UDP-Glucuronosyltransferase-Mediated Metabolic Activation of the Tobacco Carcinogen 2-Amino-9H-Pyrido[2,3-b]indole*

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*Running title: UGT bioactivation of AαC

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Background: 2-Amino-9H-pyrido[2,3-b]indole (AαC) is a carcinogen formed in tobacco smoke, but little is known about its metabolism in humans.

Result: UDP-Glucuronosyltransferases catalyze the binding of N-oxidized-AαC to DNA.

Conclusion: Glucuronidation, normally a detoxication pathway, contributes to the genotoxicity of AαC.

Significance: The exposure to and UGT-bioactivation of AαC provides a biochemical mechanism for the elevated risk of liver and digestive tract cancers in smokers.

SUMMARY

2-Amino-9H-pyrido[2,3-b]indole (AαC) is a carcinogenic heterocyclic aromatic amine (HAA) that arises in tobacco smoke. UDP-glucuronosyltransferases (UGTs) are important enzymes that detoxicate many procarcinogens, including HAAs. UGTs compete with P450 enzymes, which bioactivate HAAs by N-hydroxylation of the exocyclic amine group; the resultant N-hydroxy-HAA metabolites form covalent adducts with DNA. We have characterized the UGT-catalyzed metabolic products of AαC and the genotoxic metabolite 2-hydroxyamino-9H-pyrido[2,3-b]indole (HONH-AαC) formed with human liver microsomes, recombinant human UGT isoforms, and human hepatocytes. The structures of the metabolites were elucidated by ¹H-NMR and mass spectrometry. AαC and HONH-AαC underwent glucuronidation, by UGTs, to form, respectively, N(β-D-glucosiduronyl)-2-amino-9H-pyrido[2,3-b]indole (AαC-N²-Gl) and N(β-D-glucosiduronyl)-2-hydroxyamino-9H-pyrido[2,3-b]indole (AαC-HON²-Gl). HONH-AαC also underwent glucuronidation to form a novel O-glucuronide conjugate, O(β-D-glucosiduronyl)-2-hydroxyamino-9H-pyrido[2,3-b]indole (AαC-HN²-O-Gl). AαC-HN²-O-Gl is a biologically reactive metabolite that bound to calf thymus DNA (pH 5.0 or pH 7.0) to form the N-(deoxyguanosin-8-yl)-AαC (dG-C8-AαC) adduct at 20 to 50-fold higher levels than the adduct levels formed with HONH-AαC. Major UGT isoforms were examined for their capacity to metabolize AαC and HONH-AαC. UGT1A4 was the most catalytically efficient enzyme (Vₘₐₓ/Kₘ) at forming AαC-N²-Gl (0.67 µL mg protein⁻¹ min⁻¹), UGT1A9 was most catalytically efficient at forming AαC-HN-O-Gl (77.1 µL mg protein⁻¹ min⁻¹), whereas, UGT1A1 was most efficient at forming AαC-HON²-Gl (5.0 µL mg protein⁻¹ min⁻¹). Human hepatocytes produced AαC-N²-Gl and AαC-HN²-O-Gl in abundant quantities, but AαC-HON²-Gl was
a minor product. Thus, UGTs, usually important enzymes in the detoxication of many procarcinogens, serve as a mechanism of bioactivation of HONH-AαC.

**Introduction**

Epidemiologic studies conducted over the past two decades have consistently shown that tobacco smoking is a risk factor for cancers of the gastrointestinal tract (1,2). There is also mounting evidence that tobacco smoke is an independent risk factor for hepatocellular carcinoma, the predominant form of human liver cancer (3,4). However, the causal agents of these cancers in tobacco smoke remain to be determined.

The heterocyclic aromatic amine (HAA), 2-amino-9H-pyrido[2,3-b]indole (AαC), a pyrolysis product of protein (5), occurs in mainstream tobacco smoke at levels ranging from 60 to 258 ng/cigarette (6,7). These amounts are 25- to 100-fold higher than those of the aromatic amine, 4-aminobiphenyl (4-ABP), a known human carcinogen that has been implicated in the pathogenesis of bladder cancer in smokers (8). Apart from the endocyclic nitrogen atoms, AαC has the same chemical structure as 2-aminofluorene (2-AF), perhaps the most well-studied among the carcinogenic aromatic amines (Fig. 1) (9). AαC is a liver carcinogen in mice, and both a potent lacI transgene colon mutagen and an inducer of aberrant crypt foci, early biomarkers of neoplasms, in the colon of mice (10-12). Thus, AαC may be a causal agent of liver and other digestive tract cancers in smokers.

HAAs and structurally related arylamines undergo metabolic activation by N-hydroxylation of the exocyclic amine groups, by cytochrome P450 enzymes. Phase II enzymes such as N-acetyltransferases (NATs), sulfotransferases (SULTs), or ATP-dependent enzymes (13-15) catalyze the formation of unstable esters of the N-hydroxylated metabolites, which undergo heterolytic cleavage to produce the reactive arylnitrenium ion that binds to DNA (Scheme 1). UDP-Glucuronosyltransferases (UGTs) catalyze the glucuronidation and elimination of numerous classes of xenobiotics, steroids, other endogenous compounds, and are prominently involved in the detoxication of carcinogens (16,17). The UGTs are present in the 1A, 2A, and 2B subfamilies and are expressed in liver and extrahepatic tissues (16,18). The UGT isoforms catalyze the detoxication of aromatic amines, HAAs, and their N-hydroxylated metabolites by N-glucuronidation (17,19-23) (Scheme 1).

The evaluation of the human health risk of AαC requires an understanding of the enzymes involved in bioactivation and detoxication of this procarcinogen; however, the metabolism of AαC has not been well-studied in humans (24). Recombinant human cytochrome P450 1A2 catalyzes the N-oxidation of the exocyclic amine group of AαC, to form the genotoxic metabolite 2-hydroxyamino-9H-pyrido[2,3-b]indole, (HONH-AαC) (25). P450 1A2 also catalyzes the detoxication of AαC, by ring oxidation of the C3 and C6 atoms of the heteronucleus (25,26), followed by O-glucuronidation (27,28). However, human hepatocytes efficiently bioactivate AαC to reactive metabolites that form DNA adducts (29). The propensity of AαC and structurally related compounds in tobacco smoke, to undergo bioactivation by enzymes expressed in liver and extrahepatic tissues provides a biochemical mechanism for the elevated risk of liver and digestive cancers in smokers (25,29-31).

In this study, we have characterized the metabolic products of AαC and HONH-AαC that are formed by UGT enzymes present in human liver microsomes, recombinant human UGT1A1 and UGT2B isofoms, and freshly cultured human hepatocytes. We report a novel pathway of bioactivation of HONH-AαC, where UGTs catalyze the formation an O-linked glucuronide conjugate, AαC-HN-O-Gl, which covalently binds to DNA. Thus, UGT enzymes, normally viewed as a means of detoxication of many carcinogens, serves as a mechanism of bioactivation and likely transport of HON-AαC from liver to extrahepatic tissues.

