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

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Background: The reactivity of AαC, a tobacco smoke carcinogen, was investigated with DNA and albumin of human hepatocytes.

Results: Hepatocytes bioactivate AαC to metabolites, which adduct to DNA and albumin.

Conclusion: Cys34 and Met329 of serum albumin are targets for AαC electrophiles.

Significance: AαC forms macromolecular adducts and induces oxidative stress, which may be contributing factors to liver damage and cancer risk in smokers.

2-Amino-9H-pyrido[2,3-b]indole (AαC) is a carcinogenic heterocyclic aromatic amine formed during the combustion of tobacco. AαC 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 AαC with proteins has not been studied. The genotoxic metabolites, 2-hydroxyamino-9H-pyrido[2,3-b]indole (HONH-AαC), 2-nitroso-9H-pyrido[2,3-b]indole (NO-AαC), N-acetyloxy-2-amino-9H-pyrido[2,3-b]indole (N-acetoxy-AαC), and their [13C6]AαC-labeled homologues were reacted with albumin. Sites of adduction of AαC to albumin were identified by data-dependent scanning and targeted bottom-up proteomics approaches employing ion trap and Orbitrap MS. AαC-albumin adducts were formed at Cys34, Tyr140, and Tyr150 residues when albumin was reacted with HONH-AαC or NO-AαC. Sulfenamide, sulfinamide, and sulfonamide adduct formation occurred at Cys34 (AαC-Cys34). N-Acetoxy-AαC also formed an adduct at Tyr332. Albumin-AαC adducts were characterized in human plasma treated with N-oxidized metabolites of AαC and human hepatocytes exposed to AαC. High levels of N-(deoxyguanosin-8-yl)-AαC (dG-C8-AαC) DNA adducts were formed in hepatocytes. The Cys34 was the sole amino acid of albumin to form adducts with AαC. Albumin also served as an antioxidant and scavenged reactive oxygen species generated by metabolites of AαC in hepatocytes; there was a strong reduction in increased Cys34, whereas the levels of Cys34 sulfonic acid (Cys-SO2H), Cys34 sulfonyl acid (Cys-SO3H), and Met329 sulfoxide were greatly increased. Cys34 addition products and Cys-SO2H, Cys-SO3H, and Met329 sulfoxide may be potential biomarkers to assess exposure and oxidative stress associated with AαC and other arylamine toxicants present in tobacco smoke.

Tobacco smoke is a major risk factor not only for lung cancer but also for 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 pyrolysate product of protein (6) and subsequently identified in cigarette smoke at levels ranging from 60 to 250 ng/cigarette (7, 8). These quantities are far greater than those of the aromatic amines 4-aminobiphenyl 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 amine carcinogens (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-hydroxyamino-9H-pyrido[2,3-b]indole (HONH-Aoc) (Fig. 1) (15, 16). HONH-Aoc 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 Aoc (Fig. 1) (17), which reacts with DNA to form covalent adducts, leading to mutations (18). The genotoxic potential of Aoc has been shown in human peripheral blood lymphocytes (19), Chinese hamster ovary cells (18), and human hepatocytes (20), where high levels of Aoc-DNA adducts are formed. However, long term stable biomarkers of Aoc 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 Aoc can be measured by sensitive liquid chromatography/mass spectrometry (LC/MS)-based methods (20). However, DNA from biopsy specimens is often unavailable, which restricts the use of this biomarker. The electrophilic N-oxidized metabolites of Aoc 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 has 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 Aoc 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 Aoc. Cys34 followed by Tyr140 and Tyr150 of albumin were major sites of addition of Aoc electrophiles. The Cys34 and Met299 residues of albumin also served as scavengers of reactive oxygen species (ROS) generated by metabolites of Aoc, and the Cys34 sulfenic acid (Cys-SO2H), sulfonic acid (Cys-SO3H), and Met299 sulfoxide were recovered in high yield from human hepatocytes treated with Aoc.

**Experimental Procedures**

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

**Chemicals and Materials—Aoc** was purchased from Toronto Research Chemicals (Toronto, Canada). [4b,5,6,7,8,-8a-13C4]Aoc was a gift from Dr. Daniel Doerge (National Center for Toxicological Research, Jefferson, AR). Albumin, trypsin, chymotrypsin, Pronase E, prolidase, leucine aminopeptidase, β-mercaptoethanol, iodoacetamide (IAM), diethiothreitol (DTT), acetic anhydride, and palladium on carbon were obtained from Sigma-Aldrich. LC/MS grade solvents were from Fisher. Tetrahydrofuran was obtained from Alfa Aesar (Ward Hill, MA). Amicon ultra centrifugal filters (10 kDa cut-off) were purchased from Millipore (Billerica, MA). The Pierce albumin depletion kit was purchased from Thermo Scientific (Rockford, IL). Human plasma was purchased from Bioreclamation LLC (Hicksny, NY).

