



HAL
open science

Metabolism of the Tobacco Carcinogen 2-Amino-9H-pyrido[2,3-b]indole (A α C) in Primary Human Hepatocytes

Medjda Bellamri, Ludovic Le Hegarat, Robert J Turesky, Sophie Langouët

► **To cite this version:**

Medjda Bellamri, Ludovic Le Hegarat, Robert J Turesky, Sophie Langouët. Metabolism of the Tobacco Carcinogen 2-Amino-9H-pyrido[2,3-b]indole (A α C) in Primary Human Hepatocytes. *Chemical Research in Toxicology*, 2017, 30 (2), pp.657-668. 10.1021/acs.chemrestox.6b00394 . anses-01419468

HAL Id: anses-01419468

<https://anses.hal.science/anses-01419468>

Submitted on 19 Dec 2016

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

1
2
3 **Metabolism of the Tobacco Carcinogen 2-Amino-9H-pyrido[2,3-b]indole**
4 **(A α C) in Primary Human Hepatocytes**
5
6
7

8
9 *Medjda Bellamri^{†‡}, Ludovic Le Hegarat[‡], Robert J. Turesky^{§*} and Sophie Langouët^{†*}*
10

11
12 † Institut National de la Santé et de la Recherche Médicale (Inserm), U1085, Institut de
13 Recherche en Santé Environnement et Travail (IRSET), Université de Rennes 1, UMS 3480
14 Biosit, F-35043 Rennes, France.
15
16

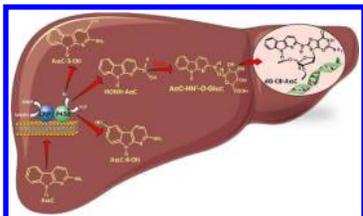
17
18 ‡ ANSES Laboratoire de Fougères, La Haute Marche-Javené, BP 90203, 350302 Fougères,
19 France.
20
21

22
23 § Masonic Cancer Center and Department of Medicinal Chemistry, Cancer and Cardiology
24 Research Building, University of Minnesota, 2231 6th Street, Minneapolis, MN 55455, USA
25
26

27
28 * Correspondence should be addressed to: Dr. Robert J. Turesky, Masonic Cancer Center and
29 Department of Medicinal Chemistry, Cancer and Cardiology Research Building, University
30 of Minnesota, 2231 6th Street, Minneapolis, MN 55455, USA. Tel: 612-626-0141; E-mail:
31 Rturesky@umn.edu, or Dr. Sophie Langouët, Institut National de la Santé et de la Recherche
32 Médicale (Inserm), U1085, Institut de Recherche en Santé Environnement et Travail
33 (IRSET), Université de Rennes 1, UMS 3480 Biosit, F-35043 Rennes, France. Tel: 02 23 23
34 48 06; E-mail: sophie.langouet@inserm.fr
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49

50 **KEYWORDS:** 2-amino-9H-pyrido[2,3-b]indole (A α C), primary human hepatocytes,
51 metabolism, P450 1A2, DNA adduct, UDP-glucuronosyltransferase.
52
53
54
55
56
57
58
59
60

Table of Content Figure



ABSTRACT

2-Amino-9*H*-pyrido[2,3-*b*]indole (AαC) is the most abundant carcinogenic heterocyclic aromatic amine (HAA) formed in mainstream tobacco smoke. AαC is a liver carcinogen in rodents, but its carcinogenic potential in humans is not known. To obtain a better understanding of the genotoxicity of AαC in humans, we have investigated its metabolism and its ability to form DNA adducts in human hepatocytes. Primary human hepatocytes were treated with AαC at doses ranging from 0.1 to 50 μM and the metabolites were characterized by UPLC/ ion trap multistage mass spectrometry (UPLC/MSⁿ). Six major metabolites were identified: a ring-oxidized doubly conjugated metabolite, *N*²-acetyl-2-amino-9*H*-pyrido[2,3-*b*]indole-6-yl-oxo-(β-D-glucuronic acid) (*N*²-acetyl-AαC-6-*O*-Gluc); two ring-oxidized glucuronide (Gluc) conjugates: 2-amino-9*H*-pyrido[2,3-*b*]indol-3-yl-oxo-(β-D-glucuronic acid) (AαC-3-*O*-Gluc) and 2-amino-9*H*-pyrido[2,3-*b*]indol-6-yl-oxo-(β-D-glucuronic acid) (AαC-6-*O*-Gluc); two sulfate conjugates, 2-amino-9*H*-pyrido[2,3-*b*]indol-3-yl sulfate (AαC-3-*O*-SO₃H) and 2-amino-9*H*-pyrido[2,3-*b*]indol-6-yl sulfate (AαC-6-*O*-SO₃H); and the Gluc conjugate, *N*²-(β-D-glucosiduronyl)-2-amino-9*H*-pyrido[2,3-*b*]indole (AαC-*N*²-Gluc). In addition, four minor metabolites were identified: *N*²-acetyl-9*H*-pyrido[2,3-*b*]indol-3-yl sulfate (*N*²-acetyl-AαC-3-*O*-SO₃H); *N*²-acetyl-9*H*-pyrido[2,3-*b*]indol-6-yl sulfate (*N*²-acetyl-AαC-6-*O*-SO₃H), *N*²-acetyl-2-amino-9*H*-pyrido[2,3-*b*]indol-3-yl-oxo-(β-D-glucuronic acid) (*N*²-acetyl-AαC-3-*O*-Gluc), and *O*-(β-D-glucosiduronyl)-2-hydroxyamino-9*H*-pyrido[2,3-*b*]indole (AαC-HN²-*O*-Gluc). The latter metabolite, AαC-HN²-*O*-Gluc is a reactive intermediate which binds to DNA to form the covalent adduct *N*-(2'-deoxyguanosin-8-yl)-2-amino-9*H*-pyrido[2,3-*b*]indole (dG-C8-AαC). Pre-incubation of hepatocytes with furafylline, a selective mechanism-based inhibitor of P450 1A2, resulted in a strong decrease in the formation of AαC-HN²-*O*-Gluc and a concomitant decrease in DNA adduct formation. Our findings describe the major pathways of metabolism of AαC in primary human hepatocytes

1
2
3 and reveal the importance of N-acetylation and glucuronidation in metabolism of A α C. P450

4
5 1A2 is a major isoform involved in the bioactivation of A α C to form the reactive A α C-HN²-

6
7
8 *O*-Gluc conjugate and A α C-DNA adducts.
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

INTRODUCTION

Epidemiologic studies conducted over the past two decades have consistently shown that smoking is a risk factor for liver and gastrointestinal tract cancer.¹⁻³ Cigarette smoking is a prominent source of exposure to a number of genotoxicants including nitrosamines, aromatic amines, polycyclic aromatic hydrocarbons, and heterocyclic aromatic amines (HAA).⁴ HAA are also formed in well-done cooked meat, poultry and fish,⁵ and some occur in diesel gas exhaust.^{6,7} 2-Amino-9*H*-pyrido[2,3-*b*]indole (AαC) is by far the most abundant HAA formed in tobacco smoke with a level range between 25 to 260 ng per cigarette.⁸⁻¹¹ These amounts are comparable to those of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), and 25-100 times higher than those of 4-aminobiphenyl (4-ABP) and benzo[*a*]pyrene (B[*a*]P),^{9,12,13} these three chemicals are recognized as human carcinogens.^{3,14}

AαC has been detected in urine of smokers of the Shanghai Cohort study.¹⁵ The urinary levels were positively correlated to the number of cigarettes smoked per day. A similar finding was reported in Zengshu, China, where AαC was present at higher levels in the urine of smokers than nonsmokers.¹⁶ Recently, a tobacco smoking cessation study conducted in the United States revealed that AαC was present in urine during the smoking phase in greater than 90% of the subjects, and the geometric mean urinary level of AαC decreased by 87% six weeks after cessation of tobacco usage.¹⁷ These urinary biomarker data demonstrate that tobacco smoking is a significant source of AαC exposure. The data reported in the literature on urinary level of AαC in human are restricted to two reports where the mean level of AαC in urine of subjects who smoked greater than 20 cigarette per day range between 12 and 35 pg/mg creatinine.¹⁵⁻¹⁷

1
2
3 AαC induces liver and blood vessel tumors in CDF1 mice,^{5,18} it induces *lacI* transgene
4 mutations in the colon of C57BL/6 mice,¹⁹ and aberrant crypt foci, an early biomarker of
5 colon neoplasia, in Big Blue mice.²⁰ However, the genotoxicity of AαC remains unknown in
6 humans and only few reports have been reported on the genotoxicity of AαC *in vitro* in
7 human cells. AαC is genotoxic in human lymphoblastoid cells (MLC-5)²¹ and in peripheral
8 blood lymphocytes cells, when assessed the by the comet and micronucleus assays.²² We also
9 showed that AαC forms high and persistent levels of DNA adducts in primary human
10 hepatocytes.^{23,24} The major DNA adduct formed by AαC is *N*-(deoxyguanosin-8-yl)-2-amino-
11 9*H*-pyrido[2,3-*b*]indole (dG-C8-AαC)^{25,26} which is regarded as a mutagenic lesion.²⁷ The
12 levels of dG-C8-AαC formed in primary human hepatocytes were greater than those dG-C8
13 adducts formed with other HAA, including 2-amino-1-methyl-6-phenylimidazo[4,5-
14 *b*]pyridine (PhIP), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), 2-amino-3-
15 methylimidazo[4,5-*f*]quinoline (IQ) or the structurally related arylamine 4-aminobiphenyl (4-
16 ABP).²⁴

17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35 AαC requires metabolism to exert its genotoxic effects. AαC undergoes metabolic
36 activation, by P450 1A2-catalyzed *N*-oxidation of the exocyclic amine group to form 2-
37 hydroxyamino-9*H*-pyrido[2,3-*b*]indole (HONH-AαC).^{28,29} Conjugation enzymes such as *N*-
38 acetyltransferases (NAT) or sulfotransferases (SULT), catalyze the conversion of HONH-
39 AαC to unstable esters, which undergo heterolytic cleavage to form the presumed short-lived
40 nitrenium ion, which covalently adducts to DNA.³⁰ In contrast to the NAT and SULT
41 enzymatic pathways of bioactivation, the UDP-Glucuronosyltransferases (UGT) are largely
42 viewed as conjugation pathways that lead to detoxication.³¹ UGT-mediated conjugation of
43 glucuronic acid (Gluc) to the exocyclic amino groups of HAA or *N*-hydroxylated HAA is a
44 detoxification pathway for HAA.³¹⁻³³ However, we discovered that the UGT also catalyze the
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3 *O*-glucuronidation of HONH-A α C to form the A α C-HN²-*O*-Gluc conjugate, a reactive
4
5 metabolite that contributes to the genotoxicity of A α C.³⁴
6
7

8
9 The abundance of A α C in tobacco smoke and its propensity to undergo bioactivation by
10
11 hepatic enzymes provides a plausible biochemical mechanism that may explain how A α C
12
13 can induce DNA damage and play a role in the development of liver and digestive tract
14
15 cancers in smokers.^{1,35,36} However stable biomarkers must be developed and implemented in
16
17 molecular epidemiological studies designed to assess the role of chemical exposures to A α C
18
19 in cancer risk. For this purpose, a better understanding of the metabolism of A α C is required.
20
21

22
23 Studies have been devoted to the metabolism of A α C *in vitro* with hepatic human liver
24
25 microsomes or recombinant human P450s^{29,30,37-39} and *in vivo* mainly in rats^{26,37,40} and mice.⁴¹
26
27 However, knowledge about the major metabolic pathways of A α C and the key enzymes
28
29 involved in bioactivation of this carcinogen in liver and extrahepatic tissues of humans are
30
31 limited. There is only one study reported the metabolism of A α C in human hepatocarcinoma
32
33 cell line HepG2.⁴⁰ This cell line is not fully metabolically competent since it does not express
34
35 some important metabolism enzymes such as UGT.⁴⁰ In contrast, primary human hepatocytes
36
37 are the gold standard to investigate different pathways of carcinogen metabolism, since this
38
39 cell model retains liver function for at least seven days and expresses phase I and phase II
40
41 enzymes as well as cofactors at physiological concentrations.⁴²
42
43
44
45