**EXPERIMENTAL PROCEDURES**

Caution: AαC and several of its 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 was purchased from Toronto Research Chemicals (Toronto, ON, Canada). Uridine-5′-diphospho-glucuronic acid (UDPGA), alamethicin, and β-
glucuronidase type IX-A from *Escherichia coli* were purchased from Sigma (St. Louis, MO). Human liver samples were from Tennessee Donor Services, Nashville, TN, and kindly provided by Dr. F. P. Guengerich, Vanderbilt University. Recombinant human UGT expressed in baculovirus-infected insect cell baculosomes (UGT1A1, -1A3, -1A4, -1A6, -1A8, -1A9, -1A10, and UGT2B7 were purchased from BD Biosciences (Woburn, MA). 2-Hydroxyamino-1-methyl-6-phenylimidazo-[4,5-b]pyridine (HONH-PhIP) and 4-hydroxyaminobiphenyl (HONH-4-ABP), N-(deoxyguanosin-8-yl)-PhIP (dG-C8-PhIP), N-(deoxyguanosin-8-yl)-4-ABP (dG-C8-4-ABP), N-(deoxyguanosin-8-yl)-AαC (dG-C8-AαC), and their [13C6]-dG isotopically labeled internal standards were synthesized as previously described (32-34).

**General Methods.** Mass spectra were acquired on a Finnigan Quantum Ultra triple stage quadrupole mass spectrometer (Thermo Fisher, San Jose, CA) with a Michrom Advance CaptiveSpray™ source (Auburn, CA). Typical instrument tuning parameters were as follows: capillary temperature, 200 °C; source spray voltage, 2 kV; tube lens offset, 95 V; capillary offset, 35 V; and source fragmentation, 10 V. Argon, set at 1.5 mTorr, was used as the collision gas. There was no sheath or auxiliary gas. All analyses were conducted in the positive ionization mode. Metabolites were also characterized with the Thermo Fisher linear quadrupole ion trap MS (LTQ) with the Advance CaptiveSpray™ ion source. The source voltage was 1.5 kV, the capillary voltage was 25 V, and the tube lens was 80 V. The isolation width was set at m/z 4 and 1, respectively, for the MS² and MS¹ scan modes, the activation Q was set at 0.35, and the activation time was 10 ms. Helium was used as the collision damping gas in the ion trap. One microscan was used for data acquisition. The automatic gain control settings were full MS target 30,000 and MS² target 10,000, and the maximum injection time was 10 ms.

**NMR Studies.** ^1^H-NMR resonance assignment experiments for the glucuronide metabolites of AαC and HNOH-AαC were conducted at 25°C with a Bruker Avance III 600 MHz spectrometer equipped with a triple resonance cryoprobe (Bruker BioSpin Corp., Billerica, MA). The ^1^H chemical shifts were referenced directly from the DMSO-d₆ multiplet at 2.50 ppm. A standard DQF-COSY experiment was employed to collect 1024 t₁ increments over a 7507 Hz spectral window. Standard rotating frame NOESY (ROESY) was employed using 400 ms mixing time.

**Synthesis of HONH-AαC and Biosynthesis of the Glucuronide Conjugates of AαC and HNOH-AαC.** and HONH-AαC as synthesized by reduction of 2-nitro-9H-pyrido[2,3-b]indole as described (31). The glucuronide metabolites were prepared by incubating AαC or HONH-AαC (1 mg in 100 μL DMSO) with 5 mg of human liver microsomal protein in 5 mL of 100 mM Tris-HCl buffer (pH 7.5), containing 10 mM MgCl₂, 0.5 mM EDTA and 5 mM UDPGA for 3 h at 37 °C. The microsomal mixture was preincubated with alamethicin (50 µg/mg protein) on ice for 30 min, to overcome the latency phenomena associated with UGT enzymes, prior to the addition of the AαC compounds. Ascorbic acid (2 mM) was added to the microsomal incubation containing NOH-AαC, to minimize oxidation of the substrate. The reactions were terminated by the addition of 1 vol ice-cold CH₃OH, and the mixtures were placed on ice for 30 min. The precipitated proteins were removed by centrifugation.

**UGT Glucuronidation and Enzyme Kinetics of AαC and HNOH-AαC with Recombinant UGT Isoforms.** UGTs were diluted to a concentration of 0.5 mg protein/mL in 100 mM Tris-HCl buffer (pH 7.5), containing 10 mM MgCl₂, 0.5 mM EDTA, 2 mM UDPGA, and 25 µg alamethicin per 0.5 mg of protein, and incubated on ice for 30 min, prior to addition of AαC substrates. The reactions were conducted under an atmosphere of argon at 37 °C. Time-dependent studies with HONH-AαC (10 or 100 µM) showed that product formation was linear over 140 min (data not shown). Enzyme kinetics experiments were conducted with HNOH-AαC at concentrations between 5 and 500 µM, and the concentrations of AαC ranged from 75 to 1500 µM. The time of incubation was 1 h. Aliquots (100 µL) were taken and added to 2 vol of ice-cold CH₃OH, to terminate the reaction, followed by centrifugation to remove protein. The methanolic extracts were analyzed by HPLC. The activities of UGT isoforms were assessed with β-estradiol (150 µM) as a substrate for UGTs 1A1 and 1A3, trifluoperazine (200 µM)
as a substrate for UGT 1A4, and 7-hydroxy-4-trifluoromethylcoumarin (50 μM) as a substrate for UGTs 1A6, 1A8, 1A9, 1A10 and 2B7. The enzyme activities were in good agreement to those values provided by BD Biosciences.

**HPLC analysis of AαC- and HONH-AαC-Glucuronide Conjugates.** Metabolites were analyzed with an Agilent model 1100 HPLC Chemstation (Palo Alto, CA) equipped with a photodiode array detector. The metabolites were separated with a Aquasil C18 column (4.6 mm × 150 mm, 5 μm particle size) from Thermo Scientific. The chromatography of the glucuronide conjugates of AαC began at 10 mM NH₄H₂CO₃ (pH 6.8) for 2 min, followed by a linear gradient over 22 min to 100% CH₃CN, at a flow rate of 1 mL/min. The chromatography of the glucuronide conjugates of HNOH-AαC also commenced at 10 mM NH₄H₂CO₃ (pH 6.8), for 2 min, followed by a linear gradient over 16 min to arrive at 40% CH₃CN, and reached 100% CH₃CN at 25 min. The estimates in formation of glucuronide conjugates of AαC and HNOH-AαC were determined by integration of the peak monitored at 338 nm. We assumed that the molar extinction coefficients of the glucuronide conjugates were comparable to the molar extinction coefficient of AαC (21560 ε (M⁻¹ cm⁻¹)).

**Kinetic Studies on AαC-N²-Gl AαC-HON-N²-Gl and AαC-HN²-O-Gl as a Function of pH or by Treatment with β-Glucuronidase.** The stabilities AαC- and HONH-AαC glucuronide conjugates were examined in 50 mM potassium phosphate buffer (pH 7.0) or 50 mM citric acid buffer (pH 5.0). The conjugates were also incubated with β-glucuronidase (240 U/mL) in 50 mM potassium phosphate buffer (pH 7.0). The compounds (4 μg, 10.7 nmol) were incubated under an atmosphere of argon at 37 °C for up to 3 h. The conjugates and hydrolysis products were assayed directly by HPLC except for studies with β-glucuronidase; the solution was diluted with 2 vol of ice-cold CH₃OH and the protein was removed by centrifugation, prior HPLC (vide supra).