**Synthesis of N-Oxidized Metabolites of Aoc—2-Nitro-9H-pyrido[2,3-b]indole (NO2-Aoc) and NO2-13C4Aoc were prepared by oxidation of Aoc with dimethyldioxirane (17). HONH-Aoc and HONH-13C4Aoc were prepared by reduction of NO2-Aoc in tetrahydrofuran with hydrazine, using palladium on carbon as a catalyst (31). HONH-Aoc was oxidized to 2-nitroso-9H-pyrido[2,3-b]indole (NO-Aoc) with potassium ferricyanide (32). N-(Deoxyguanosin-8-yl)-Aoc (dG-C8-Aoc) and the isotopically labeled internal standards [13C10]dG-C8-Aoc were synthesized as described (33).

**Modification of Albumin and Human Plasma with N-Oxidized Metabolites of Aoc—Mixed disulfides formed at Cys34 of commercial albumin (34) were reduced by treatment with β-mercaptoethanol (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 of Cys34/mol of albumin upon β-mercaptoethanol treatment. An equimolar solution of HONH-Aoc and HNOH-13C4-Aoc or N-acetoxy-Aoc and N-acetoxy-13C4-Aoc (15 nmol, in 10 μl of EtOH) or NO-Aoc (30 nmol in 10 μl of EtOH) was reacted with albumin (0.6 nmol, 40 μg) in 1 ml of 100 mM potassium phosphate buffer (pH 7.4) at 37 °C for 18 h. N-Acetoxy-Aoc and N-acetoxy-13C4-Aoc were prepared in situ by adding equimolar solution of HONH-Aoc and HONH-13C4-Aoc (15 nmol) to the solution of albumin, immediately followed by the addition of 450 μmol of acetic anhydride (36), and incubated at 37 °C for 1 h. The unreacted Aoc metabolites were removed by ethyl acetate extraction. Human plasma (5 μl, containing ~200 μg of albumin, 3 nmol) was diluted with 1 ml of PBS and reacted with N-oxidized Aoc derivatives as described above. Albumin from plasma was purified by affinity purification by Pierce albumin depletion kit. Other studies on Aoc-albumin adduct formation were carried out using lower amounts of N-oxidized Aoc (see below).

**Human Hepatocyte Cell Culture—**Human samples were obtained from the Centre de Ressources Biologiques (CRB)-Santé of Rennes. 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 × 104 viable cells/19.5-cm2 Petri dish in 3 ml of Williams’ medium with supplements as reported (20), except fetal calf serum was replaced with human albumin (1 g/liter) pretreated with β-mercaptoethanol. After 2 days, the differentiated cells were incubated with Aoc (20, 33).

**Albumin and DNA Adduct Formation with Aoc in Hepatocytes—**Metabolism studies with Aoc (0 or 50 μM in DMSO, 0.01% (v/v)) were conducted for 24 h. A solution of 1:1 Aoc and 13C4-Aoc (50 μM) was employed for characterization of Aoc-albumin adducts, whereas the DNA adduct studies employed Aoc (50 μM). The culture media containing albumin were removed after 24 h of incubation and immediately stored at −80 °C. The cells were washed with PBS, and cell pellets were collected by centrifugation at 3500 × g for 10 min at 4 °C. Cells were stored at −80 °C until further use. Cell viability was determined by 3-(4,5-dimethylazol-2-yl)-2,5-diphenyltetrazolium
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bromide test, and treatment with AαC did not decrease cell viability (37). The medium was extracted with 3 volumes 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-fold molar excess of IAM (150 nmol) at 37 °C for 1 h. Excess IAM was removed by Amicon Ultra centrifugal filters.

Trypsin/Chymotrypsin Digestion—The digestion of AαC-modified albumin (10 μg) was carried out using trypsin and chymotrypsin at a protease/protein ratio of 1.50 (w/w) and 1:25 (w/w), respectively, in 100 μl of 50 mM ammonium bicarbonate buffer (pH 8.5) containing CaCl₂ (1 mM) at 37 °C for 16–18 h (35).

Pronase E/Leucine Aminopeptidase/Prolidase Digestion—Albumin (10 μg, 150 pmol) was digested with Pronase E, leucine aminopeptidase, and prolidase at protease/protein ratios of 1:2 (w/w), 1:30 (w/w), and 1:8 (w/w), respectively, in 50 mM ammonium bicarbonate buffer (pH 8.5) containing MnCl₂ (1 mM) at 37 °C for 20 h (23). The AαC mixture of AαC adducts displaying the characteristic pattern of a 1:1 isotopic difference of 6 (singly charged), 3 (doubly charged), and 2 (triply charged) species. A mass list of precursor ions (Table 1) for AαC-amino acid and AαC-peptide adducts identified from MS tag DDA was used for targeted analysis.

The five precursor ions for CID-MS/MS analysis with dynamic exclusion for 180 s with three repeats of 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 the characteristic pattern of a 1:1 isotopic mixture of AαC and [13C6]AαC (38). The partner intensity ratio was 85–100%; m/z difference of 6 (singly charged), 3 (doubly charged), and 2 (triplly charged) species. A mass list of precursor ions (Table 1) for AαC-amino acid and AαC-peptide adducts identified from MS tag DDA was used for targeted analysis.

