Characterization of \( \text{A} \alpha \text{C} \text{-serum albumin adducts} \)

2-Amino-9\(H\)-pyrido[2,3-\(b\)]indole (A\(\alpha\)C) Adducts and Thiol Oxidation of Serum Albumin as Potential Biomarkers of Tobacco Smoke

Khyatiben V. Pathak\(^1\), Medjda Bellamri\(^2\), Yi Wang\(^1\), Sophie Langouët\(^2\), and Robert J. Turesky\(^1\)

\(^1\)Masonic Cancer Center and Department of Medicinal Chemistry, University of Minnesota, Minneapolis, MN 55455, USA

\(^2\)UMR Inserm 1085 IRSET, Rennes1 University, UMS 3480 Bosis, F-35043 Rennes, France

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To whom correspondence should be addressed: Robert J. Turesky, Masonic Cancer Center and Department of Medicinal Chemistry, University of Minnesota, Minneapolis, MN 55455, USA, Tel.: (612) 626-0141; Fax: (612) 624-3869; E-mail: rturesky@umn.edu

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**Background:** The reactivity of A\(\alpha\)C, a tobacco smoke carcinogen, was investigated with DNA and albumin of human hepatocytes.

**Results:** Hepatocytes bioactivate A\(\alpha\)C to metabolites which adduct to DNA and albumin.

**Conclusion:** Cys\(^{34}\) and Met\(^{329}\) of serum albumin are targets for A\(\alpha\)C electrophiles.

**Significance:** A\(\alpha\)C forms macromolecular adducts and induces oxidative stress, which may be contributing factors to liver damage and cancer risk in smokers.

**ABSTRACT**

2-Amino-9\(H\)-pyrido[2-3-\(b\)]indole (A\(\alpha\)C) is a carcinogenic heterocyclic aromatic amine (HAA) formed during the combustion of tobacco. A\(\alpha\)C undergoes bioactivation to form electrophilic N-oxidized metabolites that react with DNA to form adducts, which can lead to mutations. Many genotoxicants and toxic electrophiles react with human serum albumin (albumin); however, the chemistry of reactivity of A\(\alpha\)C with proteins has not been studied. The genotoxic metabolites, 2-hydroxyamino-9\(H\)-pyrido[2-3-\(b\)]indole (HONH-A\(\alpha\)C), 2-nitroso-9\(H\)-pyrido[2-3-\(b\)]indole (NO-A\(\alpha\)C), a N-acetoxy-9\(H\)-pyrido[2-3-\(b\)]indole (N-acetoxy-A\(\alpha\)C), and their \(^{13}\text{C}_2\)-A\(\alpha\)C labelled homologues were reacted with albumin. Sites of adduction of A\(\alpha\)C to albumin were identified, by data dependent scanning and targeted bottom-up proteomics approaches employing ion trap and Orbitrap MS. A\(\alpha\)C–albumin adducts were formed at Cys\(^{34}\), Tyr\(^{140}\) and Tyr\(^{150}\) residues, when albumin was reacted with HONH-A\(\alpha\)C or NO-A\(\alpha\)C. Sulfenamide, sulfinamide, and sulfonamide adduct formation occurred at Cys\(^{34}\) (A\(\alpha\)C-Cys\(^{34}\)). N-Acetoxy-A\(\alpha\)C also formed an adduct at Tyr\(^{332}\). Albumin-A\(\alpha\)C adducts were characterized in human plasma treated with N-oxidized metabolites of A\(\alpha\)C and human hepatocytes exposed to A\(\alpha\)C. High levels of N-(deoxyguanosin-8-yl)-A\(\alpha\)C (dG-C8-A\(\alpha\)C) DNA adducts were formed in hepatocytes. The Cys\(^{34}\) was the sole amino acid of albumin to form adducts with A\(\alpha\)C. Albumin also served as an antioxidant and scavenged reactive oxygen species (ROS) generated by metabolites of A\(\alpha\)C in hepatocytes: there was a strong decrease in reduced Cys\(^{34}\) while the levels of Cys\(^{34}\)-sulfinic acid (Cys-SO\(_2\)H), Cys\(^{34}\)-sulfonic acid (Cys-SO\(_3\)H) and Met\(^{329}\) sulfoxide were greatly increased. Cys\(^{34}\)-adduction products and Cys-SO\(_2\)H, Cys-SO\(_3\)H and Met\(^{329}\) sulfoxide may be potential biomarkers to assess exposure and oxidative stress.
stress associated with AαC and other arylamine toxicants present in tobacco smoke.

Tobacco smoke is a major risk factor for lung cancer, but also cancer of the liver, bladder, and gastrointestinal tract (1-4). The combustion of tobacco produces many genotoxicants including polycyclic aromatic hydrocarbons, nitrosamines, aromatic amines, and heterocyclic aromatic amines (HAAs), which are potential human carcinogens (5). AαC was originally discovered as a mutagenic pyrolysis product of protein (6) and subsequently identified in cigarette smoke at levels ranging from 60 - 250 ng/cigarette (7,8). These quantities are far greater than those of the aromatic amines 4-aminobipheynl and 2-naphthylamine, which are implicated in the pathogenesis of bladder cancer in smokers (1,9). Apart from the endocyclic nitrogen atoms, AαC shares the same structure as 2-aminofluorene, one of the most well studied aromatic amines, and heterocyclic aromatic amines (10). Significant levels of AαC were detected in the urine of male smokers of the Shanghai cohort in China, providing evidence that tobacco smoke is a major source of AαC exposure (11). AαC is a liver carcinogen in mice, a transgene colon mutagen and an inducer of colonic aberrant crypt foci, an early biomarker of colon neoplasia (12-14). Therefore, AαC could play a role in the incidence of liver or digestive tract cancers of smokers.

AαC undergoes metabolic activation by N-oxidation of the exocyclic amine group, by cytochrome P450 (P450) enzymes, to form 2-hydroxyaminono-9H-pyrido[2-3-b]indole (HONH-AαC) (Fig.1) (15,16). HONH-AαC can undergo conjugation reactions with N-acetyltransferases or sulfotransferases, to form unstable esters. These metabolites undergo heterolytic cleavage to form the proposed short-lived nitrenium ion of AαC (Fig.1) (17), which reacts with DNA to form covalent adducts, leading to mutations (18). The genotoxic potential of AαC has been shown in human peripheral blood lymphocytes (19), Chinese hamster ovary cells (18), and human hepatocytes (20), where high levels of AαC-DNA adducts are formed. However, long-term stable biomarkers of AαC must be developed for implementation in molecular epidemiology studies that seek to address a role for this chemical in human cancer risk.

DNA adducts of AαC can be measured by sensitive liquid chromatography/mass spectrometry (LC/MS)-based methods (20). However, DNA from biopsy specimens is often unavailable, and restricts the use of this biomarker. The electrophilic N-oxidized metabolites of AαC are also expected to react with proteins (21). The biomonitoring of protein-carcinogen adducts is an alternative approach to assess exposure to hazardous chemicals. Stable carcinogen protein adducts do not undergo repair and are expected to follow the kinetics of the lifetime of the protein (22,23). The major proteins in blood are hemoglobin (Hb) with a life-span of 60 days, and human serum albumin with a half-life of 21 days. The chemistry of reactivity of Hb and albumin with various genotoxicants and toxic electrophiles have been reported (24-26), and several protein-carcinogen adducts have been employed to assess human exposures (23,27,28).

Our goal is to develop and implement protein-based biomarkers of AαC and other HAAs (29,30) in molecular epidemiological studies designed to assess the role of HAAs in human cancers. In this study, we have examined the reactivity of albumin with N-oxidized metabolites of AαC. Cys34, followed by Tyr140 and Tyr150 of albumin were major sites of adduction of AαC electrophiles. The Cys34 and Met329 residues of albumin also served as scavengers of reactive oxygen species (ROS) generated by metabolites of AαC, and the Cys34 sulfonic acid (Cys-SO3H), sulfonic acid (Cys-SO2H), and Met329 sulfoxide were recovered in high yield from human hepatocytes treated with AαC.