**DNA Binding Studies with HONH-AaC, AαC-HON-N²-Gl, and AαC-HN²-O-Gl.** Calf thymus DNA (0.6 mg/mL) was incubated with HONH-AαC (10 μg, 50 nmol), HONH-PhIP (11 μg, 46 nmol) or HONH-4-ABP (13 μg, 70 nmol) or AαC-HN²-O-Gl and AαC-HON-N²-Gl (6 μg, 16 nmol) in 1 mL of 50 mM potassium citrate buffer (pH 5.0) or 50 mM potassium phosphate buffer (pH 7.0). AαC-HN²-O-Gl and AαC-HON-N²-Gl were also incubated with DNA (0.6 mg/mL) and β-glucuronidase (240 U/mL) in 50 mM potassium buffer (pH 7.0). The DNA solutions were incubated at 37°C for 2 h, under an atmosphere of argon. The reactions were terminated by three solvent extractions with an equal volume of ethyl acetate, and the DNA samples were precipitated from solution by addition of 0.1 volume of 5 M NaCl, followed by 1.5 mL of C₂H₅OH. The DNA filament was washed with a C₂H₅OH:H₂O mixture (7:3) and air-dried.

**UGT-Mediated Binding of HONH-AαC to DNA.** Human liver microsomal protein (0.5 mg) in 0.5 mL of 100 mM Tris-HCl buffer (pH 7.5), containing salts, ascorbic acid, 5 mM UDPGA (vide supra), and calf thymus DNA (0.3 mg) was preincubated with alamethicin (25 μg/0.5mg protein) on ice for 30 min, followed by the addition 1-naphthol (0, 100 or 1,000 μM), and HONH-AαC (10 μM). The incubation proceeded at 37 °C for 30 min. The reaction was terminated by the addition of CaCl₂ (50 mM final concentration), to precipitate the protein. Following centrifugation, the supernatant was retrieved, and 0.1 vol of 5 M NaCl and 2 vol C₂H₅OH were added, to precipitate DNA. The supernatants containing HONH-AαC glucuronide conjugates were measured by HPLC (vide supra). The pelleted DNA was washed with C₂H₅OH:H₂O mixture (7:3) and digested enzymatically as described above.

**Metabolism Studies of AαC with Human Hepatocytes.** Human liver samples were obtained from patients undergoing liver resection for primary or secondary hepatomas through the Biological Resource Center (CHRU Pontchaillou, Rennes, France). The research protocol was conducted under French legal guidelines and fulfilled the requirements of the local institutional ethics committee. This study was approved by the Institutional Review Board at the Wadsworth Center. Hepatocytes were isolated by a two-step collagenase perfusion procedure and parenchymal cells were seeded in Petri dishes at a density of 3 x 10⁶ viable cells/19.5 cm² dish, in 3 mL of Williams’ modified medium prior to incubation with AαC (10 or 50 μM) as previously described (29).
LC-ESI/MS/MS Measurements of DNA Adducts. DNA (5 μg) in 0.1 ml of 5 mM Bis-Tris-HCl buffer (pH 7.1) was spiked with 100 pg internal standards (\(^{13}\text{C}_{10}\)-dG-C8-AαC, \(^{13}\text{C}_{10}\)-dG-C8-PhIP or \(^{13}\text{C}_{10}\)-dG-C8-4-ABP) or 1.5 adducts per \(10^5\) DNA bases. The DNA was subjected to enzymatic digestion as previously reported (33,34). Analyses of adducts were performed with a NanoAcquity™ UPLC system (Waters Corporation, Milford, MA) interfaced with a LTQ MS. A Waters Symmetry trap column (180 µm x 20 mm, 5 µm particle size) was employed for online solid phase enrichment of the DNA adducts. The analytical column was a C18 AQ (0.3 x 150 mm, 3 µm particle size) from Michrom Bioresources Inc., (Auburn, CA). The UPLC conditions have been reported (35). Adducts were measured at the MS\(^3\) scan stage, which produced the aglycone adducts [BH\(^+\)]\(^{+}\) (36). The ions monitored in MS > MS\(^2\) > MS\(^3\) scan modes were as follows: dG-C8-PhIP (m/z 490.1 > 374.1 >); \(^{13}\text{C}_{10}\)-dG-C8-PhIP (m/z 500.1 > 379.1 >); dG-C8-AαC (m/z 449.1 > 333.1 >); \(^{13}\text{C}_{10}\)-dG-C8-AαC (m/z 459.1 > 338.1 >); dG-C8-4-ABP (m/z 435.1 > 319.1 >); \(^{13}\text{C}_{10}\)-dG-C8-4-ABP (m/z 445.1 > 324.1 >). Total ions were measured at the MS\(^3\) scan stage. The normalized collision energies were set at 32 and 40, and the isolation widths were set at m/z 3.0 and 1.0, respectively, for the MS\(^2\) and MS\(^3\) scan modes. The source voltage was 2.5 kV, the capillary voltage was 25 V, and the tube lens voltages was 80 V. The activation Q was set at 0.35, and the activation time was 10 ms, for both scan modes.

LC-ESI/MS/MS Analysis of AαC-Glucuronide Metabolites in Human Hepatocytes. Cell extracts were assayed by LC-ESI/MS/MS with the TSQ Quantum Ultra triple stage quadrupole mass spectrometer in the selected reaction monitoring mode and employed the following transitions for the glucuronide conjugates of oxidized AαC: 376.1 > 183.1, 184.1, and 200.1, with a collision energy of 35 eV. The sample preparation, chromatographic conditions, and MS tuning parameters were previously described (29).

Data Analysis. GraphPad Prism 5 software (La Jolla, CA) was employed to calculate enzyme kinetic values. Apparent \(K_m\) and \(V_{\text{max}}\) values for glucuronidation by each enzyme were derived from the Michaelis-Menten equation

\[
v = \frac{(V_{\text{max}} \times [S])/(K_m + [S])}
\]

or by the substrate inhibition equation

\[
v = \frac{(V_{\text{max}} \times [S])/(K_m + [S] \times (1 + [S]/K_{\text{si}}))}
\]

where \(v\) is the initial velocity, \(V_{\text{max}}\) is the maximum enzyme velocity (pmol/mg microsomal protein/min), \(K_m\) is the Michaelis constant, [S] is the initial substrate concentration, \(K_{\text{si}}\) is the dissociation constant for the substrate from the enzyme inhibitor complex. Catalytic efficiency (\(K_m/V_{\text{max}}\)) was expressed as \(\mu\)L mg microsomal protein\(^{-1}\) min\(^{-1}\). The data were fitted using nonlinear regression, employing the least squares, to obtain the best curve.