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the isotopic pair (i.e., for the radical cation (AαC\(^{+}\) at \(m/z\) 183.1, \([^{13}\text{C}_6]\text{AαC}\^{+}\) at \(m/z\) 189.1) and protonated ions (AαC\(^{+}\) at \(m/z\) 184.1 and \([^{13}\text{C}_6]\text{AαC}\) at \(m/z\) 190.1)) (Fig. 3). Nine AαC-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-AαC; an additional adduct was formed at Tyr332 with N-acetoxy-AαC. The adduction of NO-AαC 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 AαC-modified albumin with trypsin/chymotrypsin produced peptides containing adducts at 31LQQCPEDHVK\(^{-}\) and 32QQCPEDHVK\(^{-}\). 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\(^{-}\)CPEDHVK-AαC Adducts**—The single missed cleavage peptide containing the proposed sulfonamide adduct LQQ\(^{-}\)C\(^{[\text{SO}_2\text{AαC}]^{-}}\)PFEDHV (P2) (1555.7 Da), eluted at \(t_r = 17.7\) min and occurred as a triply charged species [M + 3H]\(^{3+}\) at \(m/z\) 519.6 (Fig. 4A). The 213-Da increase in mass over the non-modified peptide corresponds to the addition of AαC (182 Da) and two oxygen atoms (32 Da) minus one proton (1 Da) on the -SH moiety. The product ion spectrum of [M + 3H]\(^{3+}\) displayed a series of -b ions and -y ions confirming the sequence assignment. The shift in masses at the \(b_4\) and \(y_8\) ions identify the site of adduction at the Cys34 residue. The proposed sulfonamide adduct LQQ\(^{-}\)C\(^{[\text{SO}_2\text{AαC}]^{-}}\)PFEDHVK (P3) (1539.7 Da) at \(t_r = 17.8\) min occurred as a triply charged species [M + 3H]\(^{3+}\)
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**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 AαC (B), NO-AαC (C), and HONH-AαC (D) were acquired by ion trap mass spectrometry.

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 (AαC^−) and 184.1 (AαC^−), with a base peak at m/z 679.9, corresponding to the sulfonium ion [M + 2H-AαC]^2+ (Fig. 4B). The MS^3 scan stage product ion spectrum of the proposed LQQC[^SO]PFEDHVK sulfoxide ion at m/z 679.9 [M'] showed -b and -y ion series and supports the sequence assignment. Doubly charged product ion at m/z 649.9 attributed to the loss of CH₂SO from the M' ion and ^b_6-SO, and ^y_3-SO ions supports the proposed structure (Fig. 4C).

**LQQC[^SO]PF-AαC 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 AαC sulfonamide LQQC[^SO]PF (P4) ([M + H]^+ at m/z 948.4, [M + 2H]^+ at m/z 474.7, t_r = 17.9 min) (Fig. 4D), AαC sulfonamide LQQC[^SO]PF (P7) ([M + H]^+ at m/z 932.4, [M + 2H]^+ at m/z 466.7, t_r = 20.4 min) (Fig. 4, E and F), and AαC sulfonamide LQQC[^SO]PF (P8) ([M + H]^+ at m/z 916.4, [M + 2H]^+ at m/z 458.7 t_r 21.5 min) (Fig. 4G).

The doubly charged peptide precursor ion [M + 2H]^2+ of LQQC[^SO]PF (P8) [M + H]^+ 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 AαC (182 kDa) minus one proton (1 kDa) from the -SH moiety. The increase in mass of the peptide is consistent with the proposed sulfonamide linkage. The product ion spectrum of [M + 2H]^2+ at m/z 458.7 (Fig. 4G) shows the -b ion and -y ion series, where ^b_x, ^y_x ions provide evidence that adduction of AαC occurred at the sulphydrl group of Cys^34. The product ion at m/z 439.3 (^b_2-AαC-SH) occurs via the cleavage of the C-S bond (41, 42). The prominent base peak observed at m/z 184.1 is attributed to protonated AαC (Fig. 4G). It is noteworthy that most arylsulfonamide adducts undergo hydrolysis during proteolytic digestion, which generally makes these adducts difficult to detect (30, 43, 44).

**MS Characterization of *Cys-AαC Adducts**—The Cys sulfenamide- and sulfonamide-linked adducts of AαC underwent hydrolysis to produce AαC during proteolysis of albumin with Pronase E, leucine aminopeptidase, and prolinease. Two peaks attributed to Cys-AαC adducts containing the S-dioxide linkage were identified (t_r = 10.9 and 11.5 min) (A1) in the UPLC/MS chromatogram (Fig. 5, A and B). The precursor ions [M + H]^+ were observed at m/z 335.0806, a value that 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-AαC and in a 2:8 ratio when albumin was modified with N-ace-toxy-AαC (Fig. 5, A and B). The adducts were formed at ~5-fold higher levels in albumin treated with N-ace-toxy-AαC than in albumin treated with HONH-AαC. The earlier eluting isomer underwent CID to preferentially form a radical cation at m/z 183.0790 (AαC^+), whereas the second adduct favored the formation of the even electron ion at m/z 184.0867, the m/z of protonated ion of AαC^+ (Fig. 5 and D) and Table 2). Other notable product ions were detected for both adducts but with different relative abundances: m/z 230.0382 ([AαC + SO]^+); m/z 254.0922 ([M + H-NH₂SO₄]⁺); m/z 271.1189 ([M + HSO₂]⁺); and m/z 318.0547 ([M + H₂O]) (Fig. 5 and D and Table 2).

Arylsulfonamides 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-AαC adducts at elevated temperature, suggest that the adducts are not conformational isomers.