**EXPERIMENTAL PROCEDURES**

**Caution — AαC is a potential human carcinogen.** AαC and its derivatives must be handled in a well-ventilated fume hood with proper use of gloves and protective clothing.

**Chemicals and Materials—** 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). Albumin, trypsin, chymotrypsin, pronase E, prolidase, leucine amino peptidase, β-mercaptoethanol (βME), iodoacetamide (IAM), dithiothreitol (DTT), acetic anhydride, and Pd/C were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). LC-MS grade solvents were from Fisher Scientific (Pittsburg, PA). Tetrahydrofuran
N-acetoxy-[\textsuperscript{13}C\textsubscript{6}]A\textalpha C (34) were reduced by treatment with albumin contained 0.98 mol Cys\textsubscript{34}/mol albumin upon Amicon Ultra centrifugal filters. The reduced albumin was recovered in 100 mM \textbeta ME (35). The reduced albumin was alkylated with a 100 molar excess of IAM (150 nmol) at 37 ºC for 1 h. Excess of IAM was removed by Amicon Ultra centrifugal filters.

Modification of albumin and human plasma with N-oxidized metabolites of A\textalpha C— Mixed disulfides formed at Cys\textsuperscript{34} of commercial albumin (34) were reduced by treatment with \textbeta ME (35). The reduced albumin was recovered in 100 mM potassium phosphate buffer (pH 7.4) employing Amicon Ultra centrifugal filters. The reduced albumin contained 0.98 mol Cys\textsuperscript{34}/mol albumin upon \textbeta ME treatment. An equimolar solution of HONH-A\textalpha C and HNOH-[\textsuperscript{13}C\textsubscript{6}]A\textalpha C or N-acetoxy-A\textalpha C and N-acetoxy-[\textsuperscript{13}C\textsubscript{6}]A\textalpha C (15 nmol, in 10 µL EtOH) or NO-A\textalpha C (30 nmol in 10 µL EtOH) was reacted with albumin (0.6 nmol, 40 µg) in 1 ml of 100 mM potassium phosphate buffer (pH 7.4) at 37 ºC for 18 h. N-Acetoxy-A\textalpha C and N-acetoxy-[\textsuperscript{13}C\textsubscript{6}]A\textalpha C were prepared in situ by adding the HONH-A\textalpha C (15 nmol) to the solution of albumin, immediately followed by the addition of 450 nmol of acetic anhydride (36), and incubated at 37 ºC for 1 h. N-Acetoxy-A\textalpha C and N-acetoxy-[\textsuperscript{13}C\textsubscript{6}]A\textalpha C were prepared in situ by adding the HONH-A\textalpha C (15 nmol) to the solution of albumin, immediately followed by the addition of 450 nmol of acetic anhydride (36), and incubated at 37 ºC for 1 h. The unreacted A\textalpha C metabolites were removed by ethyl acetate extraction. Human plasma (5 µL, containing ~200 µg of albumin, 3 nmol) was diluted with 1 ml PBS and reacted with N-oxidized A\alpha C derivatives as described above. Albumin from plasma was purified affinity purification by Pierce albumin depletion kit. Other studies on A\alpha C-albumin adduct formation were carried out using lower amounts of N-oxidized A\alpha C (vide infra).

Human hepatocyte cell culture— Human samples were obtained from the Centre de Ressources Biologiques (CRB)-Santé of Rennes (http://www.crbsante-rennes.com). The research protocol was conducted under French legal guidelines and approved by the local institutional ethics committee. Hepatocytes were isolated by a two-step collagenase perfusion, and the parenchymal cells were seeded at a density of ~ 3 x 10\textsuperscript{6} viable cells /19.5 cm\textsuperscript{2} Petri dish in 3 mL of Williams’ medium with supplements as reported (20), except fetal calf serum was replaced with human albumin pretreated with \textbeta ME (1 g/L). After two days, the differentiated cells were incubated with A\alpha C (20,33).

Albumin and DNA adduct formation with A\textalpha C in Hepatocytes— Metabolism studies with A\alpha C (0 or 50 µM in DMSO, 0.01% v/v) were conducted for 24 h. A solution of 1:1 A\alpha C and [\textsuperscript{13}C\textsubscript{6}]A\textalpha C (50 µM) was employed for characterization of A\alpha C-adducts, whereas the DNA adduct studies employed A\alpha C (50 µM). The culture media containing albumin were removed after 24 h incubation and immediately stored at -80 ºC. The cells were washed with PBS and cell pellets were collected by centrifugation a 3500 g for 10 min at 4 ºC. Cells were stored at -80 ºC until further use. Cell viability was determined by methylthiazol-tetrazolium test and treatment with A\alpha C did not decrease cell viability (37). The media was extracted with 3 vol of ethyl acetate and then the albumin was recovered with >85% purity, by ethanol precipitation. For some analyses, the albumin (100 µg, 1.5 nmol) was alkylated with a 100 molar excess IAM (150 nmol) at 37 ºC for 1 h. Excess of IAM was removed by Amicon Ultra centrifugal filters.

Trypsin/chymotrypsin digestion— The digestion of A\alpha C-modified albumin (10 µg) was carried out using trypsin and chymotrypsin at protease:protein ratio, respectively, at 1:50 (w/w) and 1:25 (w/w), in 100 µL 50 mM ammonium bicarbonate buffer (pH 8.5) containing CaCl\textsubscript{2} (1 mM) at 37 ºC for 16-18 h (35)

Pronase E/leucine aminopeptidase/prolidase digestion— albumin (10 µg, 150 pmol) was digested with pronase E, leucine aminopeptidase and prlidase at a protease to protein ratios, respectively, at 1:2 (w/w), 1:30 (w/w) and 1:8 (w/w), in 50 mM ammonium bicarbonate buffer (pH 8.5) containing MnCl\textsubscript{2} (1 mM) at 37 ºC for 20 h (23). The A\alpha C-
Characterization of AaC-serum albumin adducts

UPLC-Mass Spectrometry Parameters for Peptide Analyses—Peptides were resolved with an Atlantis C18 nanoACQUITY column (0.3 mm x 150 mm, 3 µm particle size, 100 Å) (Waters Corp., Milford, MA) using Solvent A (5% acetonitrile, 94.99% water, 0.01% formic acid) and Solvent B (95% acetonitrile, 4.99% water, 0.01% formic acid) as mobile phases with Thermo Dionex Ultimate 3000 Nano/Cap LC System connected to an Orbitrap Elite Mass spectrometer (Thermo Scientific, San Jose, CA) using an Advance CaptiveSpray source (Auburn, CA) in the positive ionization mode.

A 60 min gradient (99% A solvent to 60% B solvent for 45 min, 60 to 99% B solvent at 45-60 min) and 25 min gradient (99% A solvent to 60% B solvent for 20 min and 60% to 90% B solvent for 20-25 min) at 5 µL/min flow rate were used, respectively, for data dependent (DDA) and targeted data acquisition.

The top five precursor ions for CID-MS/MS analysis with dynamic exclusion for 180 s with a 3 repeats for 60 s repeat duration were selected for DDA. Mass-tag DDA (MS-tag DDA) was employed to trigger MS/MS on peptides and amino acid adducts displaying characteristic pattern of 1:1 to trigger MS/MS on peptides and amino acid adducts identified from MS-tag DDA were used for targeted analysis.

The tune parameters were: capillary tube temperature, 270 °C; spray voltage, 2.5 kV; S-lens RF level, 68 (%); in-source fragmentation, 5 V; collision gas, Helium; normalized collision energy, 35 eV; activation Q 0.3; HCD collision gas, Argon; HCD collision energy, 20 - 35. Full scan data were acquired over a mass range of 150-1800 m/z at a resolving power of 120,000 (at 400 m/z) and 200,000 (at 2000 m/z). The ions were monitored with the MS3 scan mode. For dG-C8-AαC, ions at m/z 449.1 (MS) > 333.1 (MS²) > 209.2, 291.4, 316.4 (MS³) and for the internal standard, [13C10]-dG-C8-AαC, ions at m/z 459.1 (MS) > 338.1 (MS²) > 210.2, 295.4, 321.5 (MS³) were monitored (40).