46
47 The aim of our study was to characterize the major metabolites of A α C formed in human
48
49 liver using primary cultured human hepatocytes. The metabolites were characterized by
50
51 UPLC/MSⁿ in conjunction with HPLC and ultraviolet detection, and by treatment with the
52
53 deconjugation enzymes arylsulfatase and β -glucuronidase. Our findings show that A α C
54
55 undergoes multiple pathways of metabolism that include *N*²-acetylation, *N*²-glucuronidation,
56
57
58
59
60

1
2
3 as well as ring-oxidation at the C-3 and C-6 atoms of the heterocyclic ring of A α C. The
4
5 metabolic activation of A α C through *N*-oxidation was shown to occur by formation of A α C-
6
7 HN²-*O*-Gluc, a genotoxic metabolite that reacts with DNA.³⁴ Preincubation of hepatocytes
8
9 with furafylline, a selective inhibitor of P450 1A2⁴³ resulted in a strong decrease in the
10
11 formation of A α C-HN²-*O*-Gluc with a concomitant decrease in DNA adducts. Our data
12
13 describes for the first time, the metabolism of A α C in human liver , and the importance of
14
15 P450 1A2 in formation of A α C-HN²-*O*-Gluc and DNA adducts.
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

MATERIALS AND METHODS

Caution. *AαC and its derivatives are potential human carcinogens. These chemicals must be handled in a well-ventilated fume hood with proper use of gloves and protective clothing.*

Chemicals. AαC was purchased from the Toronto Research Chemicals (Toronto, ON, Canada). [4b,5,6,7,8,8a-¹³C₆]AαC was a gift from Dr. Daniel Doerge, National Center for Toxicological Research (Jefferson, AR). Human liver microsomes were obtained from the Tennessee Donor Services, Nashville, TN, and kindly provided by Prof. F. P. Guengerich, Vanderbilt University. DMSO, ethoxyresorufin, methoxyresorufin, methanol, acetonitrile, ascorbic acid, hydrochloric acid, ammonium acetate, sulfatase from *Helix pomatia* (≥10,000 units/g solid) and β-Glucuronidase from *Helix pomatia* (≥30,000 units/g solid) were purchased from Sigma Aldrich (St. Louis, MO, USA). AαC-3-OH and AαC-6-OH were prepared with human liver microsomes or rat liver microsomes and spectroscopically characterized as previously reported.^{17,34} *N*-(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.²⁷

Cell Isolation, Culture, and Treatment. Human liver samples were obtained from patients undergoing liver resection for primary or secondary hepatomas through the Centre de Ressources Biologiques (CRB)-Santé of Rennes (<http://www.crbsante-rennes.com>) The demographics information is provided in supporting information (Table S1). The research protocol was conducted under French legal guidelines and fulfilled the requirements of the local institutional ethics committee. Hepatocytes were isolated by a two-step collagenase perfusion procedure and seeded in petri dishes at a density of 3×10⁶ viable cells/19.5 cm² dish, in William's medium (Gibco, Life technologies, Carlsbad, CA, USA), supplemented by bovine serum albumin (1 g/L) (Life technologies), glutamine (2 mM) (Life technologies), bovine insulin (5 µg/mL) (Life technologies), penicillin (10 U/mL) (Life technologies),

1
2
3 streptomycin (10 $\mu\text{g}/\text{mL}$) (Life technologies) and 10% fetal calf serum (v/v) (Life
4 technologies). After 18 h of cell seeding, the media was replaced with cell media lacking fetal
5 calf serum and containing hydrocortisone hemisuccinate (54 μM) (Laboratoire SERB, Paris,
6 France). After 36 h of culture, the differentiated cells were incubated with A α C in DMSO
7 (0.1% v/v) for 24 h. At the end of treatment, the supernatants were collected and the cells
8 were washed with PBS. Cell pellets were collected by centrifugation at 3500 g for 10 min at 4
9 $^{\circ}\text{C}$. Both supernatants and cell pellets were stored at -80 $^{\circ}\text{C}$ until further use. To assess the
10 role of P450 1A2 in the metabolism of A α C, the cells were pre-treated with furafylline (5
11 μM) or 0.1% DMSO (v/v) for 24 h. The media was then renewed with furafylline (5 μM) or
12 0.1% DMSO (v/v), and the cells were incubated with A α C (0.1, 1, 10 or 50 μM) for an
13 additional 24 h. At the end of each time point, the cell media was collected for metabolite
14 analysis and the cellular pellets were collected after PBS washing for DNA adducts
15 measurements.

16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33 **EROD/MROD Activity.** Ethoxyresorufin O-deethylase (EROD) and methoxyresorufin O-
34 demethylase (MROD) activities associated with P450 1A1/2⁴⁴ and P450 1A2,⁴⁵ respectively,
35 were measured in all primary cultured hepatocytes used in this study as described
36 previously.²⁴ The reaction rates were linear over the reaction time and proportional to protein
37 concentration.⁴⁶

38
39
40
41
42
43
44
45 **Samples preparation and HPLC analysis of the major metabolites of A α C.** Cell media
46 was treated by 3 volumes of cold methanol and incubated on ice for 30 min, followed by a
47 centrifugation at 20 000 g for 10 min at 4 $^{\circ}\text{C}$. The supernatant was collected and evaporated
48 to dryness, and the extracts were dissolved in HPLC grade water. The metabolites were
49 separated and collected with an Agilent model 1100 HPLC Chemstation (Palo Alto, CA)
50 equipped with UV/Vis detector. The metabolites were separated with Aquasil C18 column
51
52
53
54
55
56
57
58
59
60

1
2
3 (4.6 x 250 mm, 5 μ m particle size) from Thermo Scientific (Bellefonte, PA). The
4
5 chromatography commenced isocratically at 95% A solvent (20 mM ammonium acetate and
6
7 5% acetonitrile) for 10 min, followed by a linear gradient over 20 min to 50% B (acetonitrile)
8
9 at a flow rate of 1 mL/min. The column was then washed with 100% acetonitrile and re-
10
11 equilibrated at starting solvent conditions. Once collected, the metabolites were evaporated to
12
13 dryness and stored at -80 °C until LC/MS analysis.

14
15
16
17 **UV Spectral Characterization of A α C metabolites by treatment with deconjugating**
18 **enzymes or acid hydrolysis.** The proposed ring oxidized sulfate conjugates (A α C-3-*O*-SO₃H
19 and A α C-6-*O*-SO₃H) and the ring oxidized Gluc conjugates (A α C-3-*O*-Gluc and A α C-6-*O*-
20 Gluc) were hydrolyzed, respectively with sulfatase or β -glucuronidase. The metabolites
21 purified by HPLC (~25 ng) were concentrated to dryness by vacuum centrifugation,
22 resuspended in 25 μ L of 50 mM potassium phosphate buffer (pH 7.0) and incubated with 25
23 μ L of sulfatase (200 units/mL) or 25 μ L of β -glucuronidase (300 units/mL) for 1 h at 37 °C.
24 The enzymes solutions and buffers were purged with argon before use, and the incubations
25 were conducted in tightly closed Eppendorf tubes to minimize oxidation of the deconjugated
26 metabolites. The hydrolysis was terminated by the addition of 1 volume of ice-cold methanol.
27 The precipitated proteins were removed by centrifugation and the supernatants were analyzed
28 by HPLC as described above. The proposed *N*-acetylated ring-oxidized A α C Gluc conjugate
29 (*N*²-acetyl-A α C-6-*O*-Gluc) was subjected to enzymatic hydrolysis by β -glucuronidase under
30 the same conditions as described above, followed by acid hydrolysis (0.1 M HCl) for 3 h at
31 60 °C. After addition of 1 volume of ice-cold methanol and incubation for 5 min on ice, the
32 precipitated protein was eliminated by centrifugation. The supernatant was dried and re-
33 suspended in HPLC grade water. The hydrolysis products were analyzed by HPLC as
34 described above. The UV spectra of each hydrolysis products was compared to those spectra
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3 of A α C-6-OH and A α C-3-OH, which were produced with human liver microsome as
4
5 previously reported.^{34,41}
6
7

8 **Solid-phase extraction (SPE) of hepatocyte metabolites for UPLC-ESI-MSⁿ.** The cell
9
10 media containing A α C metabolites were processed by SPE using Oasis HLB 1 cc (30 mg)
11
12 cartridge (Waters, Milford, MA, USA), prior to UPLC-ESI-MSⁿ analysis. After the
13
14 conditioning the cartridge with 1 mL of methanol, followed by 1 mL of LC/MS grade water,
15
16 the cell media (100-500 μ L) diluted in 2 mM ammonium acetate (added up to 1 mL) were
17
18 loaded in the cartridge. The cartridge was washed with 1 mL of 2 mM ammonium acetate,
19
20 and metabolites were eluted with 1 mL of methanol. The eluates were evaporated to dryness
21
22 by vacuum centrifugation at 42 °C. The residues were dissolved in mobile phase buffer (2
23
24 mM ammonium acetate).
25
26
27

28
29 **UPLC-ESI-MSⁿ analysis.** Ultraformance liquid chromatography electrospray ionization
30
31 mass spectrometry (UPLC-ESI-MS) analyses was performed with NanoAcquity UPLC
32
33 system (Waters Corp., New Milford, MA) equipped with a Waters Symmetry C18 Trap
34
35 Column (180 μ m x 20 mm, 5 μ m particle size) (Waters Corp., New Milford, MA) and an
36
37 Advance CaptiveSpray ion source (Michrom Bioresources, Auburn, CA) interfaced with a
38
39 linear quadrupole ion trap mass spectrometer (LTQ Velos, Thermo Fisher, San Jose, CA).
40
41 Solvent A was 2 mM ammonium acetate and solvent B was 95% acetonitrile and 5% H₂O.
42
43 After 3 min at 5% of B, a linear gradient was employed, starting at 5% B, arriving at 50% B
44
45 in 20 min and ended at 99% B at 25 min at a flow rate of 5 μ L/min. The transitions were as
46
47 follow: A α C-*O*-SO₃H ([M+H]⁺ at m/z 280.1 > 200.1 >); A α C-*O*-Gluc ([M+H]⁺ at m/z
48
49 376.1 > 200.1 >); *N*²-acetyl-A α C-*O*-SO₃H ([M+H]⁺ at m/z 322.1 > 241.1 >); *N*²-acetyl-
50
51 A α C-*O*-Gluc ([M+H]⁺ at m/z 418.1 > 242.1 >); A α C-*N*²-Gluc ([M+H]⁺ at m/z 360.1 > 183.1
52
53
54
55
56
57
58
59
60

1
2
3 >); A α C-HN²-O-Gluc ([M+H]⁺ at *m/z* 376.1 > 200.1 >); A α C ([M+H]⁺ at *m/z* 184.1 > 167.1
4
5 >).
6
7

8 **Isolation and Digestion of DNA for Adduct Measurements.** Cell pellets containing
9 3 \times 10⁶ primary human hepatocytes were homogenized in 400 μ L of TE buffer at pH 8.0 (50
10 mM Tris-HCl and 10 mM EDTA) and incubated with RNase T1 (319 U) and RNase A (20
11 μ g) for 30 min at 37 °C. Then proteinase K (200 μ g) and SDS (0.05% final concentration)
12 were added and the mixture was incubated for 1 h at 37 °C. The DNA was purified by the
13 phenol/chloroform extraction, followed by precipitation with ethanol.⁴⁷ DNA was
14 resuspended in 200 μ L of sterile water and quantified with a NanoDrop 1000
15 Spectrophotometer (Thermo Fisher Scientific). Each DNA sample was spiked with
16 isotopically labeled internal standards at a level of 1 adduct per 10⁷ bases. DNA digestion
17 was performed in 5 mM Bis-Tris-HCl buffer (pH 7.1). DNA was incubated for 3.5 h with
18 DNase I and Nuclease P1 at 37 °C followed by 18 h incubation with alkaline phosphatase and
19 phosphodiesterase at 37 °C.⁴⁸ After vacuum centrifugation to the dryness, digested DNA was
20 resuspended in 30 μ L of 1:1 water/DMSO (v/v) and sonicated for 5 min. Samples were then
21 centrifuge for 5 min at 21 000 g and the supernatant was transferred to LC vials.
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39

40 **UPLC/MS³ measurement of DNA adducts.** DNA adduct measurements were performed
41 with the NanoAcquity UPLC system (Waters Corp, New Milford, MA) equipped with a
42 Waters Symmetry trap column (180 μ m \times 20 mm, 5 μ m particle size), a Michrom C18 AQ
43 column (0.3 mm \times 150 mm, 3 μ m particle size), and a Michrom Captive Spray source
44 interfaced with LTQ Velos. Chromatographic conditions were described previously.²⁴ The
45 ions were monitored at the MS³ scan stage as follows: dG-C8-A α C (*m/z* 449.1 > 333.1 >
46 209.2, 291.4, 316.4); [¹³C₁₀]-dG-C8-A α C (*m/z* 459.1 > 338.1 > 210.2, 295.5, 321.5).
47
48
49
50
51
52
53
54
55
56
57
58
59
60

RESULTS

Identification of the major metabolites of A α C formed in human hepatocytes. Six major metabolites were produced by incubating primary human hepatocytes with of A α C (50 μ M) and arbitrarily named M1 to M6 as function of the order of their elution time by HPLC (Figure 1). All of the metabolites retained the characteristic UV chromophore of A α C with slight changes in the spectral properties. The metabolites were collected and infused into the LTQ Velos ion trap MS for characterization.