RESULTS

Glucuronide Metabolites of AαC and HONH-AαC Produced by Human Liver Microsomes. The HPLC profile of glucuronide conjugates of AαC and HONH-AαC produced by human liver microsomes under elevated substrate concentrations (1 mM), are shown in Fig. 2. One major glucuronide conjugate was formed with AαC (t\(r\) 9.4). The on-line UV spectrum of the metabolite displayed a chromophore that was very similar to the spectrum of AαC, suggesting the metabolite was the AαC-N\(^2\)-Gl. Two glucuronide conjugates were formed with HONH-AαC (t\(r\) 14.9 and 15.8 min). The UV spectra of both conjugates strongly resembled the UV spectrum of HONH-AαC. The ratio of the peak area between the conjugates was about 2.4:1.

\(^1\text{H}-\text{NMR Spectroscopy of Glucuronide Conjugates of AαC and HONH-AαC.}\) The glucuronide conjugate of AαC (120 µg), and the major (210 µg) and minor (80 µg) conjugates of HONH-AαC were produced in sufficient quantities, to analyze by \(^1\text{H}-\text{NMR spectrometry (Figs. 3 - 5). The chemical shift values for the metabolites are summarized in Table 1. All of the protons of the heterocyclic ring and the endocyclic N9 atom were observed in the \(^1\text{H}-\text{NMR spectrum of the glucuronide conjugate of AαC.}\) The resonance signal at 7.18 ppm had the intensity of one proton and
metabolites were distinguished by electrospray ionization multistage tandem mass spectrometry (ESI/MS/MS²).

ESI/MS/MS² Product Ion Spectra of Glucuronide Conjugates of AαC and HONH-AaC. The product ion spectra of the glucuronide conjugates were acquired in the negative ionization mode. The product ion spectrum of AαC-HON²-Gl ([M-H]⁺ at m/z 358.1), shown in Fig. 6A, displays fragment ions at m/z 296.0 ([M-H₂CO₂H]⁻) and m/z 278.0 ([M-H₂2H₂O-CO₂]⁻). The proposed negatively charged acetyl derivative of AαC at m/z 224.2 [M-H₂C₆H₄O₃]⁻ and the deprotonated AαC at m/z 182.1 [M-H₆C₆H₈O₆]⁻ are also observed.

The product ion spectrum of AαC-HON²-Gl, [M-H]⁻ at m/z 374.1, is shown in Fig. 6B. Prominent ions are observed at m/z 282.1 [M-H₂C₆H₄O₃]⁻ attributed to deprotonated HONH-AaC containing the C1-C4 atoms of glucuronic acid, and at m/z 222.1 [M-H₂C₆H₄O₃]⁻ attributed to deprotonated HONH-AaC containing the C1 and C2 atoms of glucuronic acid (Fig. 6B). The fragment ion at m/z 198 [M-H₂C₆H₄O₃]⁻ is attributed to the negatively charged glucuronic acid, which occurred by cleavage of the glucuronide linkage, and the ion at m/z 182.0 [M-H₂C₆H₈O₆]⁻ is attributed to the loss of oxygen from HONH-AaC.

The product ion spectrum of AαC-HON²-O-Gl, [M-H]⁻ at m/z 374.1, is shown in Fig. 6C. One predominant ion is observed at m/z 193 [M-H-C₁₁H₂N₇]⁻ and is proposed to occur by cleavage of the N₂-O bond of HONH-AaC, with the oxygen atom remaining attached to the C₁ atom of the glucuronate. The second generation product ion spectrum of AαC-HON²-O-Gl acquired on m/z 193 shows the typical collision-induced dissociation fragmentation pattern previously reported for the glucuronate (Fig. 6D) (43), and proves that the linkage formed between HONH-AaC and glucuronic acid occurred at the oxygen atom of HONH-AaC. The product ion spectra of O-glucuronide conjugates of aryloxidation acids typically display a prominent ion at m/z 193 in the negative ion mode; this fragment ion is diagnostic for this class of N-O-glucuronide aryloxidation conjugates (44).

Glucuronidation of AαC and HONH-AaC with Recombinant Human UGTs. Recombinant human UGT isoforms 1A1, 1A3, 1A4, 1A6, 1A8, 1A9, 1A10 and 2B7 were screened for
their capacities to catalyze AαC-N-glucuronidation, AαC-HON-glucuronidation, and AαC-HN-glucuronidation formation. UGT1A4 was the only isoform that showed detectable N-glucuronidation activity for AαC (93.4 ± 1.5 pmol mg protein⁻¹ min⁻¹), and only under elevated substrate concentrations (Kₘ = 2060 μM). HONH-AαC underwent N-glucuronidation, by UGT 1A1, 1A4, 1A9 and 2B7, but with different specificities in product formation (Supplementary Information, Table 1S). AαC-HON-glucuronidation was the predominant conjugate formed with UGT2B7, whereas AαC-HN-glucuronidation was the major conjugate produced by UGTs 1A1 and 1A9. UGT1A4 catalyzed the formation AαC-HON-glucuronidation but did not form AαC-HN-glucuronidation. UGTs 1A1, 1A9 and 2B7 produced both AαC-HON-glucuronidation and AαC-HN-glucuronidation. The activity observed for UGT1A8 was below the detectable level of metabolite detection by HPLC (<7 pmol min⁻¹ mg⁻¹ microsomal protein).

Steady-State Enzyme Kinetic Parameters of AαC-N-glucuronidation, AαC-HON-glucuronidation, and AαC-HN-glucuronidation Formation. The steady-state enzyme kinetic parameters were determined for UGTs 1A1, 1A4, 1A9, and 2B. The data are summarized in Table 2, and the non-linear regression Michaelis-Menten curves are provided in Supplementary Information, Fig. 1S. In the case of UGT1A4, very high concentrations of AαC and HONH-AαC (Kₘ values > 1000 μM for both substrates) were required to observe product formation. Therefore, the apparent Vₘₐₓ and Kₘ values for AαC-N-glucuronidation and AαC-HON-glucuronidation formation were estimated, by non-linear regression, at concentrations of substrates below the Kₘ values. UGT 1A1 displayed the highest rate of catalysis of AαC-HN-glucuronidation formation (Vₘₐₓ = 575 pmol/min/mg microsomal protein, Kₘ = 21.4 μM); however, UGT 1A9 displayed the lowest apparent Kₘ (Kₘ = 0.7 μM), and it was the most catalytically efficient UGT isoform in producing AαC-HN-glucuronidation (77.1 μL mg protein⁻¹ min⁻¹). Moreover, an atypical substrate inhibition was observed for UGT1A9: the rate of formation of AαC-HN-glucuronidation, but not the rate of formation of AαC-HON-glucuronidation, was diminished when the initial substrate concentration of HONH-AαC exceeded 20 μM. The diminution in activity may be attributed to the binding of HONH-AαC or AαC-HN-glucuronidation with the enzyme-UDP complex, leading to a nonproductive dead-end complex that slows the catalytic cycle (45). The substrate inhibition effect may not be relevant in vivo given that the exposure to AαC via tobacco smoke or cooked meat is on the order of several μg per day. On the basis of the enzyme kinetic data, we expect the formation of AαC-HN-glucuronidation to be greater than that of AαC-HON-glucuronidation, under the low substrate concentrations of AαC that occur in vivo. The preferred formation of AαC-HN-glucuronidation over AαC-HON-glucuronidation is supported by studies with human hepatocytes that are described below.