RESULTS

The metabolic activation of AαC in hepatocytes, and reaction of N-oxidized AαC metabolites with DNA and albumin, and the scavenging of ROS by albumin are summarized in Fig. 1.

The UV spectra of AαC, HONH-AαC, and NO-AαC are displayed in Fig 2A. NO-AαC displays a redshift in its maximal absorbance compared to
AaC and HOHN-AaC. The product ion spectrum of AaC ([M+H]+ at m/z 184.1) displays fragment ions at m/z 167.0 and 157.1, attributed to the loss of NH3 and HCN (Fig 2B) (9). The product ion spectrum of NO-AaC ([M+H]+; m/z 198.1) displays a sole ion at m/z 168.0 and assigned to the loss of the NO group (Fig 2C). The product ion spectrum of HONH-AaC ([M+H]+; m/z 200.1) displays prominent fragment ions at m/z 182.1 and 183.1, which are attributed to the losses of H2O and OH+. The product ion at m/z 155.1 is attributed to a loss of H2O and HCN (Fig 2D).

**MS-tag DDA mapping of AaC-albumin peptides and AaC-amino acid adducts**— The assignment of AaC-peptide adducts are based on CID and higher-energy collision dissociation (HCD) tandem MS.

The chromatograms of the MS-tag DDA experiments of albumin modified with a 50-fold molar excess of N-oxidize AaC and [13C6]AaC (1:1 ratio) were filtered using m/z values of the isotopic pair, i.e. for the radical cation (AaC++ at m/z 183.1, [13C6]AaC++ at m/z 189.1) and protonated ions (AaC+ at m/z 184.1 and [13C6]AaC at m/z 190.1). (Fig. 3). Nine AaC-peptide adducts (P1-P9) were detected in the tryptic/chymotryptic digest. The assignments of the peptide adduct sequences and accurate mass measurements of the amino acid adducts, following digestion with pronase E, leucine aminopeptidase, and prolidase, are listed in Table 1. Cys34, followed by Tyr140 and Tyr150 formed adducts with HONH-AaC; an additional adduct was formed at Tyr332 with N-acetoxy-AaC. The adduction of NO-AaC primarily occurred at Cys34, followed by much lower levels of adduct formation at Tyr150.

**MS characterization of adducts formed at Cys34 of albumin**— The digestion of AaC-modified albumin with trypsin/chymotrypsin produced peptides containing adducts at 31LQQCPFPEDHVK41 and 31LQQCPF EDHVK41. A total of six adducts with different oxidation states of the sulfur atom were identified. The product ion spectra of several adducts peptides are described in detail below. **LQQ*CPFEDHVK-AaC adducts**— The single missed cleavage peptide containing the proposed sulfonamide adduct LQQ*C[S=O]PF EDHVK (P2) (1555.7 Da), eluted at tR = 17.7 min and occurred as a triply charged species [M+3H]+ at m/z 519.6 (Fig. 4A). The 213 Da increase in mass over the non-modified peptide corresponds to the addition of AaC (182), and two oxygen atoms (32) minus one proton (1) on the –SH moiety. The product ion spectrum of m/z 519.6 displayed a series of -b ions and -y ions confirming the sequence assignment. The shift in masses at the *b2 and (*y3)2+ ions identify the site of adduction at the Cys34 residue. The proposed sulfanamide adduct LQQ*C[S=O]C=PF EDHVK (P3) (1539.7 Da) at tR = 17.8 min occurred as a triply charged species [M+3H]+ at m/z 514.2, a mass 16 Da less than the sulfonamide adduct. The product ion spectrum of m/z 514.2 contains minor fragment ions at m/z 183.1 (AaC**) and 184.1 (AaC+), with a base peak at m/z 679.9, corresponding to the sulfonium ion [M+2H-AaC]+ (Fig. 4B). The MS3 scan stage product ion spectrum of the proposed LQQ*[^S=O]PFEDHVK sulfoxide ion at m/z 679.9 [M'] showed -b and -y ions series in MS3 scan stage product ion spectrum at m/z 679.9 [M'] supports LQQ*[^S=O]PFEDHVK sulfoxide structure. Doubly charged product ion at m/z 648.9 attributed to the loss of CH3SO from the M’ ion and *b2-So,*b3,*y8 and *y9 ions support the proposed structure (Fig. 4C).

**LQQ*CPF-AaC peptide adducts**— Three additional S-N linked peptide adducts were identified. The product ion mass spectra of the doubly protonated ions are consistent with the AaC sulfonamide LQQ*C[S=O2]PF (P4) ([M+H]+ at m/z 948.4, [M+2H]+ at m/z 474.7, tR 17.9 min) (Fig. 4D), AaC sulfanamide LQQ*C[S=O]PF (P7) ([M+H]+ at m/z 932.4, [M+2H]+ at m/z 466.7, tR 20.4 min) (Fig. 4E-F), and AaC sulfenamide LQQ*[^AaC]PF (P8) ([M+H]+ at m/z 916.4, [M+2H]+ at m/z 458.7, tR 21.5 min) (Fig. 4G).

The doubly charged peptide precursor ion [M+2H]+ at m/z 458.7 is a 181 Da increase in mass over the non-modified protonated peptide (m/z 735.3). This increase in mass corresponds to the addition of AaC (182) minus one proton (1) from the –SH moiety. The increase in mass of the peptide is consistent with the proposed sulfanamide linkage. The product ion spectrum of [M+2H]+ at m/z 458.7 (Fig. 4G) shows the -b ion and -y ion series, where *b4,*y5 ions provide evidence that addition of AaC occurred at the sulfhydryl group of Cys34. The product ion at m/z 439.3 (*b4-AaC-SH) occurs via the cleavage of the C-S bond (41,42).
prominent base peak observed at \( m/z \) 184.1 is attributed to protonated AaC (Fig. 4G). It is noteworthy that most arsulfenamide adducts undergo hydrolysis during proteolytic digestion, which generally makes these adducts difficult to detect (30, 43, 44).

**MS characterization **Cys-AaC adducts — The Cys sulfenamide and sulfinamide linked adducts of AaC underwent hydrolysis to produce AaC during proteolysis of albumin with pronase E, leucine aminopeptidase, and prolidase. Two peaks attributed to Cys-AaC adducts containing the S-dioxide linkage were identified (\( t_R = 10.9 \) and 11.5 min) (A1) in the UPLC/MS chromatogram (Fig. 5A-B). The precursor ions [M+H] were observed at \( m/z \) 335.0806, a value which is within 0.6 ppm of the calculated \( m/z \) value of the proposed sulfonamide structure (Table 1). The adducts were present in an approximate ratio of 4:6 in albumin modified with HONH-AaC, and a 2:8 ratio when albumin was modified with N-acetoxy-AaC (Fig. 5A-B). The adducts were formed at ~5 fold higher levels in albumin treated with N-acetoxy-AaC than albumin treated with HONH-AaC. The earlier eluting isomer underwent CID to preferentially form a radical cation at \( m/z \) 183.0790 (AaC\( ^+\)), whereas the second adduct favored the formation of the even electron ion at \( m/z \) 184.0867, the \( m/z \) of protonated ion of AaC\( ^+\) (Fig. 5C-D, Table 2). Other notable product ions were detected for both adducts but with different relative abundances: \( m/z \) 230.0382 ([AaC+SO\( ^-\)]); \( m/z \) 254.0922 ([M+H-NH\(_3\)-SO\(_2\)])\(^-\)); \( m/z \) 271.1189 ([M+H-SO\(_2\)])\(^-\)); and \( m/z \) 318.0547 ([M+H-H\(_2\)O] (Fig. 5C-D, Table 2).

Arsulfenamides are weak acids and the nitrogen anion of the sulfonamide linkage can form several tautomeric forms with hindered rotation about the S=N bond (45). However, the prominent differences in the product ion spectra combined with the inability to interconvert these Cys-AaC adducts at elevated temperature, suggest that the adducts are not conformational isomers.