ESI-MS product ion and UV spectra of A α C metabolites. The product ion spectrum of A α C-*N*²-Gluc ([M+H]⁺ at *m/z* 360.1) (M5) at MS² stage, shown in Figure 2A, displays fragment ions at *m/z* 280.1 ([M+H-2H₂O-CO₂]⁺), *m/z* 226.1 ([M+H-C₄H₆O₅]⁺) and at *m/z* 184.1 ([M+H-C₆H₈O₆]⁺). The latter two fragment ions are attributed to the positively charged acetyl derivative of A α C and the protonated A α C. These results are consistent with a previous study.⁴⁹

The two A α C-*O*-Gluc isomers (M2 and M3) are readily distinguished by their product ion spectra at MS³ scan stage. The product ion spectrum of A α C-6-*O*-Gluc ([M+H]⁺ at *m/z* 376.1) (M2) at MS³ scan stage displays two fragments ions: one ion at *m/z* 200.1 ([M+H-C₆H₈O₆]⁺) attributed to the positively charged A α C-6-OH, which formed by the cleavage of the Gluc moiety, and the second ion at *m/z* 183.0 ([M+H-C₆H₈O₆-NH₃]⁺) is attributed to the loss of NH₃ from A α C-6-OH (Figure 2B). In the case of A α C-3-*O*-Gluc, ([M+H]⁺ at *m/z* 376.1) (M3) the fragment ions at *m/z* 200.1 and *m/z* 183.0 are also observed, in addition to a prominent fragment ion at *m/z* 155.1 ([M+H-C₆H₈O₆-NH₃-CO]⁺), which is proposed to occur by the successive loss of NH₃ and CO from A α C-3-OH (Figure 2C). The ion at *m/z* 155.1 is a prominent product ion of A α C-3-OH, but not for A α C-6-OH, when these hydroxylated A α C derivatives undergo CID with a triple quadrupole MS, or by ion trap at the MS³ scan stage

(unpublished results, R. Turesky). The differences in the pattern of fragmentation of A α C-*O*-Gluc isomers lead us to tentatively assign M3 as A α C-3-*O*-Gluc and M2 as A α C-6-*O*-Gluc.

The hydrolysis products of M2 and M3 obtained by treatment with β -glucuronidase showed that M2 co-eluted with A α C-6-OH and displayed an UV spectrum identical to that of A α C-6-OH, whereas the hydrolysis product of M3 co-eluted with A α C-3-OH and exhibited the same UV spectrum as A α C-3-OH (Figure S1). The A α C-6-OH and A α C-3-OH reference compounds produced by human liver microsomes were characterized by $^1\text{H-NMR}$, which unambiguously identified the sites of ring-oxidation of A α C.^{17,34} Taken together these results lead us to identify M2 as A α C-6-*O*-Gluc and M3 as A α C-3-*O*-Gluc.

As was observed for the *O*-Gluc conjugates, the isomeric sulfate conjugates can be distinguished by their product ion spectra. The product ion spectrum of A α C-6-*O*-SO₃H (M6) ($[\text{M}+\text{H}]^+$ at m/z 280.0) at MS³ stage displays two fragments ions at m/z 200.0 ($[\text{M}+\text{H}-\text{SO}_3]^+$) and m/z 183.0 ($[\text{M}+\text{H}-\text{SO}_3-\text{NH}_3]^+$) (Figure 2D). The product ion spectrum of A α C-3-*O*-SO₃H (M4) ($[\text{M}+\text{H}]^+$ at m/z 280.0) at MS³ stage also displays the fragment ions at m/z 200.1 and 183.0, and a major fragment ion at m/z 155.0 ($[\text{M}+\text{H}-\text{SO}_3-\text{NH}_3-\text{CO}]^+$) attributed to the loss of NH₃ and CO from A α C-3-OH (Figure 2E). These results lead to the tentative identification of M4 as A α C-6-*O*-SO₃H and M6 as A α C-3-*O*-SO₃H. The sulfatase hydrolysis product of M4 and M6 co-eluted by HPLC and exhibited the characteristic UV spectra of A α C-3-OH and A α C-6-OH, respectively support the assignment of M4 as A α C-6-*O*-SO₃H and M6 as A α C-3-*O*-SO₃H (Figure S1).

The product ion spectrum of *N*²-acetyl-A α C-6-*O*-Gluc (M1) ($[\text{M}+\text{H}]^+$ at m/z 418.1) at MS³ scan stage is shown in Figure 3A. A major fragment ion is observed at m/z 200.1 ($[\text{M}+\text{H}-\text{C}_6\text{H}_8\text{O}_6-\text{C}_2\text{H}_3\text{O}]^+$) attributed to the cleavage of the Gluc and the acetyl linkages. Fragment ions at m/z 183.1 ($[\text{M}+\text{H}-\text{C}_6\text{H}_8\text{O}_6-\text{C}_2\text{H}_3\text{O}-\text{NH}_3]^+$) and m/z 172.1 ($[\text{M}+\text{H}-\text{C}_6\text{H}_8\text{O}_6-$

1
2
3 C₂H₃O-CO]⁺) are also observed. A fragment ion at *m/z* 155.1 attributed to the successive loss
4
5 of NH₃ and CO from protonated AαC-6-OH was also observed. However, this ion is
6
7 relatively minor in abundance. The position of oxidation and identification of the metabolite
8
9 as *N*²-acetyl-AαC-6-*O*-Gluc was determined by comparing the UV spectrum of hydrolysis
10
11 product to those UV spectra of AαC-6-OH and AαC-3-OH. The *N*²-acetyl-AαC-6-*O*-Gluc
12
13 hydrolysis product (enzymatic hydrolysis using β-glucuronidase followed by acid hydrolysis)
14
15 co-eluted and exhibited an UV spectrum identical to that of AαC-6-OH, whereas the UV
16
17 spectrum and retention time of AαC-3-OH were different. These data confirm the
18
19 identification of M1 as *N*²-acetyl-AαC-6-*O*-Gluc (Figure 3B).
20
21
22
23

24
25 **Characterization of minor metabolites of AαC in human hepatocytes.** We assessed the
26
27 metabolite formation in primary human hepatocytes treated with various concentrations of
28
29 AαC (0.1, 1, 10 and 50 μM) over 24 h followed by UPLC/MSⁿ analysis. In addition to the
30
31 previously described metabolites, several minor metabolites were identified. There were two
32
33 *N*-acetylated ring-oxidized AαC sulphate conjugates (*N*²-acetyl-AαC-6-*O*-SO₃H, *N*²-acetyl-
34
35 AαC-3-*O*-SO₃H), one *N*-acetylated ring-oxidized AαC Gluc conjugate (*N*²-acetyl-AαC-3-*O*-
36
37 Gluc) and the genotoxic metabolite, AαC-HN²-*O*-Gluc (Figure 4).
38
39
40

41
42 The product ion spectra of the two *N*²-acetyl-AαC-*O*-SO₃H conjugates ([M+H]⁺ at *m/z*
43
44 322.1) at the MS³ scan stage revealed a prominent fragment ion at *m/z* 224.1 ([M+H-
45
46 H₂SO₄]⁺) which occurs by cleavage of the sulfate linkage. In addition, fragment ions were
47
48 observed at *m/z* 200.1 ([M+H-SO₃-C₂H₃O]⁺) attributed to the loss of SO₃ and the acetyl
49
50 linkage to form a protonated AαC-OH species (Figure 4A and 4B). We were unable to
51
52 distinguish the identities of these two isomers based on mass spectral data; however, based on
53
54 their polarity and the fact that the retention time for the AαC-6-*O*-SO₃H metabolite was
55
56 characteristically shorter than that of AαC-3-*O*-SO₃H, the metabolite observed at *t*_R 15.2 min
57
58
59
60

1
2
3 was tentatively assigned as *N*²-acetyl-AαC-6-*O*-SO₃H and the metabolite observed at *t*_R 16.9
4
5 min assigned as *N*²-acetyl-AαC-3-*O*-SO₃H (Figure 5).
6
7

8
9 In the case of *N*²-acetyl-AαC-3-*O*-Gluc ([M+H]⁺ at *m/z* 418.1), The product ion spectra at
10
11 MS³ stage shows a fragment ion at *m/z* 224.1 ([M+H-C₆H₈O₆-H₂O]⁺), which is attributed to
12
13 the cleavage of the Gluc linkage and the loss of water. In addition, we observed a fragment
14
15 ion at *m/z* 200.1 attributed to the protonated AαC-3-OH resulting from the concomitant
16
17 cleavages of the Gluc and the acetyl moieties (Figure 4D). Based on its polarity and its longer
18
19 retention time in comparison to its *N*²-acetyl-AαC-6-*O*-Gluc isomer described above, we
20
21 tentatively identified this metabolite as *N*²-acetyl-AαC-3-*O*-Gluc (Figure 5).
22
23
24

25
26 The product ion spectrum of the previously reported AαC-HN²-*O*-Gluc ([M+H]⁺ at *m/z*
27
28 376.0) at MS³ stage shown in Figure 4C displays two fragment ions: the ion at *m/z* 184.0
29
30 ([M+H-C₆H₈O₇]⁺) is attributed to the positively charged AαC, which is formed by the
31
32 cleavage of the Gluc linkage. The ion at *m/z* 167.0 ([M+H-C₆H₈O₇-NH₃]⁺) is attributed to the
33
34 loss of NH₃ from the protonated AαC. The online LC-ESI-MSⁿ consecutive reaction product
35
36 ion spectra acquired in the negative mode of AαC-HN²-*O*-Gluc [M-H]⁻ at *m/z* 374.1 at the
37
38 MS² and MS³ scan stages support the proposed structure with an *O* linkage formed between
39
40 the HONH-AαC and Gluc. The base peak ion in the product ion spectrum at the MS² scan
41
42 stage was observed at *m/z* 193.0 [M-H-AαC]. The second generation product ion spectrum of
43
44 AαC-HN²-*O*-Gluc acquired on *m/z* 193.0 shows the typical CID fragmentation pattern
45
46 previously reported for the glucuronate,⁵⁰ and proved that the linkage formed between the
47
48 glucuronic acid and HONH-AαC occurred through the oxygen atom of HONH-AαC (Figure
49
50 S2). These spectral data are consistent with the published data on the metabolite produced by
51
52 recombinant UGTs.³⁴
53
54
55
56
57
58
59
60

1
2
3 In addition to these fully characterized metabolites, we observed the formation of several
4 other minor metabolites, which are isomers of those characterized above and include two N^2 -
5 acetyl-A α C-*O*-Gluc, one N^2 -acetyl-A α C-*O*-SO₃H, and one A α C-*N*-Gluc.
6
7
8

9
10 **A α C metabolism at low concentrations in human hepatocytes.** The biotransformation of
11 A α C was examined as a function of concentration in primary human hepatocytes treated with
12 0.1, 1 and 10 μ M of A α C. As shown in Figure 6, the concentration of A α C did not greatly
13 impact its metabolism; indeed the patterns of metabolites formed were comparable at all of
14 the concentrations studied. Based on the total ion counts of each metabolite in positive ion
15 mode, the N^2 -acetyl-A α C-6-*O*-Gluc is the most prominent metabolite, followed by the two
16 ring-oxidized A α C glucuronide conjugates (A α C-3-*O*-Gluc and A α C-6-*O*-Gluc) and the two
17 ring-oxidized A α C sulfate conjugates (A α C-3-*O*-SO₃H and A α C-6-*O*-SO₃H). However, these
18 estimates are approximate as the ionization efficiencies, particularly those of the sulfate
19 conjugates in the positive ion mode, may result in underestimation of product formation. The
20 findings show that the ring oxidation of C3 and C6 position constitutes a major pathway of
21 metabolism of A α C. It is noteworthy that the formation of the genotoxic metabolite A α C-
22 HN²-*O*-Gluc occurred at all the concentrations studied.
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40