Kinetic Studies on AαC-N-glucuronidation, AαC-HON-glucuronidation, and AαC-HN-glucuronidation as a Function of pH or by Treatment with β-Glucuronidase. The stabilities of AαC-N-glucuronidation, AαC-HO-N-glucuronidation, and AαC-HN-glucuronidation were determined in potassium citrate buffer (pH 5.0) and potassium phosphate buffer (pH 7.0) (Supplementary Information, Fig. 2S). AαC-N-glucuronidation underwent hydrolysis to form AαC. The half-life (t₁/₂) of AαC-N-glucuronidation was estimated at 72 min (pH 5.0) and 58 h (pH 7.0). AαC-HN-glucuronidation slowly underwent hydrolysis to form HONH-AαC. There was no evidence for the formation of HONH-AαC, NO₂-AαC, or ozoxys products by HPLC analysis (data not shown). The t₁/₂ of AαC-HN-glucuronidation was 6.8 h at pH 5.0 and 19 h at pH 7.0. AαC-HN-glucuronidation underwent hydrolysis to yield AαC, demonstrating that the N-glucuronide linkage is labile and that AαC-HN-glucuronidation may undergo nucleophilic substitution reactions with DNA or protein (20).

AαC-HN-glucuronidation was substrate for β-glucuronidase (E. Coli). The sole, initial product formed was HONH-AαC (56 pmol min⁻¹ unit⁻¹ β-glucuronidase, t₁/₂=2 min) in 50 mM potassium phosphate buffer (pH 7.0), whereas the rates of hydrolysis of AαC-N-glucuronidation and AαC-HO-N-glucuronidation were considerably slower (93 fmol min⁻¹ unit⁻¹ β-glucuronidase, t₁/₂ not determined) (Supplementary Information, Fig. 2S). These differences in rates of enzymatic hydrolysis of AαC- and HONH-AαC-glucuronide conjugates are consistent with the known fact that O-glucuronide conjugates are superior substrates than N-glucuronide conjugates for β-glucuronidase (E. Coli) (46).

Reactivity of HONH-AαC, AαC-HON-glucuronidation, and AαC-HN-glucuronidation with Calf Thymus DNA. The reactivity of HONH-AαC and its glucuronide...
conjugates to DNA under different pH conditions was compared to the DNA binding of the N-hydroxy derivative of 4-ABP, a tobacco carcinogen (8), and the N-hydroxy derivative of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), a carcinogen formed in cooked meat (12). The amounts of the dG-C8-AαC, dG-C8-4-ABP, and dG-C8-PhIP adducts formed by these reactive carcinogenic metabolites were determined by LC-ESI/MS/MS. The mass chromatograms are shown in Supplementary Information, Fig. 3S. The highest level of DNA binding occurred for HONH-4-ABP, followed by HONH-AαC, and lastly by HONH-PhIP, under acidic (pH 5.0) and neutral (pH 7.0) pH conditions (Fig. 7). The reactivity of HONH-4-ABP with DNA was about 25-fold greater under acidic pH than neutral pH conditions, whereas the acidic pH increased the level of HONH-AαC DNA adduct formation by 10-fold, and by 5-fold for HONH-PhIP. The enhanced reactivity of arylnitrenium ions with DNA under acidic pH conditions has been ascribed to the formation of the arylnitrenium ion (47).

AαC-HN2-O-Gl reacted with DNA (pH 7.0) to form the dG-C8-AαC adduct at levels that were ~50-fold higher than the adduct levels formed by reaction of HONH-AαC or AαC-HON2-Gl with DNA. The O-glucuronide linkage of AαC-HN2-O-Gl was critical for dG-C8-AαC formation, and the addition of β-glucuronidase into the reaction mixture diminished the level of dG-C8-AαC formation by ~50-fold. Moreover, the binding of AαC-HN2-O-Gl to DNA at pH 5.0 was still 3.5-fold greater than the level of DNA binding of HONH-AαC. It is also worthy to note that AαC-HO-N2-Gl reacted with DNA at pH 5.0 and formed an appreciable level of adducts. The acidic pH results in hydrolysis of AαC-HON2-Gl to form reactive HONH-AαC.

**UGT-Mediated Binding of HONH-AαC to DNA.** UGT isoforms expressed in human liver microsomes produced AαC-HON2-Gl and AαC-HN2-O-Gl, and catalyzed the binding of HONH-AαC to calf thymus DNA (Figs. 8A and 8B). The presence of 1-naphthol, a substrate for multiple UGTs (18) in the incubation medium, led to a dose-dependent decrease in AαC-HN2-O-Gl and AαC-HON2-Gl conjugates and also in dG-C8-AαC adduct formation. These findings show that UGTs catalyze the binding of HONH-AαC to DNA.

**AαC-N2-Gl, AαC-HN2-O-Gl, and AαC-HON2-Gl Formation in Human Hepatocytes.** We have shown that AαC undergoes extensive metabolism and forms DNA adducts at high levels in hepatocytes (29). In this study, we have characterized several glucuronide conjugates of AαC produced in hepatocytes. The product ion spectra of AαC-HN2-O-Gl, and AαC-HON2-Gl metabolites formed in hepatocytes are shown in Supplementary Information, Fig. 5S. The 1H NMR of the P450-ring oxidized metabolites of AαC, 2-amino-3-hydroxy-9H-pyrido[2,3-b]indole (3-HO-AαC), and 2-amino-6-hydroxy-9H-pyrido[2,3-b]indole (6-HO-AαC), and their UV and mass spectral data and those of their O-glucuronide conjugates formed in human hepatocytes are presented Supplementary Information, Figs. 6S and 7S. The mass chromatograms of AαC-HO-N2-Gl, AαC-HN2-O-Gl, and the O-glucuronide conjugates of 3-HO-AαC and 6-HO-AαC are shown in Fig. 9A. Prominent peaks attributed to AαC-HN2-O-Gl, 2-amino-3-(β-D-glucosiduronyloxy)-9H-pyrido[2,3-b]indole (AαC-O2-Gl), and 2-amino-6-(β-D-glucosiduronyloxy)-9H-pyrido[2,3-b]indole (AαC-O3-Gl) are readily observed, whereas only trace levels of AαC-HON2-Gl are formed in hepatocytes exposed to AαC (10 μM). Similar findings were obtained with hepatocytes from two other donors (data not shown). The formation of the AαC-O2-Gl and AαC-O3-Gl metabolites progressed during the 24 h incubation period (10 or 50 μM AαC). AαC-N2-Gl formation also increased with time, occurring at levels comparable to the ring-oxidized conjugates (data not shown). AαC-HON2-Gl was primarily detected in hepatocytes treated with AαC (50 μM), and metabolite formation continued over 24 h. However, the biosynthesis of AαC-HN2-O-Gl peaked at 3 h and then dramatically declined at 24 h, for both concentrations of AαC (Fig. 9B and Fig. 9C). These findings show that O-glucuronidation is a principal pathway of conjugation of HONH-AαC, under low exposure conditions to AαC (Fig. 9A). We surmise that a portion of AαC-HN2-O-Gl reacts with DNA and possibly protein.