The nitrenium ion of HONH-AαC can undergo charge delocalization to form the carbenium ion resonance form with electron deficiency centered at the C-3 position of the AαC skeleton (Fig. 5E) (46). We propose that one isomeric Cys-AαC adduct contains an S-N linkage, and the second adduct contains a thioether linkage, formed between the Cys34-SH group and possibly the C-3 atom of AαC (46). The S-N-sulfonamide (or sulfonamide) and thioether adducts undergo oxidation, by ROS generated by aerobic oxidation of sulfinamide and thioether adducts. The mechanism is consistent with the inability to interconvert these Cys-AαC adducts at elevated temperature, suggesting that the adducts are not conformational isomers.

**MS Characterization of Adducts Formed at Tyr140, Tyr150, and Tyr332 of Albumin**—Three adducts were formed between Tyr residues of albumin and AαC (Table 1).

| Peptide | Unlabeled ([13C6]labeled) Observed precursor ions (\(m/z\)) | AαC-peptide adduct | Site of modification | Enzyme |
|---------|-----------------------------------------------------------|---------------------|---------------------|--------|
| P1      | 481.9 (483.9)                                             |                     |                     |        |
| P2      | 778.9 (781.9)                                             |                     |                     |        |
| P3      | 770.9 (773.9)                                             |                     |                     |        |
| P4      | 104.4 (104.4)                                             |                     |                     |        |
| P5      | 616.8 (619.8)                                             |                     |                     |        |
| P6      | 932.4 (934.8)                                             |                     |                     |        |
| P7      | 916.4 (922.4)                                             |                     |                     |        |
| P8      | 1012.3 (1012.3)                                           |                     |                     |        |

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| Amino Acid | Unlabeled ([13C6]labeled) Observed precursor ions (\(m/z\)) | AαC-amino acid adduct | Site of modification | Enzyme |
|-----------|-------------------------------------------------------------|-----------------------|---------------------|--------|
| A1        | 335.0804 (341.0986)                                         | *Cys\(^{[13C6]}\) \(\text{AαC}\) |                     | Cys    |
| A2        | 363.1449 (369.1631)                                         | *Tyr\(^{[13C6]}\) \(\text{AαC}\) |                     | Tyr    |
| A3        | 361.1293 (367.1475)                                         | *Tyr\(^{[13C6]}\) \(\text{AαC}\) |                     | Tyr    |

\(^a\) HONH-AαC/PHONH-\(^{[13C6]}\) AαC-albumin-modified sample.
\(^b\) N-acetoxy-AαC/N-acetoxy-\(^{[13C6]}\) AαC-albumin-modified sample.
\(^c\) NO-AαC-modified sample.

\(\text{L}^\text{*YEIAR Peptide Adduct—}\) The product ion spectrum of the \(\text{L}^\text{*YEIAR (P5)}\) 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, which identifies the sequence as \(139L^\text{*YEIAR}^{[13C6]}\), with the site of adduction at Tyr140 (Fig. 6A). The ion at \(m/z\) 763.4 is proposed to arise by the loss of AαC as a radical cation at \(m/z\) 183.1 \([M + H - AαC]^+\), followed by the neutral loss of quinone methide (106.1 Da) [M - \(\text{C-Serum Albumin Adducts}\)] (32). 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 AαC (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 \(m/z\) 199.0741 (\(m/z\) 199.0741, calculated) and attributed to [AαC] + and [AαC-O] + , respectively (Fig. 6A and 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).

\(\text{MS characterization of an O-Linked Tyr-AαC Adduct—}\) The proteolysis of albumin adducts with Pronase E, leucine aminopeptidase, and prolidase produced two Tyr linked adducts of AαC. A first set of Tyr-AαC and Tyr-[\(^{13C6}\)labeled]AαC adducts (A2) was observed at \(m/z\) 363.1449 (\(m/z\) 363.1451, calculated) and \(m/z\) 369.1629 (\(m/z\) 369.1631, calculated) at \(t_r\) 11.8 min. The product ion spectrum of the unlabeled Tyr-AαC adduct...
FIGURE 4. A and B, product ion spectra of AαC adducts of LQQC\[^{[SO\text{A}_2\text{C}]}\]PFEDHVK (P2) \([M + 3H]^+\) at m/z 519.6 (A) and LQQC\[^{[SO\text{A}_2\text{C}]}\]PFEDHVK (P3) \([M + 3H]^+\) at m/z 514.2 (B). C, consecutive reaction monitoring at the MS\(^3\) scan stage of LQQC\[^{[SO\text{A}_2\text{C}]}\]PFEDHVK (P3) targeting m/z 679.9 product ion from second generation product ion spectrum of m/z 514.2. D, LQQC\[^{[SO\text{A}_2\text{C}]}\]PF (P4) \([M + 2H]^2+\) at m/z 474.7. E, LQQC\[^{[SO\text{A}_2\text{C}]}\]PF (P7) \([M + 2H]^2+\) at m/z 466.7. F, third generation product ion spectrum of \([M + 2H]^2+\) at m/z 466.7 > 750.4 > . G, LQQC\[^{[\text{A}_2\text{C}]}\]PF (P8) \([M + 2H]^2+\) at m/z 658.7 of the trypsin/chymotrypsin digest of albumin modified with N-acetoxy-AαC and N-acetoxy-[\(^{13}\text{C}_6\)]AαC. *, fragment ions with AαC adduction. 