41 **Role of P450 1A2 in A α C metabolism and DNA adduct formation in human**
42 **hepatocytes.** Human P450 1A2 is well recognized as the major isoform responsible for
43 bioactivation of a number of HAA.^{29,51-53} The role of P450 1A2 in the formation of A α C
44 metabolites in hepatocytes was assessed with furafylline, a selective, mechanism-based P450
45 1A2 inhibitor.⁴³ In our experimental conditions, we observe that 24 h of treatment with 5 μ M
46 of furafylline leads to more than 80% decrease of P450 1A2 activity (date not shown). As
47 shown in Figure 7, the pre-incubation of human hepatocytes with furafylline resulted in a
48 strong decrease in the levels of A α C-HN²-*O*-Gluc with a concomitant increase in the amount
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3 of unmetabolized A α C and A α C-*N*²-Gluc. These results demonstrate that P450 1A2 is the
4
5 major isoform involved in the metabolism of A α C and formation of A α C-HN²-*O*-Gluc. In
6
7 addition, we observed that P450 1A2 inhibition lead to a decrease in the levels of the two
8
9 ring-oxidized A α C sulfate conjugates (A α C-3-*O*-SO₃H and A α C-6-*O*-SO₃H), the two ring-
10
11 oxidized A α C glucuronide conjugates (A α C-3-*O*-Gluc and A α C-6-*O*-Gluc) and the two *N*-
12
13 acetylated A α C-6-OH conjugates (*N*²-acetyl-A α C-6-*O*-Gluc and *N*²-acetyl-A α C-6-*O*-SO₃H).
14
15 In contrast, the relative abundance of the *N*²-acetyl-A α C-3-*O*-SO₃H and *N*²-acetyl-A α C-3-*O*-
16
17 Gluc were increased by the inhibition of P450 1A2. Thus, other P450s may have catalysed
18
19 the C-3 oxidation *N*²-acetyl-A α C. The potency of furafylline P450 1A2 mediated inhibition
20
21 was most pronounced when A α C was incubated at low substrate concentrations, indicating
22
23 that other P450s²⁹ or oxidases¹⁷ contributed to the metabolism of A α C at higher substrate
24
25 concentrations.
26
27
28
29
30

31 The levels of dG-C8-A α C were measured in conjunction with the amounts of A α C-HN²-*O*-
32
33 Gluc formed in human hepatocytes treated with several doses of A α C (0.1, 1 and 10 μ M) in
34
35 the presence or absence of furafylline. As shown in Figure 8, A α C-HN²-*O*-Gluc and dG-C8-
36
37 A α C were formed in a dose-dependent manner. The inhibition of P450 1A2 lead to a 70 -
38
39 80% decrease in the amount of A α C-HN²-*O*-Gluc and dG-C8-A α C adduct levels. These
40
41 results signify that P450 1A2 is the major isoform involved in the bioactivation of A α C in
42
43 human hepatocytes, to form genotoxic metabolites, including A α C-HN²-*O*-Gluc, and dG-C8-
44
45 A α C adduct formation. A representative UPLC-ESI/MS³ chromatogram and product ion
46
47 spectra of DNA adduct are shown in supplementary data (Figure S3). The product ion
48
49 spectrum is in excellent agreement to our previously published data²⁷.
50
51
52
53
54
55
56
57
58
59
60

DISCUSSION

The aim of this study was to characterize the major metabolites of A α C formed in primary human hepatocytes using UPLC/MSⁿ. Our data demonstrate that A α C is subjected to direct conjugation reactions such as *N*-glucuronidation, but also double and triple biotransformation reactions by the combination of *N*-oxidation or ring-oxidation followed by *O*-sulfonation, *O*-glucuronidation, as well as *N*-acetylation. The amounts of *N*-acetyl-A α C were extremely low in hepatocytes, indicating that this metabolite undergoes further metabolism (unpublished data, R. Turesky). We detected 10 stable metabolites including six major metabolites: A α C-3-*O*-SO₃H, A α C-6-*O*-SO₃H, A α C-3-*O*-Gluc, A α C-6-*O*-Gluc, *N*²-acetyl-A α C-6-*O*-Gluc and A α C-*N*²-Gluc, and four minor products: *N*²-acetyl-A α C-6-*O*-Gluc, *N*²-acetyl-A α C-6-*O*-SO₃H, *N*²-acetyl-A α C-3-*O*-SO₃H and A α C-HN²-*O*-Gluc.

Ring oxidation of A α C at the C-3 and C-6 atoms of the heterocyclic ring are the major pathways of A α C metabolism in human hepatocytes. These oxidation pathways are similar to the data reported by Raza et al. using human liver microsomes, where three oxidized products of A α C were identified: one *N*-oxidized product (HONH-A α C) and two ring-oxidized products (A α C-3-OH and A α C-6-OH).²⁹ The ratio of ring-oxidized products to *N*-oxidized product was estimated to be 85:15 and was relatively constant across the several human liver microsomal preparations.²⁹ Since A α C-3-OH and A α C-6-OH possess no mutagenic activities,^{28,29} the predominant routes for A α C metabolism in human hepatocytes appear as detoxication pathways. However, A α C-3-*O*-SO₃H also can be produced by the rearrangement of the genotoxic *N*-sulfate ester of HONH-A α C.¹⁷ Moreover, A α C undergoes metabolism to produce higher levels of DNA adducts than 4-ABP, PhIP or MeIQx in human hepatocytes.^{23,24} Therefore, DNA adducts are important biomarkers to measure when characterizing procarcinogen metabolism in cell systems. Although A α C undergoes extensive

1
2
3 metabolism by N^2 -acetylation and oxidation, we have not yet detected DNA adducts of A α C
4
5 that possesses an *N*-acetyl group (unpublished observations, R. Turesky) as was reported for
6
7 the structurally related aromatic amine, 2-aminofluorene.^{54,55}
8
9

10
11 There are large interspecies differences in metabolism of A α C in human hepatocytes
12 compared to those previously reported *in vivo* in rats.⁴⁰ The four major metabolites of A α C
13 identified in the bile and urine of adult male Sprague-Dawley rats treated intravenously with
14 A α C were: A α C-3-*O*-SO₃H, N^2 -acetyl-A α C-3-*O*-SO₃H, N^2 -acetyl-A α C-6-*O*-SO₃H, N^2 -
15 acetyl-A α C-3-*O*-Gluc.⁴⁰ The latter three metabolites were produced in relatively minor
16 amounts in primary human hepatocytes. This discrepancy may be explained, in part by the
17 different catalytic activities and regioselectivity of human and rat enzymes orthologues
18 toward this procarcinogen. We previously reported interspecies differences in metabolism of
19 two other HAA, PhIP and MeIQx in rodent and human hepatocytes.^{52,53} Indeed, the catalytic
20 activity of human P450 1A2 was 9 to 11 fold higher than rat P450 1A2 in the *N*-oxidation of
21 MeIQx and PhIP,^{53,56} and human P450 1A2 catalyzed the *N*-oxidation as the major pathway
22 of metabolism whereas ring oxidation of these HAA was negligible.^{52,53,57,58} The
23 biotransformation of A α C was also previously investigated in the human liver HepG2 cell
24 line, where four major metabolites were identified: A α C-3-*O*-SO₃H, A α C-6-*O*-SO₃H, N^2 -
25 acetyl-A α C-6-*O*-SO₃H and N^2 -acetyl-A α C.⁴⁰ The absence of Gluc metabolites is not
26 surprising since this cell line is devoid of UGT.^{40,59} Taken together, these metabolic data
27 show that rats and HepG2 cell lines do not fully capture the metabolism of A α C that occurs
28 in human hepatocytes.
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50

51
52 The genotoxicity of A α C is dependent upon *N*-oxidation of A α C. *In vitro* studies
53 conducted with human liver microsomes or recombinant human P450s reveal that P450 1A2
54 is the primary isoform involved in *N*-oxidation and bioactivation of A α C.^{26,29} We previously
55
56
57
58
59
60

1
2
3 reported that P450 1A2 is the primary isoform involved in *N*-oxidation of A α C in primary
4 human hepatocytes and that the catalytic activity of P450 1A2 is critical for A α C derived
5 DNA adduct formation.²³ In this current study, we demonstrate that P4501A2 is also the
6 major isoform involved in metabolism of A α C in human hepatocytes, by use of furafylline, a
7 selective inhibitor of P450 1A2.⁴³ There is a strong correlation among P450 1A2 activity,
8 A α C-HN²-*O*-Gluc and dG-C8-A α C formation in human hepatocytes. A α C-HN²-*O*-Gluc is a
9 biologically reactive metabolite that binds to DNA,^{34,60} however, other reactive intermediates
10 such as the *N*-sulfooxy- or *N*-acetoxy esters of A α C also bind to DNA.^{30,61,62} In contrast to
11 the *N*-sulfooxy- or *N*-acetoxy esters of A α C, which are labile, A α C-HN²-*O*-Gluc is
12 sufficiently stable and can be isolated and measured.^{61,62} NAT1 and NAT2 and SULT1A2
13 catalyse the DNA binding of HONH-A α C to DNA *in vitro*.³⁰ The relative contributions of
14 UGT, NAT, SULT or other conjugating enzymes in the metabolic activation of A α C in
15 humans remain to be determined. Pretreatment of hepatocytes with furafylline also strongly
16 decreased the levels of sulfate and Gluc conjugates of A α C-3-OH and A α C-6-OH, but the *N*-
17 acetylated derivatives of these metabolites increased when cells were pretreated with
18 furafylline. These findings suggest that *N*-acetyl-A α C undergoes C-3 and C-6 oxidation by
19 P450s other than P4501A2.
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41

42 Recently, some oxidative products of A α C, including A α C-3-*O*-SO₃H, were detected in
43 plasma of liver specific P-450 reductase null mice, suggesting that a non-hepatic P450
44 enzymes catalysed oxidation of A α C in the mouse model.⁴¹ The level of DNA adduct
45 formation of A α C in liver was also unaffected in the liver specific P-450 reductase null mice
46 treated with high doses of A α C.⁴³ Our data show that the contribution of non-P450 oxidases
47 to the metabolism of A α C and bioactivation in human hepatocytes is minor.
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3 In summary, human hepatocytes extensively metabolize A α C into more than ten products
4 and also produce high levels of DNA adducts. Some of the metabolites of A α C may be used
5 as biomarkers of exposure to A α C in molecular epidemiological studies since A α C is
6 extensively metabolized in humans.^{17,63} A α C-3-OSO₃H has already been employed as a
7 urinary biomarker and frequently detected in the urine of smokers.¹⁷ The development of
8 A α C-HN²-O-Gluc as a urinary biomarker could provide valuable information about the
9 extent of bioactivation of A α C in humans. Given the high levels of A α C in mainstream
10 tobacco smoke,⁸⁻¹¹ and the relatively high levels of A α C DNA adducts formed in human
11 hepatocytes compared to other HAA or aromatic amines,^{23,24} further studies on the potential
12 role of A α C in tobacco-associated liver and gastrointestinal cancers are warranted.
13
14
15
16
17
18
19
20
21
22
23
24

25 26 **Funding Sources**

27
28
29 This work was supported by Inserm, la Ligue contre le Cancer, the PNREST Anses, Cancer
30 TMOI AVIESAN, 2013/1/166 (SL), and in part by National Cancer Institute of the National
31 Institutes of Health grant RO1CA134700 (R.J.T) and R01CA134700-S (R.J.T) of the Family
32 Smoking Prevention and Tobacco Control Act, and in part by National Cancer Institute
33 Cancer Center Support Grant No. CA077598 (R.J.T).
34
35
36
37
38
39
40
41
42

43 **ACKNOWLEDGEMENT**

44
45
46 MB was a recipient of Anses and UFR Pharmacie of University of Rennes 1 as well as travel
47 fellowships from Université Européenne de Bretagne
48
49
50

51 **SUPPORTING INFORMATION**

52
53
54 Regioisomer characterization of A α C-6-O-Gluc, A α C-3-O-Gluc, A α C-6-O-SO₃H and A α C-
55 3-O-SO₃H. LC/MS² product ion mass spectra of A α C-HN²-O-Gluc in negative ion mode.
56
57
58
59
60