**DISCUSSION**

The N-glucuronidation of HAAs, aromatic
amines, and their genotoxic N-hydroxylated metabolites, by UGTs, is an important mechanism of detoxication of these structurally related chemicals. The UGT enzyme pathways compete with P450 and phase II enzyme pathways, which bioactivate these procarcinogens (Scheme 1) (17,19-21,23).

To date, the direct O-glucuronidation of carcinogenic N-hydroxy-HAAs or arylhydroxylamines, by UGTs (14,17,20,47,48), has not been reported. The formation of AαC-HN\(^2\)-O-Gl, by human UGTs, is the first example of the occurrence of a O-glucuronidate conjugate from this group of structurally related carcinogens. AαC-HN\(^2\)-O-Gl is a biologically reactive metabolite, which binds to DNA (Figure 7). The binding of HONH-AαC to DNA is catalyzed, by UGTs expressed in human liver microsomes (Fig. 8).

The kinetic parameters of UGT enzymes were characterized to determine which isoforms catalyze this unique pathway of bioactivation of HONH-AαC. UGT1A9 is the most catalytically active isoform in O-glucuronidation of HONH-AαC (Table 2). UGT1A9 is also the principal UGT isoform involved in the O-linked glucuronidation of simple phenols (18), and a major isoform involved in the detoxication, by N\(^2\)-glucuronidation of HONH-PhIP, the genotoxic metabolite of the cooked meat carcinogen PhIP (21). UGT1A9 is present in human liver, colon, prostate, breast, among other tissues (16). Given the low \(K_m\) (0.9 \(\mu\)M) value of UGT1A9-mediated AαC-HN\(^2\)-O-Gl formation, we may expect that UGT1A9 catalyzes the O-glucuronidation of HONH-AαC and its binding to DNA in vivo.

O-Glucuronide conjugates of several arylhydroxamic acids are produced by UGT enzymes (20,49), including the O-glucuronide conjugate of N-hydroxy-2-acetylaminofluorene (HONH-2-AAF), which was identified in urine of rats treated with 2-acetylaminofluorene (2-AAF) (50). The metabolite was stable in urine, but it was labile under slightly alkaline pH. The investigators proposed that the N-acetyl group of the AAF moiety had migrated to an hydroxyl group of the glucuronic acid (20). The resulting O-glucuronide of 2-hydroxyaminofluorene underwent (HONH-2-AF) reaction with nucleophiles (Scheme 2) and contributed to DNA adduct formation in rodents given 2-AAF (13,20). However, the O-glucuronide conjugate of HONH-2-AF or O-glucuronide conjugates of other arylhydroxylamines have not been identified in vivo, probably because these conjugates are unstable in aqueous solutions and decompose within several minutes (48).

The chemical stability of O-glucuronide linkage of AαC-HN\(^2\)-O-Gl is considerably greater than that of the O-glucuronide conjugate of HONH-2-AF. The half-life of AαC-HN\(^2\)-O-Gl exceeds 6 h at pH 7.0, but AαC-HN\(^2\)-O-Gl undergoes a facile nucleophilic displacement by reaction with dG, to from 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 (51). Thus, AαC-HN-O-Gl is an ideal genotoxic electrophile that can react with DNA.

UGTs play several different roles in the metabolism of AαC that impacts its biological activity. \(N^2\)-glucuronidation of AαC leads to the formation of a detoxicated product. However, HONH-AαC can undergo either \(N^2\)- or O-glucuronidation. The enzyme kinetic studies with recombinant human UGT isoforms and metabolism studies with human hepatocytes reveal that AαC-HN-O-Gl is the predominant glucuronide conjugate of HONH-AαC formed under low substrate concentrations (Fig. 9 and Supplementary Information, Table 1S). Moreover, because of its long half-life, AαC-HN-O-Gl is likely to be exported from liver to extrahepatic tissues, where it can react with DNA (Scheme 2). The role of UGTs in the genotoxicity of AαC, as opposed to NAT and SULT enzymes that are normally associated with the bioactivation of HAAs (17), warrants further study.

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FOOTNOTES

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UGT bioactivation of AαC

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The abbreviations used are: HAA, heterocyclic aromatic amine; LC-ESI/MS/MS, liquid chromatography/electrospray ionization–tandem mass spectrometry; LC-ESI/MS/MS<n>, liquid chromatography–electrospray ionization/multistage tandem mass spectrometry; NAT, N-acetyltransferase; SULT, Sulfotransferase; UGT, UDP-glucuronosyltransferase; UDPGA, uridine diphosphoglucuronic acid; 2-AAF, 2-acetylaminofluorene; N-hydroxy-2-acetylaminofluorene, HONH-AAF, 2-AF, 2-aminofluorene; HONH-2-AF, N-hydroxy-2-aminofluorene; 4-ABP, 4-aminobiphenyl; HONH-ABP, 4-hydroxyaminobiphenyl; AαC, 2-amino-9H-pyrido[2,3-b]indole; 2-amino-9H-pyrido[2,3-b]indole; HONH-AαC, 2-hydroxyamino-9H-pyrido[2,3-b]indole; 3-HO-AαC, 2-amino-3-hydroxy-9H-pyrido[2,3-b]indole; 6-HO-AαC, 2-amino-6-hydroxy-9H-pyrido[2,3-b]indole; AαC-HN²-Gl, N²-(β-D-glucosiduronyl)-2-amino-9H-pyrido[2,3-b]indole; AαC-HON²-Gl, N²-(β-D-glucosiduronyl)-2-hydroxyamino-9H-pyrido[2,3-b]indole; AαC-HN²-O-Gl, O-(β-D-glucosiduronyl)-2-hydroxyamino-9H-pyrido[2,3-b]indole; AαC-O¹-Gl, 2-amino-3-(β-D-glucosiduronyl)-9H-pyrido[2,3-b]indole; AαC-O¹-Gl, 2-amino-6-(β-D-glucosiduronyloxy)-9H-pyrido[2,3-b]indole; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine; HONH-PhIP, 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-b]pyridine; dG-C8-4-ABP, N-(deoxyguanosin-8-yl)-4-ABP; dG-C8-AαC, N-(deoxyguanosin-8-yl)-AαC; dG-C8-PhIP, N-(deoxyguanosin-8-yl)-PhIP.

SCHEMES

Scheme 1. Metabolism of arylamines by P450, UGT, and other phase II enzymes

Scheme 2. UGT-mediated bioactivation of the N-hydroxy metabolites of 2-AAF and AαC. The O-glucuronide metabolite of N-hydroxy-AAF becomes labile and biologically reactive, following migration of the N-acetyl moiety to an hydroxyl group of the glucuronate. The resultant O-glucuronide conjugate, 2-AF-N²-O-Gl-Ac, is labile and reacts with DNA or protein (20). HONH-AαC undergoes direct O-glucuronidation, to form AαC-HN²-O-Gl, which forms covalent adducts with DNA and presumably proteins.