The nitrenium ion of HONH-AaC can undergo charge delocalization to form the carbenium ion resonance form with electron deficiency centered at the C-3 position of the AaC skeleton (Fig. 5E) (46). We propose that one isomeric Cys-AaC adduct contains an S-N linkage, and the second adduct contains a thioether linkage, formed between Cys\(^{34}\)-SH group and possibly the C-3 atom of AaC (46). The S-N sulfenamide (or sulfinamide) and thioether adducts undergoes oxidation, by ROS generated by aerobic oxidation of N-oxidized AaC metabolites (Fig. 1) or during proteolysis, to form the sulfone and sulfone linkages (Fig. 5E) (47). Both adducts undergo CID to form the proposed sulfonium ion at \( m/z \) 230.0385 (Fig. 5F). These adducts were not chromatographically resolved in the trypic/chymotryptic digests of albumin. Large scale syntheses and NMR studies are required to elucidate the respective structures of these AaC-Cys-S-dioxide linked isomers. The Cys\(^{34}\) of albumin was reported to react with N-acetyl-p-benzoquinoneimine, the electrophilic metabolite of acetaminophen and formed two regioisomeric adducts (41).

**MS characterization of adducts formed at Tyr\(^{40,150,332}\) of albumin—** Three adducts were formed between Tyr residues of albumin and AaC (Table 1).

**L*YEIAR peptide adduct—** The product ion spectrum of the L*YEIAR (P5) adduct with a doubly charged peptide precursor ion [M+2H]\(^{2+}\) at \( m/z \) 473.3 (\( t_R = 14.1 \) min) displayed a series of -b ions and -y ions [identifies the sequence as \(^{139}\)L*Y[AaC]EIAR\( ^{144}\)] with the site of adduction at Tyr\(^{40}\) (Fig. 6A). The ion at \( m/z \) 763.4 is proposed to arise by the loss of AaC as a radical cation at \( m/z \) 183.1 [M+H–AaC\( ^+\)], followed by the neutral loss of quinone methide (106.1 Da) [M+H–AaC\( ^+\)-C\(_7\)H\(_6\)O]\(^-\) to form the ion at \( m/z \) 657.4 (Fig. 6B). The mass spectral data support the proposed structure as an O-linked adduct formed between oxygen atom of Tyr and possibly the C-3 atom of the heterocyclic skeleton of AaC (46). Further support for the proposed O-linkage is provided by the lower region of the HCD mass spectrum: ions are observed at \( m/z \) 183.0790 (\( m/z \) 183.0791, calculated) and \( m/z \) 199.0745 (\( m/z \) 199.0741, calculated) and attributed to, respectively, [AaC\( ^+\)] and [AaC+O\( ^+\)] (Fig. 6A-B). We recently reported a similar mechanism of fragmentation of an O-linked adduct formed between tyrosine and the HAA 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) (32).

**MS characterization of an O-linked Tyr-AaC adduct—** The proteolysis of albumin adducts with pronase E, leucine aminopeptidase, and prolidase
produced two Tyr linked adducts of AaC. A first set of Tyr-AaC and Tyr-[13C6]AaC adducts (A2) were observed at m/z 363.1449 (m/z 363.1451, calculated) and m/z 369.1629 (m/z 369.1631, calculated) were observed at tR 11.8 min. The product ion spectrum of the unlabelled Tyr-AaC adduct [M+H]+ at m/z 363.1449 displayed fragment ions at m/z 346.1186, 317.1395, and 302.1285 attributed, respectively, to the losses of NH3, H2CO2, and NH3 and CO2 (Fig. 6C, Table 2). The product ions at m/z 289.1210 [M+H-C2H5NO2]+ and 288.1137 [M+H-C2H4NO2]+ are proposed to arise by cleavage of the Cα and Cβ bond of tyrosine. The fragment ion at m/z 183.0791 is assigned to AaC**, and the ion at m/z 198.0665 is tentatively assigned as the protonated ion of the 2-imino-3-oxo derivative of AaC, with the loss of phenylalanine (165.0790 Da) as a neutral fragment. The ion at m/z 198.0665 provides evidence that adduct formation occurred between the 4-HO group of tyrosine and the AaC heterocyclic ring (Fig. 6D).

F*YAPELL-AaC adducts— Two minor Tyr adducts of AaC** with the peptide sequence 10F*Y[L[AlaC]APELL (P9A, P9B Fig. 3, Table 1) were identified at tR 14.5 and 15.8 min (Fig. 7A). The full scan spectra of both adducts displayed doubly charged [M+2H]2+ at m/z 517.3. The CID fragment ions of m/z 517.3 displaced the same characteristic −b ion and −y ion series attributed to the F*Y[A&C]APELL for both peptide adducts (Fig. 7A,CID). The shift in mass between the y3 and y5 ion series proves that the site of AaC adduction occurred at Tyr150 for both peptides (Fig. 7A). CID did not provide appreciable fragment ions at the low m/z region for either adduct. The doubly protonated ions [M+2H]2+ at m/z 517.3 were subjected to HCD to examine for potential fragmentation of the bond formed between the Tyr and AaC. The extracted ion chromatograms were generated at m/z 183.0791 and m/z 184.0869 (Fig. 7A, HCD). The earlier eluting adduct (P9A) at tR 14.5 min displayed a prominent fragment ion at m/z 184.0869, an ion attributed to protonated AaC (Fig. 7A, HCD at tR 14.5). The linkage of this adduct may have occurred between the C-3 or C-5 atom of the Tyr phenyl ring and the exocyclic amino group of AaC. The HCD product ion spectrum of the second adduct (P9C) at tR 15.8 min showed a mixture of product ions assigned as the radical cation of AaC** and protonated AaC, respectively, at m/z 183.0789 and m/z 184.0868 (Fig. 7A, HCD at tR 15.8). A Tyr linkage may have formed at the C-3 or C-7 atom of the heterocyclic ring of AaC, based on studies of nucleophilic trapping agents adducting at these sites of the nitrenium ion of AaC (46,48).

MS characterization of an amine-linked AaC Tyr adduct— The second set of Tyr-AaC and Tyr-[13C6]-AaC adducts (A3) eluted at tR 12.1 min (Fig. 7B). The protonated ions [M+H]+ were observed, respectively at m/z 361.1293 and m/z 367.1475, a mass 2 Da less than the Tyr-AaC and Tyr-[13C6]-AaC adducts (m/z 363.1449 and m/z 369.1631) described above (Fig. 7B, Table 2). The structure of the adduct is proposed to be a quinoneimine linked adduct, which occurs by oxidation of P9A (Fig 7B) during proteolytic digestion with the three enzyme mixture. The product ion spectrum of [M+H]+ at m/z 361.1293 shows ions at m/z 344.1025, 315.1239, and 300.1130, which are of losses of, respectively, NH3, H2CO2, and NH3 and CO2 (Fig. 7B, Table 2). The product ion at m/z 288.1129 is proposed to arise following ring-opening of the protonated quinoneimine ring (Fig. 7C). The Tyr-[13C6]-AaC adduct displays the same product ions as the unlabeled adduct but shifted by 6 m/z.

A third peptide adduct formed between AaC and Tyr was observed only in the albumin modified with N-acetoxy-AaC. The CID-MS/MS spectrum of doubly charged protonated precursor ion at m/z 616.8 at 19.3 min displayed typical −b and −y ion pattern attributing to LGMF*KY[AlaC]EY (P6) with AaC adduction at Tyr332 (Supplemental Fig. S1, upper panel). The [13C6] homologue of this adduct at m/z 619.8 displayed the same pattern of fragmentation (Supplemental Fig. S1 lower panel).

AaC-SA adduct formation as a function of concentration of N-oxidized AaC metabolites— The LQQc*([SOaAc]PFEDHVK (P3) sulfanimide adduct at Cys74 accounted for 73-80% of the total ion counts of AaC-peptide adducts, when commercial albumin was reacted with a 50-fold molar excess of N-oxidized AaC metabolites. In vivo, the exposure to AaC occurs at much lower levels than the physiological concentration of albumin, and relative abundances of adducts formed may be different from those adducts formed at elevated exposures to AaC in vitro. Therefore, albumin was treated with HONH-AaC or N-acetyloxy-AaC over a millon fold range of carcinogen per mol albumin (1 to 106) (Fig.
8A-B). We also examined the effect of plasma matrix components on the reactivity of albumin with N-oxidized AaC metabolites. Representative UPLC-MS chromatograms of the peptide adducts recovered from commercial albumin or albumin in plasma modified with 1 molar equivalent of N-acetoxy-AaC are shown in Supplemental Fig. 2S.A-B.

