gluc conjugate of *N*-hydroxy-2-acetylaminofluorene was identified in urine of rats treated with 2-acetylaminofluorene, the *N*-acetylated derivative of 2-AF.⁶³ The metabolite was stable in urine and at neutral pH in vitro, but it was labile under slightly alkaline pH. The investigators proposed that the *N*-acetyl group of the AAF moiety had migrated to a hydroxyl group of the Gluc under alkaline pH (Scheme 2).⁶⁴ The resulting *O*-Gluc of 2-hydroxyaminofluorene was labile and reacted with protein and DNA.⁶⁰ An important distinction between 2-AF and AαC is that *O*-Gluc conjugate is formed with HONH-AαC and not its *N*-acetylated hydroxamic acid. AαC-HN²-*O*-Gluc has been detected in human hepatocytes.³² The *O*-Gluc linkage of AαC-HN²-*O*-Gluc is more stable than that of the *O*-Gluc of HONH-AF. The half-life of AαC-HN²-*O*-Gluc exceeds 6 h at pH 7.0,³² whereas the transacetylated *O*-Gluc of HONH-AF decomposes within several minutes.⁶⁵ The increased stability of AαC-HN²-*O*-Gluc linkage can be due multiple factors including the basicity of the heterocyclic aromatic ring, and obvious electronic differences between the tricyclic ring systems of AαC and AF. Moreover, DFT calculations reveal a greater N-O bond dissociation energy for AαC-HN²-*O*-Gluc than AF-HN²-*O*-Gluc ($H^{298.15}$ 38.1984 vs 36.8098 kcal/mol). Nevertheless, AαC-HN²-*O*-Gluc undergoes a facile nucleophilic displacement reaction with dG in DNA to form the dG-C8-AαC adduct. Electrophiles of intermediate reactivity have been viewed as the most genotoxic species because highly reactive electrophiles will react with weaker nucleophiles or undergo solvolysis with water before they can react with DNA.⁶⁶ Thus, AαC-HN²-*O*-Gluc is an ideal genotoxic electrophile that can react with DNA. To the best of our knowledge, *O*-Gluc conjugates of other arylhydroxylamines have not been detected in vivo in rodent models or hepatocytes probably because the conjugates are thought to be unstable in aqueous solution and rapidly decompose.⁶⁵

We previously reported that recombinant UGT1A9 was the most catalytically efficient UGT isoform to produce AαC-HN²-*O*-Gluc with an apparent K_m value of 0.7 μ M.³² In this study, we examined the formation of AαC-HN²-*O*-Gluc and its isomeric AαC-HON²-Gluc conjugate in human liver, kidney, colon, and rectum microsomes employing a low substrate concentration of HONH-AαC (1 μ M) to determine if UGT1A9-mediated conjugation of HONH-AαC also prominently occurred human microsomal samples. The highest levels of UGT activity for AαC-HN²-*O*-Gluc formation occurred in kidney, which expresses relatively high levels of UGT1A9.³⁷ This activity was paralleled by the high levels of propofol-*O*-Gluc formed in kidney microsomes; propofol is a specific substrate for UGT1A9.³⁵ The abolition of AαC-HN²-*O*-Gluc formation both in kidney and liver microsomes treated with niflumic acid, a highly selective inhibitor for UGT1A9,^{36,50} supports the enzyme kinetic data parameters obtained with recombinant UGT1A9 and reinforces the importance of UGT1A9 in the *O*-glucuronidation of HONH-AαC in human organs. In contrast, the isomeric AαC-HON²-Gluc formed in liver was not affected by niflumic acid, and UGT1A1, other UGT1A or -2B isoforms contribute to its formation in liver,³² although AαC-HON²-Gluc formation was catalyzed predominantly by UGT1A9 in kidney (Figure 5D).

Similar to our findings on UGT activity with HONH-AαC, recombinant UGT1A1 also displayed the highest catalytic efficiencies for *N*²-glucuronidation of HONH-PhIP with an apparent K_m of 19 μM.²⁷ However, UGT1A9 and UGT2B10, but not UGT1A1, contributed to the *O*-glucuronidation of HONH-PhIP (previously reported as the HON-PhIP-*N*3-Gluc), with apparent K_m values of respectively, 36 and 58 μM.²⁷ It is noteworthy that recombinant UGT1A9 was the most active UGT isoform in the *O*-glucuronidation of HONH-PhIP to form PhIP-HN²-*O*-Gluc.^{24,26,27} Recombinant UGT2B10 was not commercially available when we conducted our enzyme kinetic studies on HONH-AαC. However, the inhibition studies conducted with niflumic acid, which does not alter UGT2B10 activity,^{36,50} clearly showed that UGT1A9 was the major isoform responsible for the *O*-glucuronidation of HONH-AαC in human liver and kidney microsomes (Figure 5).