1
2
3 Second generation product ion spectrum of A α C-HN²-O-Gluc acquired on *m/z* 193 in
4
5 negative ion mode. UPLC-ESI/MS³ chromatograms of dG-C8-A α C formed in primary
6
7 human hepatocytes with or without inhibition of P4501A2 with furafylline. Primary human
8
9 hepatocytes were pre-treated for 24 h with 0.1 % DMSO, or furafylline (5 μ M) followed by
10
11 24 h of treatment with 0.1 % DMSO (Ctrl), or A α C (0.1, 1 or 10 μ M). Donor information.
12
13
14 This material is available free of charge via the Internet at <http://pubs.acs.org>.
15
16
17
18
19
20

21 ABBREVIATIONS

22
23
24 A α C, 2-Amino-9*H*-pyrido[2,3-*b*]indole; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-
25
26 butanone; 4-ABP, 4-aminobiphenyl; B[a]P, benzo[a]pyrene; PhIP, 2-amino-1-methyl-6-
27
28 phenylimidazo[4,5-*b*]pyridine; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; IQ,
29
30 2-amino-3-methylimidazo[4,5-*f*]quinolone; dG-C8-A α C, *N*-(deoxyguanosin-8-yl)-2-amino-
31
32 9*H*-pyrido[2,3-*b*]indole; NAT, *N*-acetyltransferases; SULT, Sulfotransferases; UGT, UDP-
33
34 Glucuronosyltransferases; UPLC/MSⁿ, UPLC/ion trap multistage mass spectrometry; HONH-
35
36 A α C, 2-hydroxyamino-9*H*-pyrido[2,3-*b*]indole; A α C-3-OH, 2-amino-3-hydroxy-9*H*-
37
38 pyrido[2,3-*b*]indole; A α C-6-OH, 2-amino-6-hydroxy-9*H*-pyrido[2,3-*b*]indole; A α C-3-*O*-
39
40 SO₃H, 2-amino-9*H*-pyrido[2,3-*b*]indol-3-yl-sulfate; A α C-6-*O*-SO₃H, 2-amino-9*H*-
41
42 pyrido[2,3-*b*]indol-6-yl-sulfate; A α C-3-*O*-Gluc, 2-amino-9*H*-pyrido[2,3-*b*]indol-3-yl-oxo-(β -
43
44 D-glucuronic acid); A α C-6-*O*-Gluc, 2-amino-9*H*-pyrido[2,3-*b*]indol-6-yl-oxo-(β -
45
46 D-glucuronic acid); *N*²-acetyl-A α C-3-*O*-SO₃H, *N*²-acetyl-9*H*-pyrido[2,3-*b*]indol-3-yl sulfate;
47
48 *N*²-acetyl-A α C-6-*O*-SO₃H, *N*²-acetyl-9*H*-pyrido[2,3-*b*]indol-6-yl sulfate; *N*²-acetyl-A α C-3-
49
50 *O*-Gluc; *N*²-acetyl-2-amino-9*H*-pyrido[2,3-*b*]indol-3-yl-oxo-(β -D-glucuronic acid); *N*²-
51
52 acetyl-A α C-6-*O*-Gluc, *N*²-acetyl-2-amino-9*H*-pyrido[2,3-*b*]indol-6-yl-oxo-(β -D-glucuronic
53
54 acid); A α C-*N*²-Gluc, *N*²-(β -D-glucosiduronyl)-2-amino-9*H*-pyrido[2,3-*b*]indole; A α C-HN²-
55
56
57
58
59
60

1
2
3 *O*-Gluc, *O*-(β -D-glucosiduronyl)-2-hydroxyamino-9*H*-pyrido[2,3-*b*]indole; SPE, Solid-phase
4
5 extraction
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

REFERENCES

- 1
2
3
4
5 (1) Giovannucci, E. (2001) An updated review of the epidemiological evidence that cigarette
6 smoking increases risk of colorectal cancer. *Cancer. Epidemiol. Biomarkers. Prev.* 10, 725-
7 731.
- 8
9 (2) *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans*. IARC. (2004)
10 Tobacco smoke and involuntary smoking. *Volume 83*, 1-1438.
- 11 (3) *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans*. (2012) Personal
12 habits and indoor combustions. *Volume 100*, 1-538.
- 13 (4) Hoffmann, D., Hoffmann, I., and El-Bayoumy, K. (2001) The less harmful cigarette: a
14 controversial issue. a tribute to Ernst L. Wynder. *Chem. Res. Toxicol.* 14, 767-790.
- 15 (5) Sugimura, T., Wakabayashi, K., Nakagama, H., and Nagao, M. (2004) Heterocyclic amines:
16 Mutagens/carcinogens produced during cooking of meat and fish. *Cancer. Sci.* 95, 290-299.
- 17 (6) Manabe, S., Izumikawa, S., Asakuno, K., Wada, O., and Kanai, Y. (1991) Detection of
18 carcinogenic amino-alpha-carbolines and amino-gamma-carbolines in diesel-exhaust
19 particles. *Environ. Pollut.* 70, 255-265.
- 20 (7) Manabe, S., Kurihara, N., Wada, O., Izumikawa, S., Asakuno, K., and Morita, M. (1993)
21 Detection of a carcinogen, 2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine, in airborne
22 particles and diesel-exhaust particles. *Environ. Pollut.* 80, 281-286.
- 23 (8) Zhang, L., Ashley, D. L., and Watson, C. H. (2011) Quantitative analysis of six heterocyclic
24 aromatic amines in mainstream cigarette smoke condensate using isotope dilution liquid
25 chromatography-electrospray ionization tandem mass spectrometry. *Nicotine. Tob. Res.* 13,
26 120-126.
- 27 (9) Hoffmann, D. H., I. (1998) Letters to the editor, tobacco smoke components. *Beiträge zur*
28 *Tabakforschung International/Contributions to Tobacco Research.* 18.
- 29 (10) Smith, C. J., Qian, X., Zha, Q., and Moldoveanu, S. C. (2004) Analysis of alpha- and beta-
30 carbolines in mainstream smoke of reference cigarettes by gas chromatography-mass
31 spectrometry. *J. Chromatogr. A.* 1046, 211-216.
- 32 (11) Yoshida, D., and Matsumoto, T. (1980) Amino-alpha-carbolines as mutagenic agents in
33 cigarette smoke condensate. *Cancer. Lett.* 10, 141-149.
- 34 (12) Patrianakos, C., and Hoffmann, D. (1979) Chemical Studies on Tobacco-Smoke .64. Analysis
35 of Aromatic-Amines in Cigarette-Smoke. *J. of Ana. Toxicol.* 3, 150-154.
- 36 (13) Zha, Q., Qian, N. X., and Moldoveanu, S. C. (2002) Analysis of polycyclic aromatic
37 hydrocarbons in the particulate phase of cigarette smoke using a gas chromatographic-high-
38 resolution mass spectrometric technique. *J. Chromatogr. Sci.* 40, 403-408.
- 39 (14) Hecht, S. S. (2002) Human urinary carcinogen metabolites: biomarkers for investigating
40 tobacco and cancer. *Carcinogenesis.* 23, 907-922.
- 41 (15) Turesky, R. J., Yuan, J. M., Wang, R., Peterson, S., and Yu, M. C. (2007) Tobacco smoking
42 and urinary levels of 2-amino-9H-pyrido[2,3-b]indole in men of Shanghai, China. *Cancer.*
43 *Epidemiol. Biomarkers. Prev.* 16, 1554-1560.
- 44 (16) Fu, Y., Zhao, G., Wang, S., Yu, J., Xie, F., Wang, H., and Xie, J. (2014) Simultaneous
45 determination of fifteen heterocyclic aromatic amines in the urine of smokers and nonsmokers
46 using ultra-high performance liquid chromatography-tandem mass spectrometry. *J.*
47 *Chromatogr. A.* 1333, 45-53.
- 48 (17) Konorev, D., Koopmeiners, J. S., Tang, Y., Franck Thompson, E. A., Jensen, J. A.,
49 Hatsukami, D. K., and Turesky, R. J. (2015) Measurement of the Heterocyclic Amines 2-
50
51
52
53
54
55
56
57
58
59
60

- 1
2
3 Amino-9H-pyrido[2,3-b]indole and 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine in
4 Urine: Effects of Cigarette Smoking. *Chem. Res. Toxicol.* 28, 2390-2399.
- 5
6 (18) Ohgaki, H., Matsukura, N., Morino, K., Kawachi, T., Sugimura, T., and Takayama, S. (1984)
7 Carcinogenicity in mice of mutagenic compounds from glutamic acid and soybean globulin
8 pyrolysates. *Carcinogenesis*. 5, 815-819.
- 9
10 (19) Zhang, X. B., Felton, J. S., Tucker, J. D., Urlando, C., and Heddle, J. A. (1996) Intestinal
11 mutagenicity of two carcinogenic food mutagens in transgenic mice: 2-amino-1-methyl-6-
12 phenylimidazo[4,5-b]pyridine and amino(alpha)carboline. *Carcinogenesis*. 17, 2259-2265.
- 13
14 (20) Okonogi, H., Ushijima, T., Zhang, X. B., Heddle, J. A., Suzuki, T., Sofuni, T., Felton, J. S.,
15 Tucker, J. D., Sugimura, T., and Nagao, M. (1997) Agreement of mutational characteristics of
16 heterocyclic amines in lacI of the Big Blue mouse with those in tumor related genes in
17 rodents. *Carcinogenesis*. 18, 745-748.
- 18
19 (21) Pfau, W., Martin, F. L., Cole, K. J., Venitt, S., Phillips, D. H., Grover, P. L., and Marquardt,
20 H. (1999) Heterocyclic aromatic amines induce DNA strand breaks and cell transformation.
21 *Carcinogenesis*. 20, 545-551.
- 22
23 (22) Majer, B. J., Kassie, F., Sasaki, Y., Pfau, W., Glatt, H., Meinel, W., Darroudi, F., and
24 Knasmuller, S. (2004) Investigation of the genotoxic effects of 2-amino-9H-pyrido[2,3-
25 b]indole in different organs of rodents and in human derived cells. *J. Chromatogr. B. Analyt.*
26 *Technol. Biomed. Life. Sci.* 802, 167-173.
- 27
28 (23) Nauwelaers, G., Bellamri, M., Fessard, V., Turesky, R. J., and Langouet, S. (2013) DNA
29 adducts of the tobacco carcinogens 2-amino-9H-pyrido[2,3-b]indole and 4-aminobiphenyl are
30 formed at environmental exposure levels and persist in human hepatocytes. *Chem. Res.*
31 *Toxicol.* 26, 1367-1377.
- 32
33 (24) Nauwelaers, G., Bessette, E. E., Gu, D., Tang, Y., Rageul, J., Fessard, V., Yuan, J. M., Yu,
34 M. C., Langouet, S., and Turesky, R. J. (2011) DNA adduct formation of 4-aminobiphenyl
35 and heterocyclic aromatic amines in human hepatocytes. *Chem. Res. Toxicol.* 24, 913-925.
- 36
37 (25) Pfau, W., Schulze, C., Shirai, T., Hasegawa, R., and Brockstedt, U. (1997) Identification of
38 the major hepatic DNA adduct formed by the food mutagen 2-amino-9H-pyrido[2,3-b]indole
39 (A alpha C). *Chem. Res. Toxicol.* 10, 1192-1197.
- 40
41 (26) Frederiksen, H., and Frandsen, H. (2004) Excretion of metabolites in urine and faeces from
42 rats dosed with the heterocyclic amine, 2-amino-9H-pyrido[2,3-b]indole (AalphaC). *Food.*
43 *Chem. Toxicol.* 42, 879-885.
- 44
45 (27) Turesky, R. J., Bendaly, J., Yasa, I., Doll, M. A., and Hein, D. W. (2009) The impact of
46 NAT2 acetylator genotype on mutagenesis and DNA adducts from 2-amino-9H-pyrido[2,3-
47 b]indole. *Chem. Res. Toxicol.* 22, 726-733.
- 48
49 (28) Niwa, T., Yamazoe, Y., and Kato, R. (1982) Metabolic activation of 2-amino-9H-pyrido[2,3-
50 b]indole by rat-liver microsomes. *Mutat. Res.* 95, 159-170.
- 51
52 (29) Raza, H., King, R. S., Squires, R. B., Guengerich, F. P., Miller, D. W., Freeman, J. P., Lang,
53 N. P., and Kadlubar, F. F. (1996) Metabolism of 2-amino-alpha-carboline. A food-borne
54 heterocyclic amine mutagen and carcinogen by human and rodent liver microsomes and by
55 human cytochrome P4501A2. *Drug. Metab. Dispos.* 24, 395-400.
- 56
57 (30) King, R. S., Teitel, C. H., and Kadlubar, F. F. (2000) In vitro bioactivation of N-hydroxy-2-
58 amino-alpha-carboline. *Carcinogenesis*. 21, 1347-1354.
- 59
60 (31) Turesky, R. J., and Le Marchand, L. (2011) Metabolism and biomarkers of heterocyclic
aromatic amines in molecular epidemiology studies: lessons learned from aromatic amines.
Chem. Res. Toxicol. 24, 1169-1214.
- (32) Nowell, S. A., Massengill, J. S., Williams, S., Radominska-Pandya, A., Tephly, T. R., Cheng,
Z., Strassburg, C. P., Tukey, R. H., MacLeod, S. L., Lang, N. P., and Kadlubar, F. F. (1999)