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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 HONH-[13C6]AαC or N-acetoxy-AαC and N-acetoxy-[13C6]AαC. Shown is a total ion chromatogram (TIC) at the MS² scan stage of [M + H]+ at m/z 335.0806 obtained from albumin modified with HONH-AαC (A) and N-acetoxy-AαC (bottom) (B). The product ion spectra of [M + H]+ at m/z 335.0806 of C[SO2AαC] at 10.9 min (C) and at 11.5 min (D) 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 m/z 230.0385 in product ion spectra of isomers ([M + H]+ at m/z 335.0806).

TABLE 2
Accurate mass measurements of AαC amino acid adducts formed with Tyr and Cys

| AαC-amino acid adduct | Precursor ion/product ion assignment | Observed m/z | Molecular Formula | Calculated m/z | Error ppm |
|-----------------------|-------------------------------------|--------------|-------------------|----------------|-----------|
| AαC-O-Tyr             | [M+H]+                             | 363.1449     | C20H15N6O3        | 363.1451       | 0.5       |
|                       | [M+H-H2N]+                         | 346.1184     | C20H15N6O3        | 346.1186       | 0.5       |
|                       | [M+H-CH2O]⁺                        | 317.1392     | C19H14N6O        | 317.1396       | 1.0       |
|                       | [M+H-CH3NO2]⁺                      | 302.1285     | C19H14N6O        | 302.1287       | 0.7       |
|                       | [M+H-C3H7NO2]⁺                     | 288.1129     | C18H14N6O        | 288.1131       | 0.7       |
|                       | [M+H-C3H10NO4]⁺                    | 198.0659     | C18H12N6O3       | 198.0662       | 1.0       |
|                       | [M+H-C8H16NO6]⁺                    | 183.0789     | C17H12N3         | 183.0791       | 1.1       |
| AαC-N=Tyr             | [M+H]+                             | 361.1293     | C20H15N6O3        | 361.1295       | 0.6       |
|                       | [M+H-H2N]+                         | 344.1025     | C20H15N6O3        | 344.1029       | 1.1       |
|                       | [M+H-CH2O]⁺                        | 315.1239     | C19H14N6O        | 315.1240       | 0.7       |
|                       | [M+H-CH3NO2]⁺                      | 300.1130     | C19H14N6O        | 300.1131       | 0.7       |
|                       | [M+H-C3H7NO2]⁺                     | 288.1129     | C18H14N6O        | 288.1137       | 2.8       |
|                       | [M+H-C3H10NO4]⁺                    | 183.0789     | C17H12N3         | 183.0791       | 1.1       |
|                       | [M+H-C8H16NO6]⁺                    | 184.0868     | C17H10N3         | 184.0869       | 0.5       |
| AαC-HN-SO2-Cys        | [M+H]+                             | 335.0804     | C14H15N6O4S      | 335.0808       | 1.2       |
|                       | [M+H-H2N]+                         | 318.0546     | C14H15N6O4S      | 318.0548       | 1.2       |
|                       | [M+H-CH2O]⁺                        | 271.1193     | C14H15N6O2       | 271.1190       | 0.4       |
|                       | [M+H-CH3NO2]⁺                      | 254.0928     | C14H15N6O2       | 254.0924       | -1.5      |
|                       | [M+H-C8H16NO6]⁺                    | 230.0385     | C11H7N3OS        | 230.0384       | 0.3       |
|                       | [M+H-C8H16NO4S]⁺                   | 184.0867     | C11H10N3         | 184.0869       | 1.0       |
|                       | [M+H-C8H16NO6S]⁺                   | 183.0790     | C11H10N3         | 183.0791       | 0.5       |
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[M + H]^+ at m/z 363.1449 displayed fragment ions at m/z 346.1186, 317.1395, and 302.1285 attributed to the losses of NH3, H2CO2, and NH3 and CO2, respectively (Fig. 6C and Table 2). The product ions at m/z 289.1210 [M + H-C3H5NO2]^+ and 288.1137 [M + H-C4H6NO2]^2+ 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 AαC^+, and the ion at m/z 198.0659 is tentatively assigned as the protonated ion of the 2-imino-3-oxo derivative of AαC, with the loss of phenylalanine (165.0790 Da) as a neutral fragment. The ion at m/z 198.0659 provides evidence that adduct formation occurred between the 4-HO group of tyrosine and the AαC heterocyclic ring (Fig. 6D).

P^149F-YAP^155[αα]C-PELL—Two minor Tyr adducts of AαC with the peptide sequence ^149F^155[P-Y[αα]C]PELL (P9A and P9B; Fig. 3 and Table 1) were identified at t R 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 P^149F^155[Y[αα]C]PELL for both peptide adducts (Fig. 7A, CID). The shift in mass between the y9 and y6 ion series proves that the site of AαC 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 AαC. 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 t R = 14.5 min displayed a prominent fragment ion at m/z 184.0869, an ion attributed to protonated AαC (Fig. 7A, HCD at t R = 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 AαC. The HCD product ion spectrum of the second adduct (P9C) at t R = 15.8 min showed a mixture of product ions assigned as the radical cation of AαC^+ and protonated AαC, respectively, at m/z 183.0789 and m/z 184.0868 (Fig. 7A, HCD at t R = 15.8). A Tyr linkage may have formed at the C-3 or C-7 atom of the heterocyclic ring of AαC, based on studies of nucleophilic trapping agents adducting at these sites of the nitrenium ion of AαC (46, 48).