Subjects harboring UGT1A1 functional variants with intermediate or low genotyped-based enzyme activity were reported to be at greater risk for colorectal cancer compared to high-activity genotype individuals who frequently eat meat.⁶⁷ Mechanistically, this finding can be interpreted by the capacity of individuals with low genotype activity of UGT1A1 to less efficiently conjugate, detoxicate, and eliminate HAA or polycyclic aromatic hydrocarbon procarcinogens in cooked meats than the rapid genotype subjects. In the same study, the authors reported a paradox and the strongest association for colorectal cancer was observed among the high/intermediate UGT1A9 genotype, suggesting either poor genotype-phenotype correlation, or that there was some other chemical(s) formed in pan-fried red meat other than HAA that was driving the association. The food frequency questionnaire employed in this and other epidemiology studies estimated the exposure to PhIP and 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline, another prominent HAA, but not AαC.⁶⁸ The amount of AαC formed in well-done cooked meats has often not been measured and the estimates of daily exposure to AαC are uncertain;^{69–72} however, urinary biomarker data reveal that the exposure to AαC through tobacco smoke or well-done cooked meat can be appreciable.^{73–76} Our biochemical data on UGT1A9 activity offers an alternative interpretation for the association among the high/intermediate UGT1A9 genotype and colorectal cancer risk. HAA, such as AαC and PhIP, undergo N-oxidation by P450 1A2 in the liver, followed by *O*-glucuronidation of the *N*-hydroxy-HAA metabolites by UGT1A9 and transport of the HAA-HN-*O*-Gluc via bile to the gastrointestinal tract, where ensuing binding to colonic DNA occurs.⁷⁷

Main stream tobacco smoke contains a number of aromatic amines and HAA. AαC in particular, is present at much higher levels than other carcinogenic arylamines and heteroarylamines.^{1,78} The risk of renal cell cancer is elevated in tobacco smokers.¹ Moreover, recent studies have reported that the dietary intake of cooked red meat is significantly associated with the increased occurrence of renal cell carcinoma, and PhIP and MeIQx formed in cooked meat were implicated as possible causative agents.^{79,80} Again, the questionnaire and data base employed in these studies did not consider exposure to AαC. The principal P450 enzymes involved in bioactivation of HAA (P450 1A1, 1A2, and 1B1)¹³ are not expressed or present at very low levels in kidney.⁸¹ Consistent with these data, we observed that ethoxyresorufin-*O*-deethylase, an activity associated with P450s 1A1, 1A2, and 1B1, is very low in human kidney microsomes (1.5 pmol/min/mg protein) (Table S-5), as is the rate of P450-mediated N-oxidation of AαC and PhIP (limit of detection is 50

pmol/min/mg protein). Thus, the genotoxic effects of AαC or PhIP in kidney may require the formation of HONH-AαC and HONH-PhIP in the liver, followed by systemic circulation of the *N*-hydroxylated metabolites and their uptake in kidney where *O*-glucuronidation can occur by UGT1A9, resulting in reactive intermediates that bind to DNA. Further studies on the capacity of UGT1A9 to bioactivate other *N*-hydroxylated metabolites of HAA and arylamines and the plausible role high UGT1A9 genotype as a risk factor for colorectal and renal cell cancer are warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding Sources

This work was supported by National Institutes of Health Grants 2R01 CA122320 (R.J.T.), R01CA134700 R.J.T.), R01CA134700-03S1 (R.J.T.) of the Family Smoking Prevention and Tobacco Control Act, and in part by the National Cancer Institute Cancer Center Support Grant CA 077598 (R.J.T.).

We thank Dr. Peter Villalta of the Masonic Cancer Center's Analytical Biochemistry shared resource for direction on the use of high resolution Orbitrap mass spectrometer. We thank Dr. Shun Xiao, Masonic Cancer Center, for aid in quantum chemical calculations. We thank Dr. Sharon Murphy, University of Minnesota, for her critical review of the manuscript.