- 1
2
3 Glucuronidation of 2-hydroxyamino-1-methyl-6-phenylimidazo[4, 5-b]pyridine by human
4 microsomal UDP-glucuronosyltransferases: identification of specific UGT1A family isoforms
5 involved. *Carcinogenesis*. 20, 1107-1114.
- 6
7 (33) Malfatti, M. A., and Felton, J. S. (2004) Human UDP-glucuronosyltransferase 1A1 is the
8 primary enzyme responsible for the N-glucuronidation of N-hydroxy-PhIP in vitro. *Chem.*
9 *Res. Toxicol.* 17, 1137-1144.
- 10 (34) Tang, Y., LeMaster, D. M., Nauwelaers, G., Gu, D., Langouet, S., and Turesky, R. J. (2012)
11 UDP-glucuronosyltransferase-mediated metabolic activation of the tobacco carcinogen 2-
12 amino-9H-pyrido[2,3-b]indole. *J. Biol. Chem.* 287, 14960-14972.
- 13 (35) Lee, Y. C., Cohet, C., Yang, Y. C., Stayner, L., Hashibe, M., and Straif, K. (2009) Meta-
14 analysis of epidemiologic studies on cigarette smoking and liver cancer. *Int. J. Epidemiol.* 38,
15 1497-1511.
- 16
17 (36) Vineis, P., Alavanja, M., Buffler, P., Fontham, E., Franceschi, S., Gao, Y. T., Gupta, P. C.,
18 Hackshaw, A., Matos, E., Samet, J., Sitas, F., Smith, J., Stayner, L., Straif, K., Thun, M. J.,
19 Wichmann, H. E., Wu, A. H., Zaridze, D., Peto, R., and Doll, R. (2004) Tobacco and cancer:
20 recent epidemiological evidence. *J. Natl. Cancer. Inst.* 96, 99-106.
- 21 (37) Frederiksen, H. (2005) Two food-borne heterocyclic amines: metabolism and DNA adduct
22 formation of amino-alpha-carbolines. *Mol. Nutr. Food. Res.* 49, 263-273.
- 23 (38) Frederiksen, H., and Frandsen, H. (2002) In vitro metabolism of two heterocyclic amines, 2-
24 amino-9H-pyrido[2,3-b]indole (A(alpha)C) and 2-amino-3-methyl-9H-pyrido[2,3-b]indole
25 (MeA(alpha)C) in human and rat hepatic microsomes. *Pharmacol. Toxicol.* 90, 127-134.
- 26 (39) King, R. S., Teitel, C. H., Shaddock, J. G., Casciano, D. A., and Kadlubar, F. F. (1999)
27 Detoxification of carcinogenic aromatic and heterocyclic amines by enzymatic reduction of
28 the N-hydroxy derivative. *Cancer. Lett.* 143, 167-171.
- 29 (40) Yuan, Z. X., Jha, G., McGregor, M. A., and King, R. S. (2007) Metabolites of the carcinogen
30 2-amino-alpha-carboline formed in male Sprague-Dawley rats in vivo and in rat hepatocyte
31 and human HepG2 cell incubates. *Chem. Res. Toxicol.* 20, 497-503.
- 32 (41) Turesky, R. J., Konorev, D., Fan, X., Tang, Y., Yao, L., Ding, X., Xie, F., Zhu, Y., and
33 Zhang, Q. Y. (2015) Effect of cytochrome P450 reductase deficiency on 2-amino-9H-
34 pyrido[2,3-b]indole metabolism and DNA adduct formation in liver and extrahepatic tissues
35 of mice. *Chem. Res. Toxicol.* 28, 2400-2410.
- 36 (42) Guillouzo, A., Morel, F., Langouet, S., Maheo, K., and Rissel, M. (1997) Use of hepatocyte
37 cultures for the study of hepatotoxic compounds. *J. Hepatol.* 26 Suppl 2, 73-80.
- 38 (43) Kunze, K. L., and Trager, W. F. (1993) Isoform-selective mechanism-based inhibition of
39 human cytochrome P450 1A2 by furafylline. *Chem. Res. Toxicol.* 6, 649-656.
- 40 (44) Burke, M. D., and Mayer, R. T. (1983) Differential effects of phenobarbitone and 3-
41 methylcholanthrene induction on the hepatic microsomal metabolism and cytochrome P-450-
42 binding of phenoxazone and a homologous series of its n-alkyl ethers (alkoxyresorufins).
43 *Chem. Biol. Interact.* 45, 243-258.
- 44 (45) Eugster, H. P., Probst, M., Wurgler, F. E., and Sengstag, C. (1993) Caffeine, estradiol, and
45 progesterone interact with human CYP1A1 and CYP1A2. Evidence from cDNA-directed
46 expression in *Saccharomyces cerevisiae*. *Drug. Metab. Dispos.* 21, 43-49.
- 47 (46) Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram
48 quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248-
49 254.
- 50 (47) Gupta, R. C. (1993) 32P-postlabelling analysis of bulky aromatic adducts. *IARC. Sci. Publ.*,
51 11-23.
- 52
53
54
55
56
57
58
59
60

- 1
2
3 (48) Goodenough, A. K., Schut, H. A., and Turesky, R. J. (2007) Novel LC-ESI/MS/MS(n)
4 method for the characterization and quantification of 2'-deoxyguanosine adducts of the dietary
5 carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine by 2-D linear quadrupole ion
6 trap mass spectrometry. *Chem. Res. Toxicol.* *20*, 263-276.
- 7
8 (49) Chiu, Y. T., Liu, J., Tang, K., Wong, Y. C., Khanna, K. K., and Ling, M. T. (2012)
9 Inactivation of ATM/ATR DNA damage checkpoint promotes androgen induced
10 chromosomal instability in prostate epithelial cells. *PLoS. One.* *7*, e51108.
- 11
12 (50) Levsen, K., Schiebel, H. M., Behnke, B., Dotzer, R., Dreher, W., Elend, M., and Thiele, H.
13 (2005) Structure elucidation of phase II metabolites by tandem mass spectrometry: an
14 overview. *J. Chromatogr. A.* *1067*, 55-72.
- 15
16 (51) Butler, M. A., Iwasaki, M., Guengerich, F. P., and Kadlubar, F. F. (1989) Human cytochrome
17 P-450PA (P-450IA2), the phenacetin O-deethylase, is primarily responsible for the hepatic 3-
18 demethylation of caffeine and N-oxidation of carcinogenic arylamines. *Proc. Natl. Acad. Sci.*
19 *. 86*, 7696-7700.
- 20
21 (52) Langouet, S., Welti, D. H., Kerriguy, N., Fay, L. B., Huynh-Ba, T., Markovic, J., Guengerich,
22 F. P., Guillouzo, A., and Turesky, R. J. (2001) Metabolism of 2-amino-3,8-
23 dimethylimidazo[4,5-f]quinoxaline in human hepatocytes: 2-amino-3-methylimidazo[4,5-
24 f]quinoxaline-8-carboxylic acid is a major detoxification pathway catalyzed by cytochrome
25 P450 1A2. *Chem. Res. Toxicol.* *14*, 211-221.
- 26
27 (53) Langouet, S., Paehler, A., Welti, D. H., Kerriguy, N., Guillouzo, A., and Turesky, R. J. (2002)
28 Differential metabolism of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine in rat and
29 human hepatocytes. *Carcinogenesis.* *23*, 115-122.
- 30
31 (54) Kriek, E. (1992) Fifty years of research on N-acetyl-2-aminofluorene, one of the most
32 versatile compounds in experimental cancer research. *J. Cancer. Res. Clin. Oncol.* *118*, 481-
33 489.
- 34
35 (55) Kriek, E. (1972) Persistent binding of a new reaction product of the carcinogen N-hydroxy-N-
36 2-acetylaminofluorene with guanine in rat liver DNA in vivo. *Cancer. Res.* *32*, 2042-2048.
- 37
38 (56) Turesky, R. J., Guengerich, F. P., Guillouzo, A., and Langouet, S. (2002) Metabolism of
39 heterocyclic aromatic amines by human hepatocytes and cytochrome P4501A2. *Mutat. Res.*
40 *506-507*, 187-195.
- 41
42 (57) Turesky, R. J., Constable, A., Richoz, J., Varga, N., Markovic, J., Martin, M. V., and
43 Guengerich, F. P. (1998) Activation of heterocyclic aromatic amines by rat and human liver
44 microsomes and by purified rat and human cytochrome P450 1A2. *Chem. Res. Toxicol.* *11*,
45 925-936.
- 46
47 (58) Wallin, H., Mikalsen, A., Guengerich, F. P., Ingelman-Sundberg, M., Solberg, K. E.,
48 Rosslund, O. J., and Alexander, J. (1990) Differential rates of metabolic activation and
49 detoxication of the food mutagen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine by
50 different cytochrome P450 enzymes. *Carcinogenesis.* *11*, 489-492.
- 51
52 (59) Aninat, C., Piton, A., Glaise, D., Le Charpentier, T., Langouet, S., Morel, F., Guguen-
53 Guillouzo, C., and Guillouzo, A. (2006) Expression of cytochromes P450, conjugating
54 enzymes and nuclear receptors in human hepatoma HepaRG cells. *Drug. Metab. Dispos.* *34*,
55 75-83.
- 56
57 (60) Cai, T., Yao, L., and Turesky, R. J. (2016) Bioactivation of Heterocyclic Aromatic Amines by
58 UDP Glucuronosyltransferases. *Chem. Res. Toxicol.* *29*, 879-891.
- 59
60 (61) Pathak, K. V., Bellamri, M., Wang, Y., Langouet, S., and Turesky, R. J. (2015) 2-Amino-9H-
pyrido[2,3-b]indole (AalphaC) Adducts and Thiol Oxidation of Serum Albumin as Potential
Biomarkers of Tobacco Smoke. *J. Biol. Chem.* *290*, 16304-16318.

- 1
2
3 (62) Novak, M., and Nguyen, T. M. (2003) Unusual reactions of the model carcinogen N-acetoxy-
4 N-acetyl-2-amino-alpha-carboline. *J. Org. Chem.* 68, 9875-9881.
5
6 (63) Holland, R. D., Taylor, J., Schoenbachler, L., Jones, R. C., Freeman, J. P., Miller, D. W.,
7 Lake, B. G., Gooderham, N. J., and Turesky, R. J. (2004) Rapid biomonitoring of heterocyclic
8 aromatic amines in human urine by tandem solvent solid phase extraction liquid
9 chromatography electrospray ionization mass spectrometry. *Chem. Res. Toxicol.* 17, 1121-
10 1136.
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Figure Legends

Figure 1. HPLC-UV profile (A) and chemical structures (B) of A α C metabolites formed in primary human hepatocytes incubated with 50 μ M of A α C over 24 h.

Figure 2. LC/MS² product ion mass spectra of A α C-*N*²-Gluc (*m/z* 360.1 >) (A) and LC/MS³ product ion mass spectra of A α C-6-*O*-Gluc (*m/z* 376.1 > 200.1 >) (B), A α C-3-*O*-Gluc (*m/z* 376.1 > 200.1 >) (C), A α C-6-*O*-SO₃H (*m/z* 280.1 > 200.1 >) (D), A α C-3-*O*-SO₃H (*m/z* 280.1 > 200.1 >) (E) in positive ionization mode. The proposed mechanism of formation of the prominent fragment ion of A α C-3-OH at *m/z* 155.0 is presented.