The Tyr-peptide adducts of AaC at Tyr140, 150, 332 residues were only detected when albumin was reacted with ≥ 0.01 mol N-acetoxy-AaC per mol albumin. In contrast, adducts were still formed at Cys34 with 1 x 10^{-5} molar ratio of N-acetoxy-AoC:albumin (Fig. 8A-B), and LQQ*C[SO2H]PFEDHVK sulfinamide was the major adduct. The amounts of AaC-peptide adducts formed were ~5-40 times higher in reactions of albumin conducted with N-acetoxy-AaC than those amounts of albumin adducts formed with HONH-AoC (Figure 8A-B). The level of AaC adduct formation with albumin in plasma was several fold lower than adduct levels formed with commercial albumin (data not shown). Plasma albumin adducts were not detected at Tyr140, 150, 332 residues at any concentration of N-oxidized AaC, and the sulfinamide LQQ*C[SO2H]PFEDHVK was the predominant adduct.

AaC-DNA and AaC-albumin adduct formation in human hepatocytes—Reactive N-oxidized intermediates of AaC are formed and adduct to DNA in human hepatocytes and albumin in human hepatocytes (Fig. 1) (20,33). The basal activities of P450 1A1 and 1A2, two major isoforms involved in N-oxidation of AaC (16,17), were measured in human hepatocytes from three donors using ethox-yresorufin and methoxyresorufin as substrates (33). The level of dG-C8-AaC adduct formation from the two donors (A and C) with the highest P450 1A1 and 1A2 activities produced higher levels of dG-C8-AaC than donor B with low enzyme activity (Fig. 9A).

The occurrence of the LQQ*C[SO2A]PFEDHVK as the major adduct in hepatocytes is similar to the findings of commercial albumin and albumin in plasma treated with N-oxidized AaC metabolites (Fig. 7A, Fig. 8A, Fig. 9B,C). The LQQ*C[SO2H]PF sulfinamide and LQQ*C[SO3H]PF sulfinamide were also identified, but occurred at lower ion abundances (Fig. 9B,C, Supplemental Fig. 3S.A-C). LQQ*C[SO2A]PF and LQQ*C[SO2H]PFEDHVK were not detected.

In the absence of stable, isotopically labelled internal standards, quantitative peptide adduct measurements and correlations to DNA adduct levels of AaC cannot be determined. However, the ion counts of the major albumin peptide adduct, LQQ*C[SO2A]PFEDHVK sulfinamide were greatest in donor C who also harbored the highest level of dG-C8-AaC (Fig. 9A-B,C). Very high levels of AaC also were recovered in all of the hepatocytes (Fig. 9B,C). The occurrence of AaC is attributed to hydrosyis of S-N linked albumin-AaC adducts during proteolysis and not to unmetabolized AaC bound to albumin, because the isolation procedure effectively removed all unbound AaC from albumin in the cell culture media (Unpublished observations, K. Phatak).

Oxidative status of albumin—Cys34 and albumin-Met29 in human hepatocytes exposed to AaC—N-Oxidized metabolites of arylamines generate reactive oxygen species (ROS) (49). We sought to determine if AaC had induced oxidative stress in hepatocytes by identification of oxidation products of Cys34 and Met residues of albumin (50-52). The oxidized sulfinic LQQ*C[SO2H]PF and sulfonic LQQ*C[SO3H]PFEDHVK acids of Cys34 of albumin were monitored (29,32), and LQQCPF was measured following derivatization of albumin with iodoacetamide (IAM) (29). Elevated levels of the protonated [M+H]+ peptides for LQQ*C[SO2H]PFEDHVK at m/z 688.3 and LQQ*C[SO3H]PFEDHVK at m/z 696.3 (29) were detected in all three sets of hepatocytes treated with AaC (Fig 9D). The product ion spectra at m/z 688.3 and m/z 696.3 displayed typical –b and –y ion series type fragment ions (Supplemental Fig.4S A-B) and permitted the assignments as the peptide sequences of the Cys sulfinic and sulfonic acids (29). The levels LQQ*C[SO2H]PF FEDHVK and LQQ*C[SO3H]PF.
FEDHVK in AaC[13C6]AaC-treated hepatocytes were, respectively, 6 - 8 and 4 - 6 times higher than the levels in untreated controls, and the levels of IAM-derivatized LQQ*CPF were decreased by more than 10-fold in AaC-treated hepatocytes (Fig. 9B,D). Myrimatch search for oxidation sites in albumin also identified oxidation at Met329 residue of albumin. The product ion spectrum of, trypsin/chymotryptic representing Met 329 oxidation resulted in typical –FEDHV product ions further confirmed oxidation at Met329 (Supplemental Fig. 4S). From targeted analysis, the level of DVFLGM[O]F at m/z 844.4 representing Met329 oxidation resulted in typical –b and –y type of fragment ions where, –y2 and –yb6 product ions further confirmed oxidation at Met329 (Supplemental Fig. 4SC). From targeted analysis, the level of DVFLGM[O]F was ~6 times higher in AaC[13C6]AaC-treated hepatocytes than in untreated hepatocytes (Fig. 9B,D).

DISCUSSION

Primary human hepatocytes are an ideal ex vivo model system for studying metabolism, bioactivation and mechanisms of toxicity of carcinogens, because cofactors are present at physiological concentrations and biotransformation pathways may closely simulate those which occur in vivo (53). In this study, we investigated the metabolic activation of AaC, a rodent liver carcinogen, in human hepatocytes, and examined the reactivity of its genotoxic N-oxidized metabolites of AaC with DNA and albumin. Our goal is to develop and implement albumin-based biomarkers of AaC and other HAAs in molecular epidemiological studies designed to assess the role of HAAs in human cancers (54). The Cys34 residue was the major nucleophilic site of albumin to form adducts with AaC, followed by Tyr140 and Tyr150, which formed adducts at minor levels. Another HAAs, PhiP (55), also primarily formed adducts at the Cys34 of albumin with considerably lower levels of adducts occurring at Tyr140 and Tyr150 (56). In contrast, the Tyr140 and Tyr150 residues of albumin are the preferred site for adduct formation of neurotoxic organophosphate compounds (57-59).

The preliminary characterization AaC-albumin adducts was performed in vitro. The formation of AaC-albumin adducts was greatly enhanced by the in situ generation of N-acetoxy-AaC, which undergoes heterolytic cleavage to produce the nitrenium ion (48). N-Acetoxy-AaC is a penultimate metabolite of AaC that adducts to DNA and proteins (17). The N-acetoxy intermediates of HON-AaC, and other N-hydroxylated HAAs, such as 2-hydroxyamino-3-methylimidazo[4,5-f]quinoline and 2-hydroxyamino-3,8-dimethylimidazo[4,5-f]quinoline are unstable and cannot be isolated (60). However, the in situ formation of these N-acetoxy intermediates in the presence of DNA, by reaction of the N-hydroxylated HAAs with acetic anhydride, increased the levels of DNA adducts by 10 to 30 fold (36,61,62). Using a similar reaction scheme, we showed that adduct formation of HON-AaC with albumin was also greatly enhanced by the in situ generation of N-acetoxy-AaC (Fig. 8A-B). The Lys, Arg, Cys, and Tyr residues of albumin can potentially compete with HONH-AaC and undergo acetylation with acetic anhydride and influence the formation of different AaC-albumin adducts. However, the same AaC-albumin adducts were formed by reaction of albumin with a 50 mol excess of HONH-AaC, NO-AaC, or N-acetoxy-AaC generated in situ (Table 1). Moreover, the Myrimatch search engine did not detect acetylation at Lys, Arg, Cys, or Tyr residues of albumin under these reaction conditions (100 mM potassium phosphate buffer (pH 7.4, 37 ºC). Under low reaction conditions with HONH-AaC (10,000:1; albumin:HONH-AaC), the levels of Cys34 adducts were 50-fold greater when albumin was reacted with HONH-AaC in the presence of acetic anhydride than by reaction of albumin with HONH-AaC alone; thereby, demonstrating that N-acetoxy-AaC is efficiently formed in situ and readily reacts with nucleophilic sites of albumin to form covalent adducts (Fig. 8A-B).