Abbreviations

HAA	heterocyclic aromatic amine
PAH	polycyclic aromatic hydrocarbons
2-AF	2-aminofluorene
HONH-AF	2-hydroxyaminofluorene
AαC	2-Amino-9 <i>H</i> -pyrido[2,3- <i>b</i>]indole
PhIP	2-amino-1-methyl-6-phenylimidazo[4,5- <i>b</i>]pyridine
HONH-AαC	2-hydroxyamino-9 <i>H</i> -pyrido[2,3- <i>b</i>]indole
Gluc	glucuronide
AαC-HON²-Gluc	<i>N</i> ² -(β-D-glucosiduronyl)-2-hydroxyamino-9 <i>H</i> -pyrido[2,3- <i>b</i>]indole
AαC-HN²-O-Gluc	<i>O</i> -(β-D-glucosiduronyl)-2-hydroxyamino 9 <i>H</i> -pyrido[2,3- <i>b</i>]indole
HONH-PhIP	2-hydroxyamino-1-methyl-6-phenylimidazo[4,5- <i>b</i>]pyridine
PhIP-HON²-Gluc	<i>N</i> ² -(β-D-glucosiduronyl)-2-hydroxyamino-1-methyl-6-phenylimidazo[4,5- <i>b</i>]pyridine
PhIP-HN²-O-Gluc	<i>O</i> -(β-D-glucosiduronyl)-2-hydroxyamino-1-methyl-6-phenylimidazo[4,5- <i>b</i>]pyridine
CID	collision-induced dissociation

EROD	ethoxyresorufin-O-deethylase
MS	mass spectrometry
NAT	N-acetyltransferase isoforms
SULT	sulfotransferases
SPE	solid phase extraction
UGT	UDP Glucuronosyltransferases
HCM	human colon microsome
HKM	human kidney microsome
HLM	human liver microsome
HRM	human rectum microsome.

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Figure 1.

Histogram showing the mean and standard deviation of the rates of HONH-AαC-Gluc conjugate formation catalyzed by human microsomes (liver, n=13; colon, n=4; rectum, n=4; kidney, n=6, each sample was measured in triplicate).

Figure 2.

Aligned dot plot depicting the mean and range in glucuronidation activities of UGT1A1 and UGT1A9 in human microsomes with specific substrates. A) Estradiol-3-*O*-Gluc formation as a measure of UGT1A1 activity; B) Propofol-*O*-Gluc as a measure of UGT1A9 activity (human liver, n=13; human colon, n=4; human rectum, n=13; human kidney, n=6; each sample was measured in triplicate).

Figure 3.

Aligned dot plot showing the mean and the range in levels of protein expression of UGT1A1 and UGT1A9 in human liver (n=13), colon (n=4), rectum (n=4) and kidney (n=6) microsomes.

Figure 4.

Linear regression analysis and correlations among of HONH-AαC-Gluc conjugates with UGT1A1 and UGT1A9 activities and protein expression levels in human liver microsomes (n=13).

Figure 5.

The inhibition of UGT1A9 activity by niflumic acid. A) In liver, propofol-*O*-Gluc is carried out by UGT1A9, and niflumic acid leads to a concentration-dependent decrease in propofol-*O*-Gluc formation; B) In liver, estradiol-3-*O*-Gluc formation is largely carried out by UGT1A1 and niflumic acid has no effect on estradiol-3-*O*-Gluc levels; C) The rate of A α C-HN²-*O*-Gluc formation by liver and kidney microsomes with niflumic acid; and D) The rate of A α C-HON²-Gluc formation in liver and kidney microsomes with niflumic acid. The inhibition studies in liver and kidney were carried out for 20 min. (ANOVA with Dunnett's multiple comparison test: *, $p < 0.05$; **, $p < 0.01$).

Figure 6.

The characterization of Gluc conjugates of HONH-PhIP. A) The HPLC and UV spectra of HONH-PhIP Gluc catalyzed by human liver and kidney microsomes; B) Product ion spectra of HONH-PhIP-Gluc conjugates in positive ion mode (MS² at m/z 417.1405 > MS³ at m/z 417.1405 > 241.1084 > and m/z 417.1405 > 225.1135 >); C) Product ion spectra of HONH-PhIP-Gluc conjugates in negative ion mode (MS² at m/z 415.1259 > MS³ at 415.1259 > 239.0938 > m/z 415.1259 > 223.0989 > and m/z 415.1259 > 191.0197 >).

Figure 7.
The proposed structures of HONH-PhIP-Gluc conjugates.

Figure 8. Reactivity of HONH-PhIP, HONH-AαC and their Gluc conjugates with calf thymus DNA to form dG-C8 adducts.

Scheme 1.

Metabolism pathways of AαC and HONH-AαC by P450 and UGT.

Scheme 2.

UGT-mediated bioactivation of the *N*-hydroxy metabolite of 2-acetylaminofluorene, AaC and PhIP.