Figure 3. Identification and characterization of *N*²-Acetyl-A α C-6-*O*-Gluc. (A) LC/MS³ product ion mass spectra of *N*²-Acetyl-A α C-6-*O*-Gluc (*m/z* 418.1 > 242.1 >) formed in primary human hepatocytes. *N*²-Acetyl-A α C-6-*O*-Gluc was purified by HPLC and characterized by mass spectrometry. (B) UV spectra of A α C-6-OH, A α C-3-OH, *N*²-acetyl-A α C-*O*-Gluc and its hydrolysis product obtained after β -glucuronidase followed by acid treatment of *N*²-acetyl-A α C-*O*-Gluc as described in material and method.

Figure 4. LC/MS³ product ion mass spectra of *N*²-acetyl-A α C-6-*O*-SO₃H (*m/z* 322.1 > 241.1 >) (A), *N*²-acetyl-A α C-3-*O*-SO₃H (*m/z* 322.1 > 241.1 >) (B), A α C-HN²-*O*-Gluc (*m/z* 376.1 > 200.1 >) (C) and *N*²-acetyl-A α C-3-*O*-Gluc (*m/z* 418.1 > 242.1 >) (D). Primary human hepatocytes were treated with 0.1, 1 and 10 μ M of A α C and the metabolites derived from A α C were characterized by mass spectrometry.

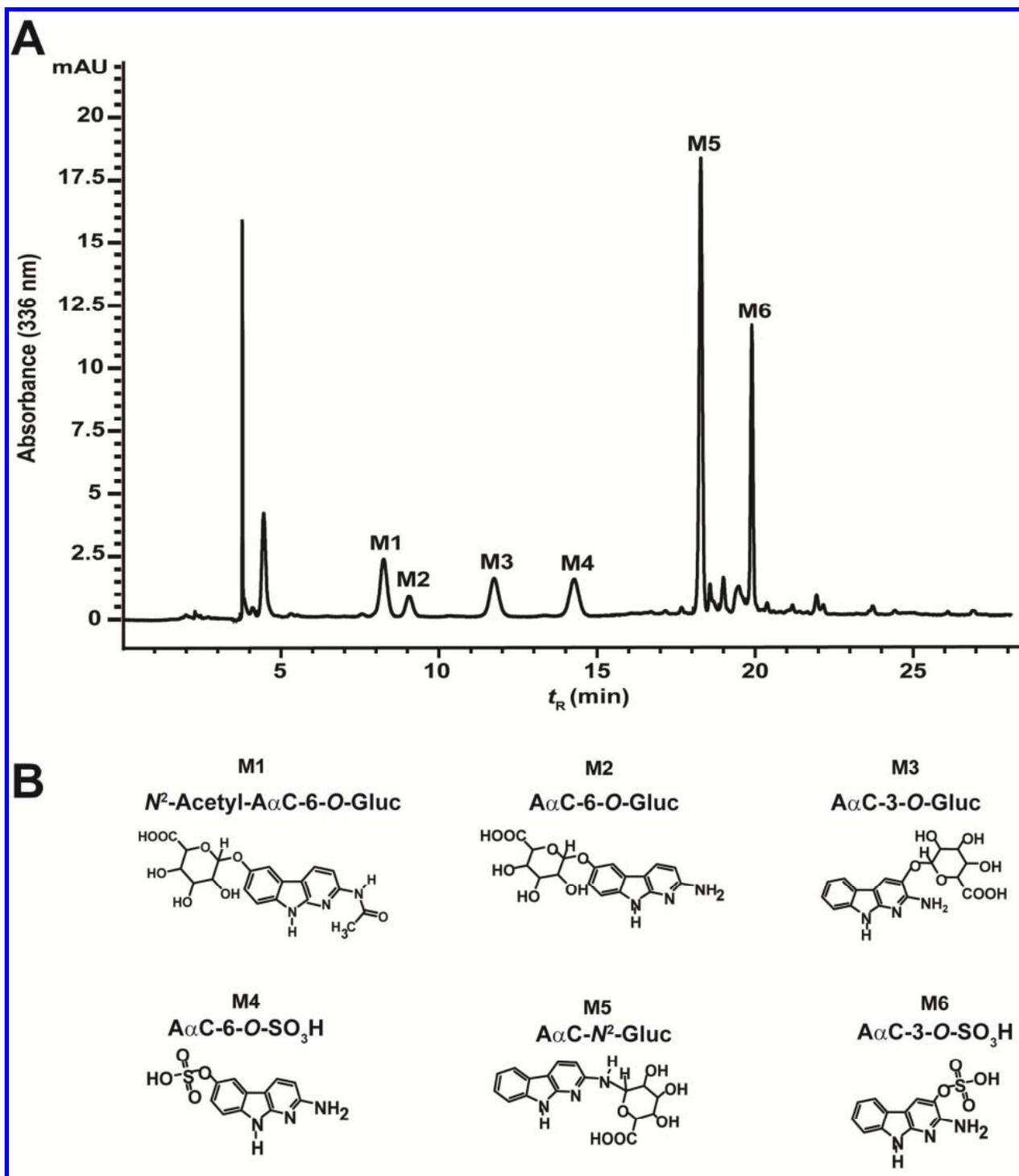
Figure 5. Mass chromatograms of A α C metabolites formed during 24h in primary human hepatocytes incubated 0.1, 1 and 10 μ M of A α C.

1
2
3 **Figure 6.** Distribution of A α C metabolites formed as a function of dose in two human
4 primary hepatocyte preparations after 24 h of incubation. Primary human hepatocytes were
5 incubated with various concentrations of A α C (0.1, 1 and 10 μ M) during 24 h and the
6 relative ion abundance of each metabolites derived from A α C formed were determined by
7 mass spectrometry.
8
9

10
11
12
13
14
15 **Figure 7.** Role of P450 1A2 in A α C metabolism in human hepatocytes. Cells were pre-
16 treated with furafylline (5 μ M) for 24h prior to treatment with A α C (0.1, 1 and 10 μ M)
17 during 24h. The relative abundance of each A α C derived metabolites was determined by
18 mass spectrometry. (Student's *t*-test, * $P < 0.05$; ** $P < 0.01$, *** $P < 0.005$ versus control).
19
20
21
22
23
24

25
26 **Figure 8.** Correlation between DNA adducts levels derived from A α C and the relative
27 abundance of A α C-HN²-O-Gluc formed in human hepatocytes. Relative abundance of A α C-
28 HN²-O-Gluc (**A**) and dG-C8-A α C (**B**) formed in human hepatocytes. Cells were pre-treated
29 with furafylline (5 μ M) for 24h prior to incubation with 0.1, 1, 10 and 50 μ M of A α C for 24h
30 and the relative abundance of A α C-HN²-O-Gluc and dG-C8-A α C levels derived from A α C
31 formed were estimated by mass spectrometry. (Student's *t*-test, * $P < 0.05$; ** $P < 0.01$,
32 *** $P < 0.005$ versus control).
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Figure 1.



1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Figure 2.

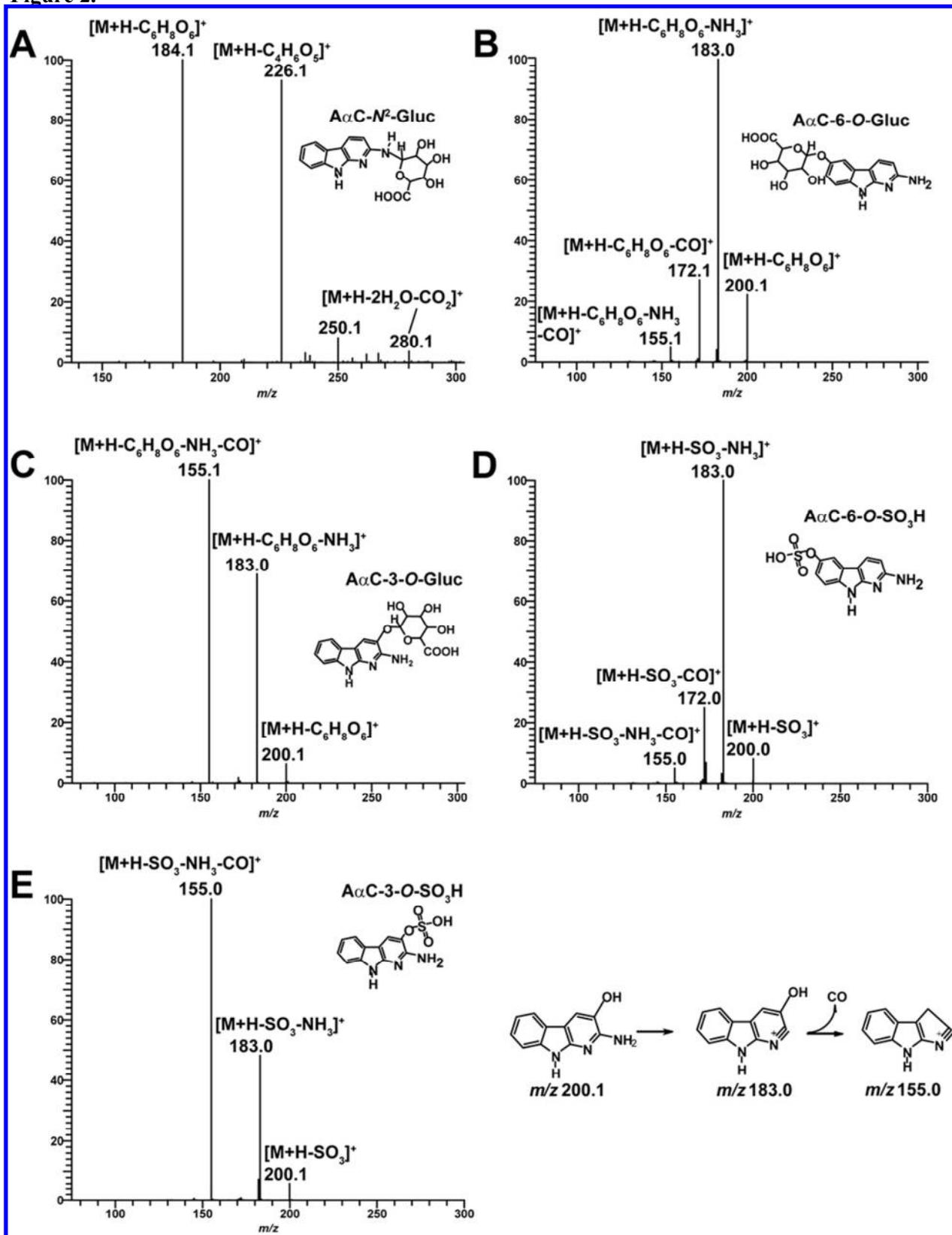


Figure 3.

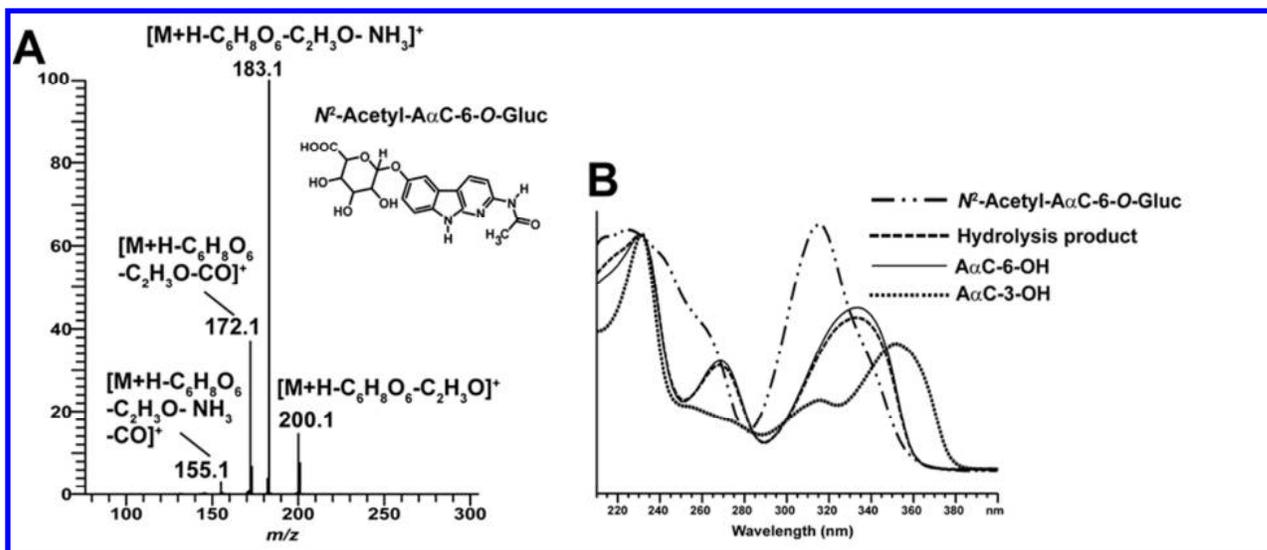


Figure 4.