Human hepatocytes efficiently bioactivated AaC to electrophilic N-oxidized metabolites, which formed covalent adducts with DNA and albumin (Fig. 9). The DNA adduct, dG-C8-AaC, was formed at relatively high levels, ranging from 2 – 12 adducts per 10⁶ DNA bases, consistent with our previous data (20,33). dG-C8-AaC was also previously identified in salivary DNA of smokers (63). The Cys34 residue was the sole site of albumin found to form adducts with AaC metabolites in hepatocytes: both sulfenamide and sulfaminate adducts were identified. However, the ion counts of AaC recovered from the albumin digest were 100-fold or greater than the ion counts of any of the AaC-Cys adducts. These findings signify that a large
Characterization of \( A_oC \)-serum albumin adducts

The proportion of the S-N linked sulfinamide adducts \( L QQ^*C^[SO_2H]P F E D H V K \) and \( L QQ^*C^[SO_3H]P F E D H V K \), or the sulfinamide adduct \( L QQ^*C^[Ac]P F \) of albumin underwent hydrolysis during proteolysis. In the absence of stable peptide adducts, or isotopically labelled \( L QQ^*C^[SO_2H]P F E D H V K \) peptides for internal standards, it is difficult to determine the relative reactivity of N-oxidized \( A_oC \) metabolites with DNA and albumin in hepatocytes.

HONH-\( A_oC \) and NO-\( A_oC \) undergo redox cycling in hepatocytes and produce ROS (Fig. 1) (49,64). Previous studies reported that structurally related aromatic amines, some of which are present in tobacco smoke (65,66), deplete glutathione levels in liver or ex-vivo in hepatocytes of rodents and induce oxidative DNA damage (67-69); however, the oxidation of albumin was not reported in those studies. In our study, we show that albumin scavenges ROS produced by metabolites of \( A_oC \) in human hepatocytes by formation of the oxidized Cys34 containing peptides \( L QQ^*C^[SO_2H]P F E D H V K \), \( L QQ^*C^[SO_3H]P F E D H V K \), and the methionine oxidation peptide, DVFLGM[O]. The level of Cys34 of albumin alkylated with IAM prior to proteolytic digestion decreased by more than 90% in hepatocytes treated with \( A_oC \) and provides strong evidence that Cys34 of albumin is scavenging ROS (Fig. 9C). Together with Cys34, six Met residues of albumin display antioxidant activity towards \( O_2^* \), \( H_2O_2 \), and HOCI (51,52). Met329 was the primary site of Met oxidation in albumin from human hepatocytes treated with \( A_oC \). The oxidation of Met residues, particularly Met329, has been observed in albumin of hemodialysis patients (52).

The major adducts of \( A_oC \) formed with albumin were determined in human hepatocytes, by employing data-dependent scanning and bottom-up proteomics approaches, and the adduction products were the same as those adducts formed in vitro with commercial albumin reacted with N-oxidized \( A_oC \) intermediates. The dose of \( A_oC \) (50 µM) employed in human hepatocytes is greater than daily human exposure to \( A_oC \), but comparable to the doses employed in studies investigating the genotoxicity of \( A_oC \) (18,19). The amount of \( A_oC \) arising in mainstream smoke is 60 - 250 ng/cigarette (7,8). Thus, the intracellular or plasma levels of \( A_oC \) found in humans exposed to this tobacco carcinogen are considerably lower than the amount of \( A_oC \) employed in our hepatocyte study. However, dG-C8-\( A_oC \) formation occurs in a concentration-dependent manner in human hepatocytes treated with \( A_oC \) over a 10,000-fold concentration range (1 nM - 10 µM), signifying that the reactive N-oxidation metabolites of \( A_oC \) are formed at physiological exposure levels (20). More sensitive mass spectrometric-based methods are required for measuring \( A_oC \)-albumin adducts and albumin oxidation products in hepatocytes at these lower exposure conditions to \( A_oC \).

The Cys34 of albumin accounts for 80% of total free thiol content in plasma and considered a major antioxidant and scavenger of electrophiles in plasma (34). A number of genotoxicants and toxic electrophiles form adducts with the Cys34 of rodent or human albumin (23). Many of these adducts have been characterized primarily in vitro and several adducts have been detected in humans. Albumin adducts have been identified with acrylamide (70), nitrogen mustard (71), \( \alpha,\beta \)-unsaturated aldehydes (26), the neurotoxin brevetoxin B (72), acetalenopihene (41), benzene (23), and several N-oxidized HAAs of diverse structures (32,35,56,73). In addition, aldehydes produced in tobacco smoke are scavenged by Cys34 of albumin in vitro (74). Immunochemical techniques have shown carbonyl residues are formed with albumin exposed to cigarette smoke extract, and the carbonylation of albumin, by tobacco smoke, has been detected in lung tissue biopsy samples of smokers (75). Spectrophotometric assays have shown a decrease in the free Cys34 content of albumin following exposure to cigarette smoke extracts in vitro (74,76). The diminution of free Cys34 content may be attributed to adducts formed with aldehydes or by oxidation with ROS (26,74,77); however, a correlation between the level of adduct formation at Cys34 of albumin and cigarette smoking constituents remains to established in vivo. Recently, elevated levels of Cys34-SO_2H of albumin were detected in plasma of smokers in a small pilot study (78). \( A_oC \), other HAAs, and structurally related aromatic amines present in tobacco smoke represent a class of chemicals in tobacco smoke that may contribute to the chemical modification or oxidation of Cys34, and the oxidation of Met residues of albumin.

In summary, \( A_oC \), a rodent liver carcinogen (12), undergoes bioactivation and forms adducts
with DNA and albumin, and induces oxidative stress in human hepatocytes. Albumin is a potent scavenger of ROS species generated by AαC metabolites. AαC, other HAAs, and many aromatic amines that arise in mainstream tobacco smoke (5,65,66) undergo N-oxidation in humans (54). Some of these metabolites form adducts with DNA and protein, and also induce oxidative stress, which may be contributing factors to liver damage and cancer risk in smokers. The Cys^{34} adducts of N-oxidized HAAs and arylamines, or their hydrolysis products, and elevated levels of Cys^{34}-SO_2H and Cys^{34}-SO_3H of albumin may be potential biomarkers to assess exposure to these hazardous chemicals in tobacco smokers.
REFERENCES


Characterization of AαC-serum albumin adducts


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FOOTNOTES

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1To whom correspondence should be addressed: Robert J. Turesky, Masonic Cancer Center and Department of Medicinal Chemistry, University of Minnesota, Minneapolis, MN 55455, USA, Tel.: (612) 626-0141; Fax: (612) 624-3869; E-mail: rturesky@umn.edu.

2The abbreviations used are: $\alpha$C, 2-Amino-9H-pyrido[2-3-b]indole; N-acetoxy-$\alpha$C, N-acetoxy-9H-pyrido[2-3-b]indole; HONH-$\alpha$C, 2-hydroxyamino-9H-pyrido[2-3-b]indole; NO-$\alpha$C, 2-nitroso-9H-pyrido[2-3-b]indole; NO2-$\alpha$C 2-nitro-9H-pyrido[2-3-b]indole; P450, cytochrome P450; HAA, heterocyclic aromatic amines; Hb, hemoglobin; ; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine; βME β-mercaptoethanol; IAM, iodoacetamide; DMSO, dimethyl sulfoxide; SPE, solid phase extraction; THF, tetrahydrofuran; DDA, data dependent acquisition; MS/MS, tandem mass spectrometry; CID, collision induced dissociation; HCD, high-energy collision dissociation; UPLC, ultra performance liquid chromatography.
Characterization of AaC-serum albumin adducts
FIGURE LEGENDS

FIGURE 1. Bioactivation of AαC, albumin adduct formation and induction of ROS in human hepatocytes. AαC undergoes bioactivation by P450 to form HONH-AαC. The HONH-AαC and its N-O esters react with albumin to form AαC-Cys\textsuperscript{34} sulphenamide and sulfinamide adducts, and also react with DNA to form dG-C8-AαC. HONH-AαC can undergo further oxidation by P450, transition metals, or by oxygen to form NO-AαC and ROS. A redox cycling mechanism catalyzed by NADPH-P450 reductase can regenerate HONH-AαC (49,64). The superoxide anion or other ROS can oxidize S-N linked of Cys-AαC to form the sulfonamide linkage. The ROS generated by N-oxidized AαC also directly oxidize albumin to form the sulfinic and sulfonic acids of Cys\textsuperscript{34} and Met residues of albumin.