FIGURE LEGENDS

Figure 1. Chemical structures of AαC, 4-ABP, and 2-AF

Figure 2. HPLC profiles and on-line UV spectra of glucuronide metabolites of AαC and HONH-AαC produced by human liver microsomes. (A) Glucuronide metabolite of AαC, (B) glucuronide metabolites of HONH-AαC. Different gradient conditions were employed for the resolution of the metabolites, as described in Experimental Procedures.

Figure 3. 1-D and 2-D ¹H-NMR spectra of the major glucuronide metabolite of AαC (in DMSO-d₆) recorded on a Bruker Avance III 600 MHz spectrometer: 1-D ¹H-NMR spectrum (top panel, 11.78 – 4.52 ppm) showing the NH9, aromatic protons of AαC moiety and H1’ proton of the glucuronide moiety. 2-D rotating frame NOESY (ROESY) contour plot (middle panel) showing NOE connectivities between protons through distance coupling around the NH9, the aromatic proton region and aliphatic region situated around the H1’ of the glucuronic acid. The portions of the 2-D COSY spectrum (bottom panel) focused on the protons having coupling through chemical bonds on the moiety of AαC. Note: x is an impurity.
Figure 4. 1- and 2-D $^1$H-NMR (ROESY and COSY) spectra of the major glucuronide metabolite of HONH-AαC (in DMSO-$d_6$) recorded on a Bruker Avance III 600 MHz spectrometer: 1-D $^1$H-NMR spectrum (top panel, 11.78 – 3.5 ppm) showing the NH9, aromatic protons of AαC moiety and H1′, H2′ proton of the glucuronide moiety. 2-D ROESY contour plot (middle panel) showing NOE connectivities between protons through distance coupling around the NH9, the aromatic proton region and aliphatic region situated around the H1′ of the glucuronic acid. The portions of the 2-D COSY spectrum (bottom panel) focused on the protons having coupling through chemical bonds on the moiety of AαC.

Figure 5. 1- and 2-D NMR (ROESY and COSY) spectra of the minor glucuronide metabolite of HONH-AαC (in DMSO-$d_6$) recorded on a Bruker Avance III 600 MHz spectrometer: 1-D $^1$H-NMR spectra (top panel, 11.78 – 3.15 ppm) showing the aromatic protons of AαC moiety and H1′, H2′ protons of the glucuronide moiety before and after addition of D$_2$O. The small amount of D$_2$O served to dilute and shift the resonances of the exchangeable protons. Previous to the addition of D$_2$O, the contour plot of a 2-D ROESY spectrum (middle panel) showed NOE connectivities between protons through distance coupling around the NH9, the aromatic proton region and aliphatic region situated around the H1′ of the glucuronic acid. The observe dimension is oriented along the Y-axis of this plot to take advantage of the stronger intensity of the NH-9-H8 crosspeak along this dimension. The portions of the 2-D COSY spectrum (bottom panel) focused on the protons having coupling through chemical bonds on the moiety of AαC.

Figure 6. ESI product ion spectra of AαC glucuronide conjugates acquired in the negative ion mode by ion trap mass spectrometry: (A) AαC-$N^2$-Gl, (B) AαC-HON$^2$-Gl, (C) AαC-HN$^2$-O-Gl and (D) the second generation product ion spectrum of AαC-HN$^2$-O-Gl acquired on m/z 193. Proposed mechanisms of formation of prominent fragment ions are presented.

Figure 7. Reactivity of HONH-AαC, AαC-HON$^2$-Gl, AαC-HN$^2$-O-Gl, HONH-4-ABP and HONH-PhIP with calf thymus DNA at pH 5.0 or 7.0, to form dG-C8 adducts. The reactivity of AαC-HON$^2$-Gl, AαC-HN$^2$-O-Gl with calf thymus DNA was also investigated at pH 7.0 in the presence of β-glucuronidase. Data are the average ± SD of 3 independent measurements.

Figure 8. UGT-mediated bioactivation of HONH-AαC in human liver microsomes fortified with UDPGA and calf thymus DNA, without or with 1-naphthol at 0, 100, or 1000 µM. (A) AαC-HON$^2$-Gl and AαC-HN$^2$-O-Gl, and (B) dG-C8-AαC adduct formation. A one-way ANOVA showed statistical significance for both metabolism and DNA adduct formation (P = 0.001), *Dunnett’s multiple comparison test showed statistical significance (P < 0.01 for 0 vs 100 µM and 0 vs 1000 µM) for both AαC-HON$^2$-Gl and AαC-HN$^2$-O-Gl formation, and dG-C8-AαC DNA adduct formation. Data are the average ± SD of 3 independent measurements.

Figure 9. (A) Mass chromatograms of AαC-HON$^2$-Gl, AαC-HN$^2$-O-Gl, AαC-O$^3$-Gl, and the AαC-O$^6$-G) in human hepatocytes incubated with AαC (10 µM) for 3 h. Levels of N-oxidized and ring-oxidized glucuronide conjugates formed as a function of time in human hepatocytes treated with (B) 10 µM AαC and (C) 50 µM AαC.
Table 1: NMR Chemical shift data (ppm) for glucuronide conjugates of $\alpha\alpha$C and HONH-$\alpha\alpha$C$^{a,b}$.

<table>
<thead>
<tr>
<th></th>
<th>$\alpha\alpha$C</th>
<th>$\alpha\alpha$C-HN$^2$-Gl</th>
<th>$\alpha\alpha$C-HON$^2$-Gl</th>
<th>$\alpha\alpha$C-HN$^2$-O-Gl</th>
</tr>
</thead>
<tbody>
<tr>
<td>H3</td>
<td>6.37</td>
<td>6.48 (d, $J=8.3$; 1.0)</td>
<td>7.02 (d, $J=8.4$, 1.0)</td>
<td>7.09 (d, $J=8.4$; 1.0)</td>
</tr>
<tr>
<td>H4</td>
<td>8.07</td>
<td>8.15 (d, $J=8.3$; 1.0)</td>
<td>8.33 (d, $J=8.4$; 1.0)</td>
<td>8.37 (d, $J=8.4$; 1.0)</td>
</tr>
<tr>
<td>H5</td>
<td>7.85</td>
<td>7.89 (d, $J=7.7$; 1.0)</td>
<td>7.98 (d, $J=7.7$; 1.0)</td>
<td>8.02 (d, $J=7.8$; 1.0)</td>
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<tr>
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<td>7.13 (t; 1.0)</td>
<td>7.17 (t; 1.0)</td>
<td>7.20 (t; 1.0)</td>
</tr>
<tr>
<td>H7</td>
<td>7.23</td>
<td>7.25 (t; 1.0)</td>
<td>7.32 (t; 1.0)</td>
<td>7.35 (t; 1.0)</td>
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<tr>
<td>H8</td>
<td>7.36</td>
<td>7.36 (d, $J=8.0$; 1.0)</td>
<td>7.40 (d, $J=8.1$; 1.0)</td>
<td>7.44 (d, $J=7.9$; 1.0)</td>
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<td>NH9</td>
<td>11.12</td>
<td>11.37 (s; 1.0)</td>
<td>11.46 (s; 1.0)</td>
<td>11.61 (s; 1.0)</td>
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<tr>
<td>H1'</td>
<td>5.09 (m; 1.0)</td>
<td>5.64 (d, $J=8.6$; 1.0)</td>
<td>4.58 (d, $J=7.7$, 1.0)</td>
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<tr>
<td>H2'</td>
<td>3.24 (m)$^{b}$</td>
<td>3.71 (m; 1.0)</td>
<td>3.28 (m)$^{b}$</td>
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<tr>
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<td>7.18 (d, $J=8.8$; 1.0)</td>
<td></td>
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</table>

$^{a}$: the protons were determined by 1-D as s (singlet), d (doublet), t (triplet) or m (multiplet) with an area integration at one proton (H4 was established as the reference for integration). $J$: J-coupling constant (unit: Hz) for doublet. The chemical shifts were reported in the average number.