MS Characterization of an Amine-linked AαC Tyr Adduct—The second set of Tyr-AαC and Tyr-[^13C6]AαC adducts (A3) eluted at t R = 12.1 min (Fig. 7B). The protonated ions [M + H]^+ were observed at m/z 361.1293 and 367.1475, respectively, a mass 2 Da less than the Tyr-AαC and Tyr-[^13C6]AαC adducts (m/z 363.1449 and 369.1631) described above (Fig. 7B and Table 2). The structure of the adduct is proposed to be a quinonemine-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 NH3, H2CO2, and NH3 and CO2, respec-
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A third peptide adduct formed between AoC and Tyr was observed only in the albumin modified with N-acetoxy-AoC. The CID-MS/MS spectrum of doubly charged protonated precursor ion at \( m/z \) 616.8 at 19.3 min displayed a typical -b and -y ion pattern attributable to LGMFL-Y[^AoC]EY (P6) with AoC adduction at Tyr^332 (supplemental Fig. S1, top). The [^13C_6]AoC homologue of this adduct at \( m/z \) 619.8 displayed the same pattern of fragmentation (supplemental Fig. S1, bottom).

**AoC-SA Adduct Formation as a Function of Concentration of N-Oxidized AoC Metabolites**—The LQQ[^C]O[^SO4]PFEDHVK (P3) sulfamidine adduct at Cys[^34] accounted for 73–80% of the total ion counts of AoC-peptide adducts, when commercial albumin was reacted with a 50-fold molar excess of N-oxidized AoC metabolites. In vivo, the exposure to AoC 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 AoC in vitro. Therefore, albumin was treated with HONH-AoC or N-acetoxy-AoC over a million-fold range of carcinogen/mol of albumin (1–10^−5) (Fig. 8, A and B). We also examined the effect of plasma matrix components on the reactivity of albumin with N-oxidized AoC metabolites. Representative UPLC/MS chromatograms of the peptide adducts recovered from commercial albumin or albumin in plasma modified with 1 molar eq of N-acetoxy-AoC are shown in supplemental Fig. S2, A and B.

The Tyr-peptide adducts of AoC at Tyr^140, Tyr^150, and Tyr^332 residues were only detected when albumin was reacted with ≥0.01 mol N-acetoxy-AoC per mol albumin. In contrast, adducts were still formed at Cys[^34] with a 1 × 10^−5 molar ratio of N-acetoxy-AoC/albumin (Fig. 8, A and B), and LQQ[^C]O[^SO4]PFEDHVK sulfamidine was the major adduct. The amounts of AoC-peptide adducts formed were ~5–40 times higher in reactions of albumin conducted with N-acetoxy-AoC than those amounts of albumin adducts formed with HONH-AoC (Fig. 8, A and B). The level of AoC adduct formation with albumin in plasma was severalfold lower than adduct levels formed with commercial albumin (data not shown). Plasma albumin adducts were not detected at Tyr^140, Tyr^150, or Tyr^332 residues at any concentration of N-oxidized AoC, and the sulfamidine LQQ[^C]O[^SO4]PFEDHVK was the predominant adduct.

**AoC-DNA and AoC-Albumin Adduct Formation in Human Hepatocytes**—Reactive N-oxidized intermediates of AoC 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 AoC (16, 17), were measured in human hepatocytes from three donors using ethoxyresorufin and methoxyresorufin as substrates (33). The level of dG-C8-AoC adduct formation...
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![Figure 8: Ion counts of \( \alpha C \)-peptide adducts targeting Cys\(^{34} \) and Tyr\(^{140} \) Tyr\(^{356} \) and free \( \alpha C \) recovered from trypsin/chymotrypsin digests of albumin modified with 1/10\(^{-2} \) molar eq of HONH-\( \alpha C \)-A or N-acetoxy-\( \alpha C \), 0.3 \( \mu g \) digest/injection (A) and albumin modified with 1/10\(^{-4} \) molar eq of HONH-\( \alpha C \) or N-acetoxy-\( \alpha C \), 1.0 \( \mu g \) digest/injection (B). Data are plotted as mean and S.D. (error bars) of ion counts (n = 3).](image)

from the two donors (A and C) with the highest P450 1A1 and 1A2 activities produced higher levels of dG-C8-\( \alpha C \) than donor B with low enzyme activity (Fig. 9A).