FIGURE 2. Characterization of AαC and its N-oxidized metabolites. (A) UV spectra of AαC, NO-AαC and HONH-AαC were obtained in methanol. Product ion spectra of (B) AαC, (C) NO-AαC, and (D) HONH-AαC were acquired by ion trap mass spectrometry.

FIGURE 3. Mass tag data dependent MS\textsuperscript{2} scanning of trypsin/chymotrypsin digest of albumin modified with 50 mol excess of an equimolar mixture of N-acetoxy-AαC and \textsuperscript{[13}C\textsubscript{6}] N-acetoxy-AαC. Reconstructed ion chromatograms for AαC at \textit{m/z} 183.1 and 184.1 from digests of albumin modified with N-acetoxy-AαC/ [\textsuperscript{13}C\textsubscript{6}] N-acetoxy-AαC (upper panel), or digests of non-modified albumin (lower panel). The chromatograms were acquired on ions exhibiting mass difference of \textit{m/z} 6 (for singly charged ions), \textit{m/z} 3 (for doubly charged ions) and \textit{m/z} 2 (for triply charged ions). The ion intensities were normalized to the same scale.

FIGURE 4. Product ion spectra of AαC adducts of (A) LQQC\textsuperscript{[SO\textsubscript{2}AαC]}PFEDHVK (P2) [M+3H]\textsuperscript{3+} at \textit{m/z} 519.6, (B) LQQC\textsuperscript{[SO\textsubscript{2}AαC]}PFEDHVK (P3) [M+3H]\textsuperscript{3+} at \textit{m/z} 514.2, (C) Consecutive reaction monitoring at the MS\textsuperscript{3} scan stage of LQQC\textsuperscript{[SO\textsubscript{2}AαC]}PFEDHVK (P3) targeting \textit{m/z} 679.9 product ion from second generation product ion spectrum of LQQC\textsuperscript{[SO\textsubscript{2}AαC]}PF (P4) [M+2H]\textsuperscript{2+} at \textit{m/z} 474.7, (E) LQQC\textsuperscript{[SO\textsubscript{2}AαC]}PF (P7) [M+2H]\textsuperscript{2+} at \textit{m/z} 466.7, (F) third generation product ion spectrum of [M+2H]\textsuperscript{2+} at \textit{m/z} 466.7 > 750.4 > , and (G) LQQC\textsuperscript{[AαC]}PF (P8) [M+2H]\textsuperscript{2+} at \textit{m/z} 658.7 of the trypsin/chymotrypsin digest of albumin modified with N-acetoxy-AαC and [\textsuperscript{13}C\textsubscript{6}] N-acetoxy-AαC. *denotes the fragment ions with AαC adduction.

FIGURE 5. AαC-Cys S-dioxide adducts of albumin obtained from pronase E/ prolidase/ leucine amino peptidase digest of albumin modified with HONH-AαC and [\textsuperscript{13}C\textsubscript{6}] HONH-AαC or N-acetoxy-AαC and [\textsuperscript{13}C\textsubscript{6}] N-acetoxy-AαC. Total ion chromatogram (TIC) at the MS\textsuperscript{2} scan stage of [M+H]\textsuperscript{+} at \textit{m/z} 335.0806 obtained from albumin modified with (A) HONH-AαC and (B) \textit{N}-acetoxy-AαC (lower panel). The product ion spectra of [M+H]\textsuperscript{+} at \textit{m/z} 335.0806 of C\textsuperscript{[SO\textsubscript{2}AαC]} at (C) at 10.9 min and (D) at 11.5 min, and proposed structures of isomers are shown. (E) Proposed formation of Cysteine-S-yl-dioxide-AαC isomeric adducts by reaction of Cys with the nitrenium – carbenium ion resonance forms of HONH-AαC, and CID fragmentation mechanism of sulfonium ion at \textit{m/z} 230.0385 in product ion spectra of isomers ([M+H]\textsuperscript{+} at \textit{m/z} 335.0806).

FIGURE 6. Product ion spectrum of AαC-Tyr peptide and mono amino acid adduct of albumin modified with N-acetoxy-AαC and [\textsuperscript{13}C\textsubscript{6}] N-acetoxy-AαC. TIC, HCD and CID product ion spectra of (A)
LY[^Ac][C]EIAR (P5) [M+2H]^2+ at m/z 473.3, tR 14.1 min, (B) proposed CID fragmentation mechanism of LY[^Ac][C]EIAR (P5) adduct [M+2H]^2+ at m/z 473.3. (C) TIC and (D) product ion spectrum of [M+H]^+ at m/z 363.1449 (A2) at tR 11.8 min, and (E) proposed CID fragmentation mechanism of AαC-Tyr adducts [M+H]^+ at m/z 363.1452.

**FIGURE 7.** Characterization of isomeric FY[^Ac][C]APELL peptides and an AαC-amine-linked Tyr amino acid adduct of albumin modified with N-acetoxy-AαC and[^13C6]N-acetoxy-AαC. TIC, HCD and CID product ion spectra of (A) FY[^Ac][C]APELL (P9) [M+2H]^2+ at m/z 517.3, tR 14.5 min and 15.8 min (CID spectra for both peptides were identical, only the spectrum of peptide at tR 14.5 min is shown). (B) TIC and CID product ion spectra of AαC-amine-linked Tyr amino acid adduct [M+H]^+ at m/z 361.1299 (A2) at tR 12.1 min. (C) Proposed CID fragmentation mechanism of AαC-Tyr adduct [M+H]^+ at m/z 361.1299. *Denotes the fragment ions with AαC adduction.

**FIGURE 8.** Ion counts of AαC-peptide adducts targeting at Cys^{34} and Tyr^{140,150} and free AαC recovered from trypsin/chymotrypsin digests of (A) albumin modified with 1/10^-2 molar equivalent of HONH-AαC or N-acetoxy-AαC, 0.3 µg digest/injection (B) albumin modified with 1/10^-4 molar equivalent of HONH-AαC or N-acetoxy-AαC, 1.0 µg digest/injection. Data are plotted as mean and standard deviation of ion counts (N = 3).

**FIGURE 9.** (A) Estimates of dG-C8-AαC adduct formation in hepatocytes of Donor A, B and C treated with 50 µM of AαC. UPLC-ESI/MS^{3} chromatograms of dG-C8-AαC from hepatocytes treated with 50 µM AαC and with DMSO (control). For dG-C8-AαC, ions at m/z 449.1 (MS) > 333.1 (MS^{2}) > 209.2, 291.4, 316.4 (MS^{3}) (upper level) and for the internal standard,[^13C10]-dG-C8-AαC, ions at m/z 459.1 (MS) > 338.1 (MS^{2}) > 210.2, 295.4, 321.5 (MS^{3}) (lower level) were monitored. (B) UPLC-ESI/MS^{2} chromatograms of AαC-peptide adducts and Cys and Met oxidation products recovered from trypsin/chymotrypsin digests of albumin from hepatocytes of Donor A treated with AαC and[^13C6] AαC (50 µM) and Donor A treated with DMSO (Control). Ion counts of (C) AαC-peptide adducts and AαC, (D) Cys^{34} sulfinic acid, Cys^{34} sulfonic acid, Met^{329} sulfoxide, and LQQC^{34}PF alkylated with IAM obtained from trypsin/chymotrypsin digests of albumin of hepatocytes of Donor A, B and C treated with 50 µM equimolar mixture of AαC and[^13C6] AαC for 24 h or treated with DMSO (control). (Values are reported as the mean and standard deviation, N=3). *P < 0.01 using one way ANOVA for Donors A, B and C treated with 50 µM of AαC, **P < 0.05 for comparison between Donor A to B and Donor B to C using Tukey’s multiple comparison test for DNA adducts. *P < 0.01 and **P < 0.05 AαC treated versus control (DMSO treated) (2-tailed t test) for peptide adducts.
TABLE 1  AαC-peptide adducts identified using mass-tag data dependent acquisition