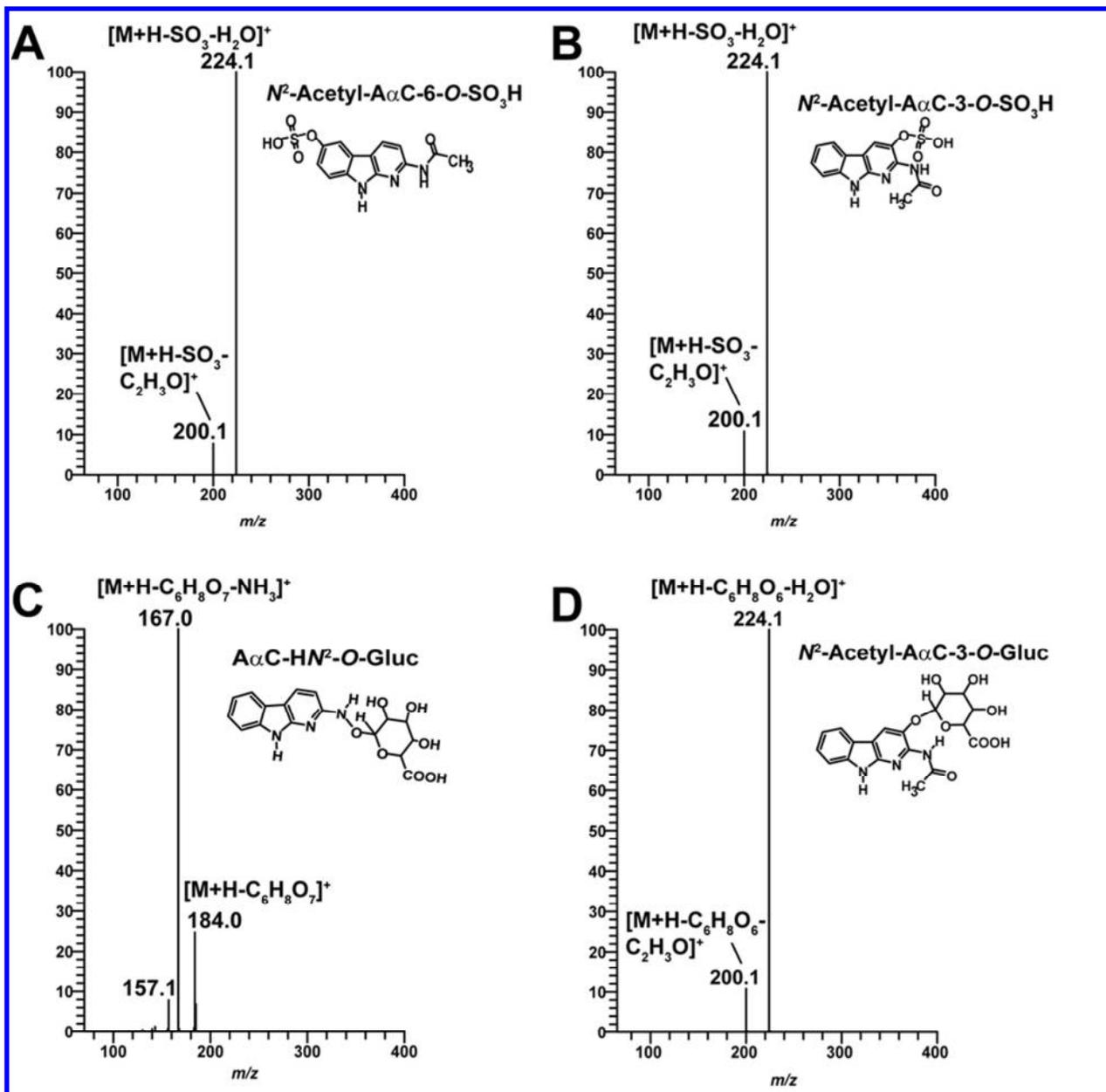


Figure 5.

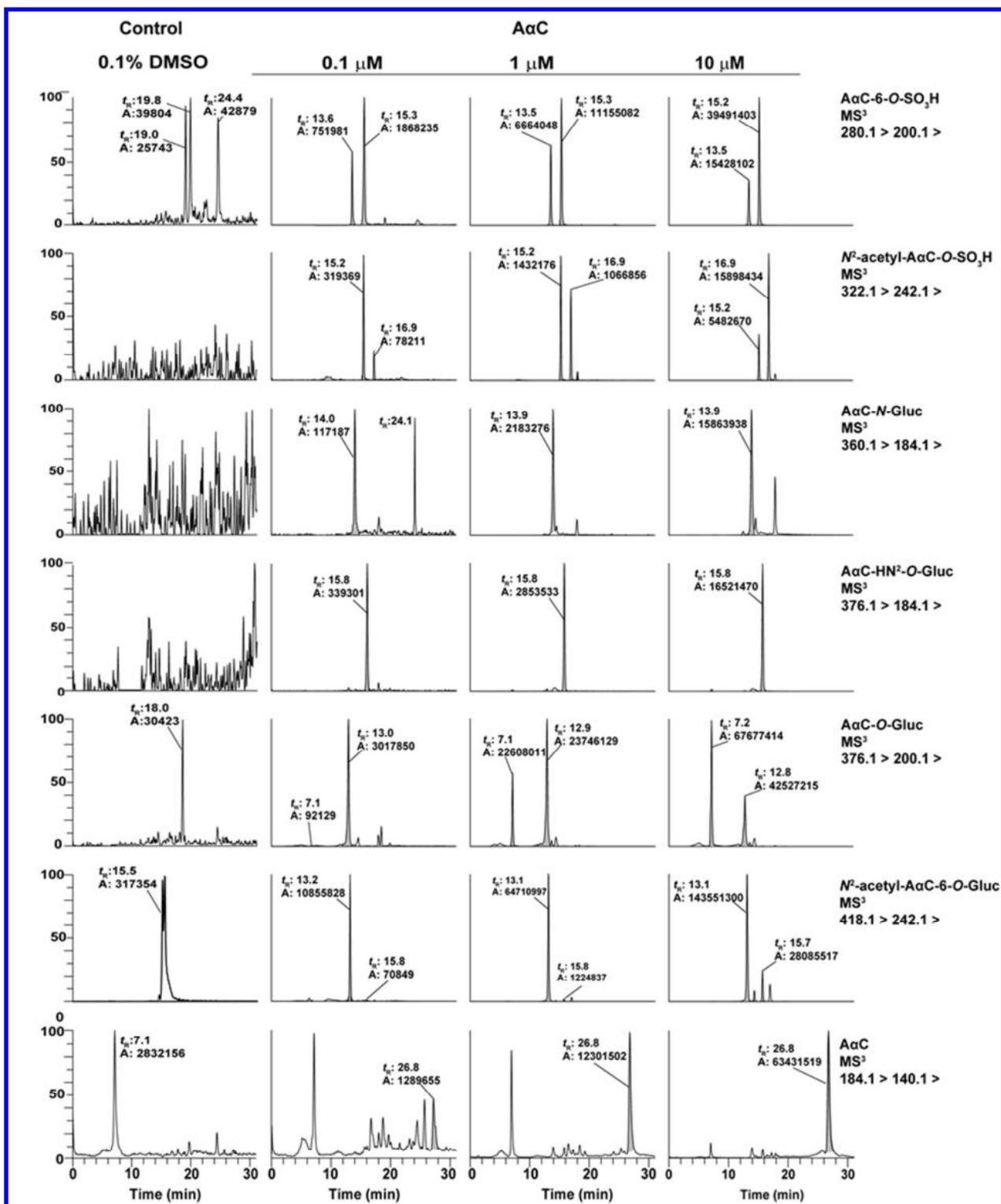


Figure 6.

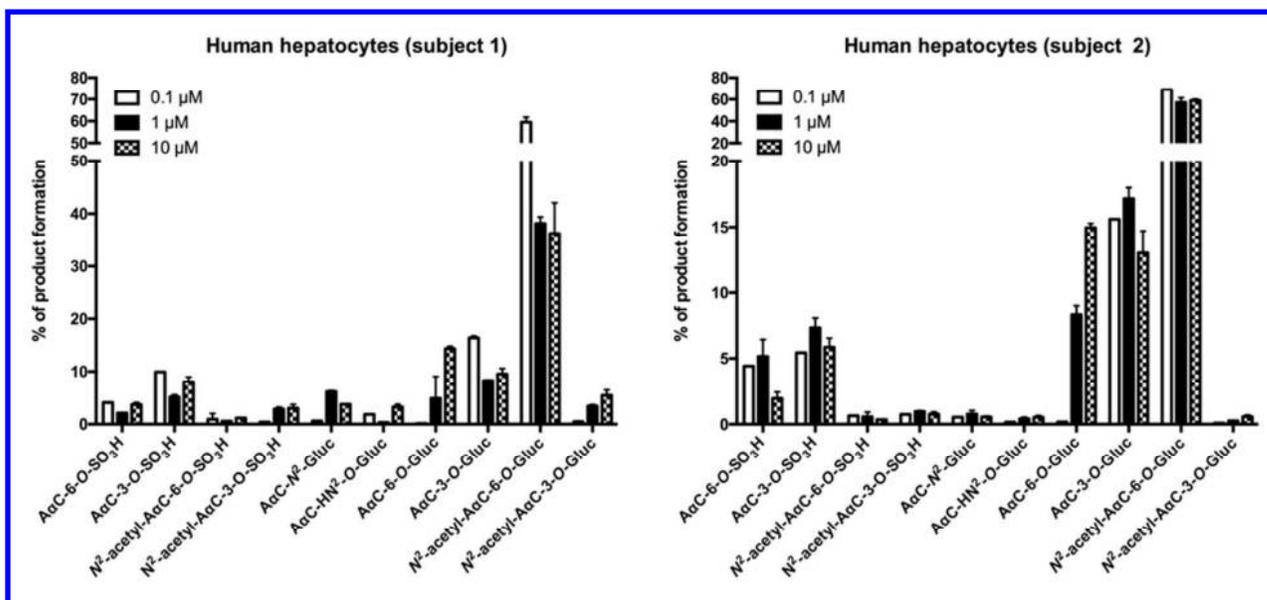


Figure 7.

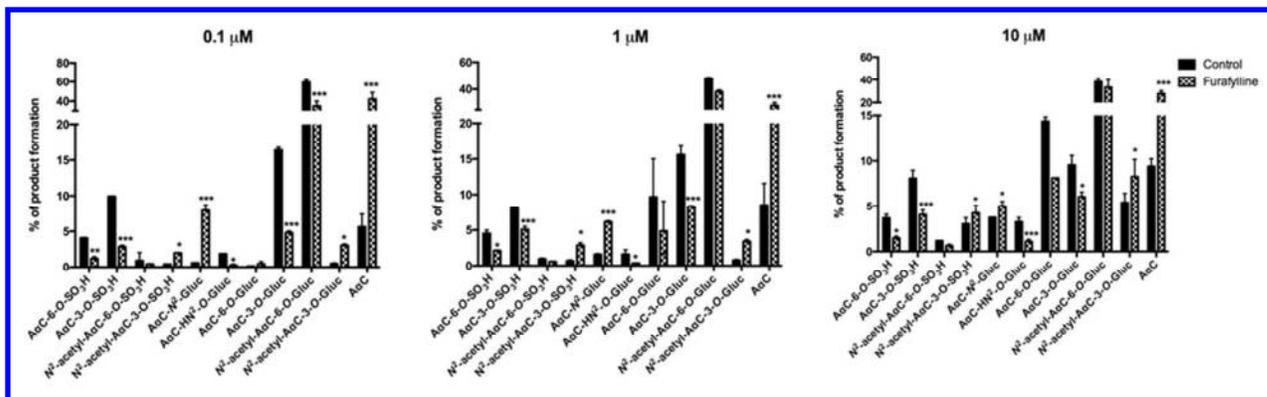


Figure 8.