$^{b}$: those protons were determined by 2-D COSY as multiplet.
**Table 2: Steady-state enzyme kinetic parameters for glucuronidation of AαC and HONH-AαC by recombinant UGT isoforms**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Glucuronide</th>
<th>UGT Isoform</th>
<th>$V_{max}$</th>
<th>$K_m$</th>
<th>$V_{max}/K_m$</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>pmol min^{-1}mg^{-1} protein</td>
<td>$\mu$M</td>
<td>$\mu$L min^{-1}mg^{-1} protein</td>
</tr>
<tr>
<td>HONH-AαC</td>
<td>AαC-HON$_2$-Gl</td>
<td>UGT1A1</td>
<td>248 ± 14.5</td>
<td>49.8 ± 7.9</td>
<td>4.99</td>
</tr>
<tr>
<td></td>
<td></td>
<td>UGT1A4$^c$</td>
<td>3960 ± 606</td>
<td>1140 ± 227</td>
<td>3.48</td>
</tr>
<tr>
<td></td>
<td></td>
<td>UGT1A9</td>
<td>15.0 ± 1.8</td>
<td>6.3 ± 4.5</td>
<td>2.37</td>
</tr>
<tr>
<td></td>
<td></td>
<td>UGT2B7</td>
<td>447 ± 60.9</td>
<td>173 ± 43.2</td>
<td>2.58</td>
</tr>
<tr>
<td>AαC-HN$_2$-O- Gl</td>
<td>UGT1A1</td>
<td>575 ± 33.7</td>
<td>21.4 ± 4.4</td>
<td>26.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>UGT1A9$^d$</td>
<td>57.5 ± 3.5</td>
<td>0.7 ± 0.6</td>
<td>77.1</td>
</tr>
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<td></td>
<td></td>
<td>UGT2B7</td>
<td>60.5 ± 5.9</td>
<td>78.4 ± 18.5</td>
<td>0.77</td>
</tr>
<tr>
<td>AαC</td>
<td>AαC-N$_2$-Gl</td>
<td>UGT1A4$^c$</td>
<td>1390 ± 170</td>
<td>2060 ± 379</td>
<td>0.67</td>
</tr>
</tbody>
</table>

$^a$ The glucuronidation activity (unit: pmol min^{-1} mg^{-1}microsomal protein) was examined for all UGT isoforms using recommended methods provided by BD Bioscience: estradiol was a substrate for UGTs 1A1 (750) and 1A3 (150); 7-hydroxy-4-trifluoromethylcoumarin was a substrate for UGTs 1A10 (128), 1A8 (388), 2B7 (780), 1A6 (6700), and 1A9 (7000); trifluoperazine was a substrate for UGT1A4 (820).

$^b$ There was no detectable glucuronidation activity of either AαC or HONH-AαC for UGTs 1A3, 1A6 and 1A10. UGT1A8 displayed low glucuronidation activity of HONH-AαC (<7 pmol min^{-1} mg^{-1}microsomal protein). Only UGT1A4 displayed activity for N$_2$-glucuronidation of AαC.

$^c$ Kinetic constants could not be determined with confidence because of the high substrate concentrations required for analysis.

$^d$ Substrate inhibition was observed, $K_i= 255 ± 58 \mu$M.
Scheme 1
Scheme 2
Figure 1

2-Amino-9H-pyrido[2,3-b]indole (A{\alpha}C)  
4-Aminobiphenyl (4-ABP)  
2-Aminofluorene (2-AF)
UGT bioactivation of AαC

Figure 2
Figure 4

1-D $^1$H-NMR
No $D_2O$

2-D ROESY

2-D COSY
UGT bioactivation of AaC

Figure 5

1-D 'H-NMR
D₂O added

1-D 'H-NMR
No D₂O

2-D ROESY

2-D COSY
Figure 7

![Graph showing the effect of pH on dG-C8 adduct formation per nmol of substrate.

- The graph compares the formation of dG-C8 adducts at pH 7, pH 5, and pH 7 with β-glucuronidase.
- The x-axis represents the substrates: dG-C8-4-ABP, dG-C8-AoC, dG-C8-PhIP.
- The y-axis shows the dG-C8 adduct (per 10^6 nucleotides) per nmol of substrate.

Chemical structures of dG-C8-4-ABP, dG-C8-AoC, and dG-C8-PhIP are depicted below the graph.

- dG-C8-4-ABP: Aromatic ring with a nitrogen atom at the 8-position.
- dG-C8-AoC: Aromatic ring with two nitrogen atoms at the 8-position.
- dG-C8-PhIP: Aromatic ring with a nitrogen atom and a methyl group at the 8-position.

The graph and chemical structures indicate the bioactivation of AoC under different pH conditions.
Figure 8
UGT bioactivation of AαC

Figure 9

- Relative Abundance
- Time (min)
- Total Ion Counts
- m/z: 376.1 > 183.1, 184.1, 200.1

- $A\alpha C\text{-O}^\text{I} \text{-Gl}$
  - $t_R$: 11.8
  - $A$: 7000687

- $A\alpha C\text{-O}^\text{II} \text{-Gl}$
  - $t_R$: 8.3
  - $A$: 1332997

- $A\alpha C\text{-HN}^\text{I} \text{-O} \text{-Gl}$
  - $t_R$: 14.3
  - $A$: 4117880

- $A\alpha C\text{-HN}^\text{II} \text{-O} \text{-Gl}$
  - $t_R$: 13.3
  - $A$: 170829

- $A\alpha C\text{-HO} \text{-N}^\text{II} \text{-Gl}$
  - $t_R$: 11.8
  - $A$: 7000687

- $A\alpha C\text{-HO} \text{-N}^\text{I} \text{-Gl}$
  - $t_R$: 8.3
  - $A$: 1332997

- $A\alpha C\text{-O} \text{-Gl}$
  - $t_R$: 14.3
  - $A$: 4117880

- $A\alpha C\text{-O} \text{-Gl}$
  - $t_R$: 11.8
  - $A$: 7000687

For 10 µM $A\alpha C$:

- Total Ion Counts

For 50 µM $A\alpha C$:

- Total Ion Counts