The mass chromatograms of \( \alpha C \)-albumin peptide adducts, following tryptic/chymotryptic digestion of albumin recovered from hepatocytes, are shown in Fig. 9B. The relative ion abundances of the peptide adducts are summarized in Fig. 9C, and product ion spectra are presented in supplemental Fig. S3. The major adduct, based on ion counts, is LQQ\(^{34} \)C\(^{[SO_2A\alpha C]} \)PFEDHVK sulfinamide, followed by LQQ\(^{34} \)C\(^{[SO_2A\alpha C]} \)PF and LQQ\(^{34} \)C\(^{[A\alpha C]} \)PF. The occurrence of the LQQ\(^{34} \)C\(^{[SO_3A\alpha C]} \)PFEDHVK as the major adduct in hepatocytes is similar to the findings of commercial albumin and albumin in plasma treated with N-oxidized \( \alpha C \) metabolites (Figs. 7A, 8A, and 9 (B and C)). The LQQ\(^{34} \)C\(^{[A\alpha C]} \)PF sulfinamide and LQQ\(^{34} \)C\(^{[SO_2A\alpha C]} \)PF sulfinamide were also identified but occurred at lower ion abundances (Fig. 9 (B and C) and supplemental Fig. S3 (A–C)).

LQQ\(^{34} \)C\(^{[SO_2A\alpha C]} \)PF and LQQ\(^{34} \)C\(^{[SO_3A\alpha C]} \)PFEDHVK were not detected.

In the absence of stable, isotopically labeled internal standards, quantitative peptide adduct measurements and correlations to DNA adduct levels of \( \alpha C \) cannot be determined. However, the ion counts of the major albumin peptide adduct, LQQ\(^{34} \)C\(^{[SO_3A\alpha C]} \)PFEDHVK sulfinamide, were greatest in donor C, who also harbored the highest level of dG-C8-\( \alpha C \) (Fig. 9, A–C). Very high levels of \( \alpha C \) also were recovered in all of the hepatocytes (Fig. 9, B and C). The occurrence of \( \alpha C \)-adducts is attributed to hydrolysis of S-N-linked albumin-\( \alpha C \)-adducts during proteolysis and not to unmethylized \( \alpha C \) bound to albumin, because the isolation procedure effectively removed all unbound \( \alpha C \) from albumin in the cell culture media.\(^3 \)

Oxidative Status of Albumin-Cys\(^{34} \) and Albumin-Met\(^{329} \) in Human Hepatocytes Exposed to \( \alpha C \)---N-Oxidized metabolites of arylamines generate ROS (49). We sought to determine if \( \alpha C \) had induced oxidative stress in hepatocytes by identification of oxidation products of Cys\(^{34} \) and Met residues of albumin (50–52). The oxidized sulfenic LQQ\(^{34} \)C\(^{[SO_2H]} \)PF and sulfonic LQQ\(^{34} \)C\(^{[SO_3H]} \)PFEDHVK acids of Cys\(^{34} \) of albumin were monitored (29, 32), and LQQCPF was measured following derivatization of albumin with IAM (29). Elevated levels of the protonated [M + H]\(^{+} \) peptides for LQQ\(^{34} \)C\(^{[SO_3H]} \)PFEDHVK at m/z 688.3 and LQQ\(^{34} \)C\(^{[SO_3H]} \)PFEDHVK at m/z 696.3 (29) were detected in all three sets of hepatocytes treated with \( \alpha C \) (Fig. 9D). The product ion spectra at m/z 688.3 and 696.3 displayed typical -b and -y ion series type fragment ions (supplemental Fig. S4, A and B) and permitted the assignments as the peptide sequences of the Cys sulfenic and sulfonic acids (29). The levels of LQQ\(^{34} \)C\(^{[SO_2H]} \)PFEDHVK and LQQ\(^{34} \)C\(^{[SO_3H]} \)PFEDHVK in \( \alpha C / [{}^{13}C_6] \alpha C \)-treated hepatocytes were 6–8 and 4–6 times higher, respectively, than the levels in untreated controls, and the levels of IAM-derivatized LQQ\(^{34} \)CPF were decreased by more than 10-fold in \( \alpha C \)-treated hepatocytes (Fig. 9, B and D). A Myrimatch search for oxidation sites in albumin also identified oxidation at the Met\(^{329} \) residue of albumin. The product ion spectrum of trypsin/chymotryptic peptide DVFLGM\(^{[O]} \)F at [M + H]\(^{+} \) at m/z 844.4 representing Met\(^{329} \) oxidation resulted in typical -b and -y types of fragment ions, where \(-y_6\) and \(-b_6\) product ions further confirmed oxidation at Met\(^{329} \) (supplemental Fig. S4C). From targeted analysis, the level of DVFLGM\(^{[O]} \)F was ~6 times higher in \( \alpha C / [{}^{13}C_6] \alpha C \)-treated hepatocytes than in untreated hepatocytes (Fig. 9, B and 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 that occur in vivo (53). In this study, we investigated the metabolic activation of \( \alpha C \), a rodent liver carcinogen, in human hepatocytes and examined the reactivity of its genotoxic N-oxidized metabolites of \( \alpha C \) with DNA and