<table>
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<th>Peptide</th>
<th>Unlabeled (13C6labeled) observed precursor ions (m/z)</th>
<th>Z</th>
<th>M.W</th>
<th>tR (min)</th>
<th>AαC-peptide adduct</th>
<th>Site of modification</th>
<th>Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>481.9 (483.9)</td>
<td>3</td>
<td>1442.6</td>
<td>16.5</td>
<td>32QQ*C[AαC]PFEDHVK 41 sulfonamide a,b,c</td>
<td>Cys34</td>
<td></td>
</tr>
<tr>
<td>P2</td>
<td>778.9 (781.9)</td>
<td>2</td>
<td>1555.7</td>
<td>17.7</td>
<td>31LQQ*C[AαC]PFEDHVK 41 sulfonamide a,b,c</td>
<td>Cys34</td>
<td></td>
</tr>
<tr>
<td>P3</td>
<td>770.9 (773.9)</td>
<td>2</td>
<td>1539.7</td>
<td>17.8</td>
<td>31LQQ*C[AαC]PFEDHVK 41 sulfinamide a,b,c</td>
<td>Cys34</td>
<td></td>
</tr>
<tr>
<td>P4</td>
<td>948.4 (954.4)</td>
<td>1</td>
<td>947.4</td>
<td>17.9</td>
<td>31LQQ*C[AαC]PF37 sulfinamide a,b,c</td>
<td>Cys34</td>
<td></td>
</tr>
<tr>
<td>P5</td>
<td>473.3 (476.3)</td>
<td>2</td>
<td>944.5</td>
<td>19.0</td>
<td>139L*Y[AαC]EIAR144 a,b</td>
<td>Tyr140 Trypsin- chymotrypsin</td>
<td></td>
</tr>
<tr>
<td>P6</td>
<td>616.8 (619.8)</td>
<td>2</td>
<td>1231.5</td>
<td>19.3</td>
<td>327LGMoxiFL*Y[AαC]EY334 a,b</td>
<td>Tyr332</td>
<td></td>
</tr>
<tr>
<td>P7</td>
<td>932.4 (938.4)</td>
<td>1</td>
<td>931.4</td>
<td>20.4</td>
<td>31LQQ*C[AαC]PF37 sulfinamide a,b,c</td>
<td>Cys34</td>
<td></td>
</tr>
<tr>
<td>P8</td>
<td>916.4 (922.4)</td>
<td>1</td>
<td>915.4</td>
<td>21.5</td>
<td>31LQQ*C[AαC]PF37 sulfenamide a,b,c</td>
<td>Cys34</td>
<td></td>
</tr>
<tr>
<td>P9(A,B)</td>
<td>517.3 (520.3)</td>
<td>2</td>
<td>1032.5</td>
<td>22.8</td>
<td>149F*Y[AαC]APELL155 a,b,c</td>
<td>Tyr150</td>
<td></td>
</tr>
</tbody>
</table>

Z – Charge state; tR - Retention time; M.W- Molecular weight; o HONH-AαC/ HONH-[13C6]AαC-albumin modified sample; ν N-acetoxy-AαC/ N-acetoxy-[13C6]-AαC-albumin modified sample; c NO-AαC- modified sample; 3-Enzyme mixture: pronase E, leucine aminopeptidase, and prolidase.
### Table 2 Accurate mass measurements of AaC amino acid adducts formed with Tyr and Cys

<table>
<thead>
<tr>
<th>AaC-amino acid adduct</th>
<th>Precursor ion/product ion assignment</th>
<th>Observed m/z</th>
<th>Molecular Formula</th>
<th>Calculated m/z</th>
<th>Error ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AaC-O-Tyr</strong></td>
<td>[M+H]+</td>
<td>363.1449</td>
<td>C_{20}H_{19}N_{4}O_{3}</td>
<td>363.1451</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>[M+H-H_3N]^+</td>
<td>346.1184</td>
<td>C_{20}H_{16}N_{3}O_{3}</td>
<td>346.1186</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>[M+H-CH_2O_2]^+</td>
<td>317.1392</td>
<td>C_{19}H_{17}N_{4}O</td>
<td>317.1396</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>[M+H-CH_3NO_2]^+</td>
<td>302.1285</td>
<td>C_{19}H_{16}N_{3}O</td>
<td>302.1287</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>[M+H-C_2H_5NO_2]^+</td>
<td>288.1129</td>
<td>C_{18}H_{14}N_{3}O</td>
<td>288.1131</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>[M+H-C_9H_11NO_2]^+</td>
<td>198.0659</td>
<td>C_{11}H_{8}N_{3}O</td>
<td>198.0662</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>[M+H-C_9H_10NO_3]^+</td>
<td>183.0789</td>
<td>C_{11}H_{9}N_{3}</td>
<td>183.0791</td>
<td>1.1</td>
</tr>
</tbody>
</table>

| **AaC-N=Tyr**         | [M+H]^+                             | 361.1293     | C_{20}H_{17}N_{4}O_{3} | 361.1295       | 0.6       |
|                      | [M+H-H_3N]^+                         | 344.1025     | C_{20}H_{14}N_{3}O_{3} | 344.1029       | 1.1       |
|                      | [M+H-CH_2O_2]^+                      | 315.1239     | C_{19}H_{15}N_{4}O  | 315.1240       | 0.7       |
|                      | M+H-CH_3NO_2^+                       | 300.1130     | C_{19}H_{14}N_{3}O  | 300.1131       | 0.7       |
|                      | [M+H-C_2H_5NO_2]^+                   | 288.1129     | C_{18}H_{14}N_{3}O  | 288.1137       | 2.8       |
|                      | [M+H-C_9H_7NO_3]^+                   | 183.0789     | C_{11}H_{8}N_{3}   | 183.0791       | 1.1       |
|                      | [M+H-C_9H_6NO_3]^+                   | 184.0868     | C_{11}H_{9}N_{3}   | 184.0869       | 0.5       |

| **AaC-HN-SO_2-Cys**   | [M+H]^+                             | 335.0804     | C_{14}H_{13}N_{2}O_3S | 335.0808       | 1.2       |
|                      | [M+H-H_3N]^+                         | 318.0546     | C_{14}H_{12}N_{2}O_3S | 318.0548       | 1.2       |
|                      | [M+H-O_3S]^+                         | 271.1193     | C_{14}H_{13}N_{4}O   | 271.1190       | 0.4       |
|                      | [M+H-H_3NO_3S]^+                     | 254.0928     | C_{14}H_{12}N_{3}O   | 254.0924       | -1.5      |
|                      | [M+H-C_9H_6O_4NS]^+                  | 230.0385     | C_{11}H_{8}N_{3}O   | 230.0384       | 0.3       |
|                      | [M+H-C_9H_5O_4NS]^+                  | 184.0867     | C_{11}H_{9}N_{3}   | 184.0869       | 1.0       |
|                      | [M+H-C_9H_6O_4NS]^+                  | 183.0790     | C_{11}H_{9}N_{3}   | 183.0791       | 0.5       |
Characterization of AaC-serum albumin adducts

Figure 1
Characterization of AaC-serum albumin adducts

Figure 2

![Graph and chemical structures](image-url)
Characterization of AaC-serum albumin adducts

Figure 3
Characterization of AαC-serum albumin adducts

Figure 4
Characterization of AoC-serum albumin adducts

Figure 5
Figure 6

A

B

C

D

Characterization of AaC-serum albumin adducts
Characterization of AaC-serum albumin adducts

Figure 7

A

B

C

[Image of Figure 7 with chemical structures and data]
Figure 8
Characterization of AaC-serum albumin adducts

Figure 9