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\(^3\) K. Phatak, unpublished observations.
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albumin. Our goal is to develop and implement albumin-based biomarkers of AαC 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 AαC, followed by Tyr140 and Tyr150, which formed adducts at minor levels. Another HAA, PhIP (55), also primarily formed adducts at the Cys34 of albumin to form adducts with AαC (50 μM) and donor A treated with DMSO (control). Shown are ion counts of AαC-peptide adducts and AαC (C) and Cys34 sulfonic acid, Cys34 sulfenic acid, Met329 sulfoxide, and LQQC34PF alkylated with IAM obtained from trypsin/chymotrypsin digests of albumin of hepatocytes of donor A, B, and C treated with a 50-fold molar excess of AαC (50 μM) and donor A treated with DMSO (control) (D). Values are reported as the mean and S.D. (error bars) (n = 3). *, p < 0.01; **, p < 0.05 for comparison between donors A and B and donors B and C using Tukey’s multiple-comparison test for DNA adducts. *, p < 0.01; **, p < 0.05; AαC-treated versus control (DMSO-treated) (two-tailed Student’s t test) for peptide adducts.
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$\alpha$C in the presence of acetic anhydride than by reaction of albumin with HONH-$\alpha$C alone, thereby demonstrating that N-acetoxy-$\alpha$C is efficiently formed in situ and readily reacts with nucleophilic sites of albumin to form covalent adducts (Fig. 8, A and B).

Human hepatocytes efficiently bioactivated $\alpha$C to electrophilic N-oxidized metabolites, which formed covalent adducts with DNA and albumin (Fig. 9). The DNA adduct, dG-C8-$\alpha$C, was formed at relatively high levels, ranging from 2 to 12 adducts/10^{6} DNA bases, consistent with our previous data (20, 33). dG-C8-$\alpha$C was also previously identified in salivary DNA of smokers (63). The Cys^{34} residue was the sole site of albumin found to form adducts with $\alpha$C metabolites in hepatocytes; both sulfenamide and sulfonamide adducts were identified. However, the ion counts of $\alpha$C recovered from the albumin digest were 100-fold or greater than the ion counts of any of the $\alpha$C-Cys adducts. These findings signify that a large proportion of the S-N-linked albumin-$\alpha$C adducts underwent hydrolysis during proteolysis. In the absence of stable peptide adducts or isotopically labeled LQQ^{C[SO_2H]}PFEDHKVK peptides for internal standards, it is difficult to determine the relative reactivity of $\alpha$C metabolites with DNA and albumin in hepatocytes.

HONH-$\alpha$C and NO-$\alpha$C 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 (1, 66), deplete glutathione levels in the 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 $\alpha$C in human hepatocytes by formation of the oxidized Cys^{34}-containing peptides LQQ^{C[SO_2H]}PFEDHKVK, LQQ^{C[SO_2H]}PFEDHKVK, and the methionine oxidation peptide DVFGLGM^{[O]}F. The level of Cys^{34} of albumin alkylated with IAM prior to proteolytic digestion decreased by more than 90% in hepatocytes treated with $\alpha$C and provides strong evidence that Cys^{34} of albumin is scavenging ROS (Fig. 9C). Together with Cys^{34}, six Met residues of albumin display antioxidant activity toward $\text{O}_2^\bullet$, $\text{H}_2\text{O}_2$, and HOCl (51, 52). Met^{329} was the primary site of Met oxidation in albumin from human hepatocytes treated with $\alpha$C. The oxidation of Met residues, particularly Met^{329}, has been observed in albumin of hemodialysis patients (52).

The major adducts of $\alpha$C 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 $\alpha$C intermediates. The dose of $\alpha$C (50 $\mu$M) employed in human hepatocytes is greater than daily human exposure to $\alpha$C but comparable with the doses employed in studies investigating the genotoxicity of $\alpha$C (18, 19). The amount of $\alpha$C arising in mainstream smoke is 60–250 ng/cigarette (7, 8). Thus, the intracellular or plasma levels of $\alpha$C found in humans exposed to this tobacco carcinogen are considerably lower than the amount of $\alpha$C employed in our hepatocyte study. However, dG-C8-$\alpha$C formation occurs in a concentration-dependent manner in human hepatocytes treated with $\alpha$C over a 10,000-fold concentration range (1 nM to 10 $\mu$M), signifying that the reactive N-oxidation metabolites of $\alpha$C are formed at physiological exposure levels (20). More sensitive mass spectrometry-based methods are required for measuring $\alpha$C-albumin adducts and albumin oxidation products in hepatocytes at these lower exposure conditions to $\alpha$C.

The Cys^{34} of albumin accounts for 80% of total free thiol content in plasma and is considered a major antioxidant and scavenger of electrophiles in plasma (34). A number of genotoxicants and toxic electrophiles form adducts with the Cys^{34} 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), acetaminophen (42), benzene (23), and several N-oxidized HAAs of diverse structures (32, 35, 56, 73). In addition, aldehydes produced in tobacco smoke are scavenged by Cys^{34} of albumin in vitro (74). Immunochemical techniques have shown carbonyl residues are formed with albumin exposed to cigarette smoke extract, and the carboxylation 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 Cys^{34} 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 Cys^{34} of albumin and cigarette smoking constituents remains to established in vivo. Recently, elevated levels of Cys^{34}-SO_{2}H of albumin were detected in plasma of smokers in a small pilot study (65). $\alpha$C, 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 Cys^{34} and the oxidation of Met residues of albumin.

In summary, $\alpha$C, 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 $\alpha$C metabolites. $\alpha$C, other HAAs, and many aromatic amines that arise in mainstream tobacco smoke (1, 5, 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_{2}H and Cys^{34}-SO_{3}H of albumin may be potential biomarkers to assess exposure to these hazardous chemicals in tobacco smokers.

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