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DNA Adducts of the Tobacco Carcinogens 2-Amino-9*H*-pyrido[2,3-*b*]indole and 4-Aminobiphenyl are Formed at Environmental Exposure levels and Persist in Human Hepatocytes

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Abstract

Aromatic amines and structurally related heterocyclic aromatic amines (HAAs) are produced during the combustion of tobacco or during the high-temperature cooking of meat. Exposure to some of these chemicals may contribute to the etiology of several common types of human cancers. 2-Amino-9*H*-pyrido[2,3-*b*]indole (AαC) is the most abundant HAA formed in mainstream tobacco smoke: it arises in amounts that are 25–100 times greater than the levels of the arylamine, 4-aminobiphenyl (4-ABP), a human carcinogen. 2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) is a prevalent HAA formed in cooked meats. AαC and MeIQx are rodent carcinogens; however, their carcinogenic potency in humans is unknown. A preliminary assessment of the carcinogenic potential of these HAAs in humans was conducted by examining the capacity of primary human hepatocytes to form DNA adducts of AαC and MeIQx, in comparison to 4-ABP, followed by the kinetics of DNA adduct removal by cellular enzyme repair systems. The principal DNA adducts formed were *N*-(deoxyguanosin-8-yl) (dG-C8) adducts. Comparable levels of DNA adducts were formed with AαC and 4-ABP, whereas adduct formation was ~5-fold lower for MeIQx. dG-C8-AαC and dG-C8-4-ABP were formed at comparable levels in a concentration-dependent manner in human hepatocytes treated with procarcinogens over a ten thousand-fold concentration range (1 nM – 10 μM). Pretreatment of hepatocytes with furafylline, a selective inhibitor of cytochrome P450 1A2, resulted in a strong diminution of DNA adducts signifying that P450 1A2 is a major P450 isoform involved in bioactivation of these procarcinogens. The kinetics of adduct removal varied for each hepatocyte donor. Approximately half of the DNA adducts were removed within 24 h of treatment; however, the remaining lesions persisted over 5 days. The high levels of AαC present in tobacco smoke and

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SUPPORTING INFORMATION:

Level of adducts of AαC and 4-ABP formed in hepatocytes (Table S-1). Amount of unmetabolized AαC, 4-ABP, and MeIQx present in cell culture media (Figure S-1). UPLC-ESI/MS^3 chromatogram for dG-C8-MeIQx adduct formation in human hepatocytes (Figure S-2). The product ion spectra for dG-C8-AαC, dG-C8-4-ABP, and dG-C8-MeIQx (Figure S-3). This material is available free of charge via the internet at http//pubs.acs.org.

its propensity to form persistent DNA adducts in human hepatocytes, suggests that AαC can contribute to DNA damage and the risk of hepatocellular cancer in smokers.

Keywords

Heterocyclic aromatic amines; DNA adducts; DNA repair

INTRODUCTION

Certain aromatic amines present in tobacco smoke, including 4-aminobiphenyl (4-ABP) and 2-naphthylamine (2-NA), are classified as human carcinogens (Group 1), and several prevalent heterocyclic aromatic amines (HAAs) formed in tobacco smoke or cooked meats are classified as probable or possible human carcinogens (Group 2A and 2B), based on toxicity data reviewed by the International Agency for Research on Cancer.[1,2] There are extensive epidemiological data reported in the literature on 4-ABP and 2-NA, which are human urinary bladder carcinogens.[1,2] Many epidemiological studies have implicated frequent consumption of well-done cooked meats containing HAAs with an increased risk in the development of cancers of the digestive tract, prostate gland, or mammary gland of women.[3–6]

The HAA 2-amino-9*H*-pyrido[2,3-*b*]indole (AαC) is an experimental animal carcinogen.[7] AαC was discovered in a pyrolysate of soy bean globulin,[8] and subsequently detected in well-done cooked meat,[9] and in environmental fumes.[10] AαC also arises in mainstream cigarette smoke at levels ranging from 37 to 258 ng/cigarette.[11,12] These levels are 25 to 100-fold greater than the amounts of 4-ABP present in tobacco smoke.[13] 4-ABP has been implicated in the pathogenesis of bladder cancer in smokers.[1] Recently, we detected AαC in the urine of individuals of the Chinese cohort study.[14] The number of cigarettes smoked per day was positively and significantly related to urinary levels of AαC in study subjects. This data signifies that cigarette smoking is a major point source of exposure to AαC. Apart from the endocyclic nitrogen atoms, AαC has the same chemical structure as 2-aminofluorene, one of the most well-studied carcinogenic aromatic amines.[15] 2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), is another prevalent HAA, which is formed in well-done cooked meats and fish,[16] and it induces cancer of the liver and multiple extrahepatic organs of rodents.[7] MeIQx has been identified in urine of meat-eaters.[17]

Arylamines and HAAs are bioactivated via a cytochrome P450 (P450) mediated N-oxidation of the exocyclic amine group.[18] The N-hydroxylated metabolites undergo conjugation by phase II enzymes to form unstable esters, which hydrolyze to form the presumed nitrenium ions, reactive electrophiles that covalently bind to DNA.[17,19] The bladder carcinogenicity of 4-ABP is thought to be attributed to the ability of 4-ABP to form DNA adducts in bladder cells, probably by its P450-mediated bioactivation in the liver and transport to the bladder.[19] The major DNA adduct of 4-ABP, *N*-(deoxyguanosin-8-yl)-4-aminobiphenyl (dG-C8-ABP), has been detected in human urinary bladder, by ^{32}P-postlabeling[20] and LC/MS methods.[21] 4-ABP is also a hepatic carcinogen in rodents.[22] One case-control study measured 4-ABP-DNA adducts, by immunohistochemistry methods, in hepatocytes of subjects with hepatocellular carcinoma. A statistically significant increase in risk for hepatocellular carcinoma was reported with increasing levels of adducts.[23] Because cigarette smoking is a major source of exposure to 4-ABP in humans, this molecular epidemiologic study strengthened the notion that tobacco smoke is a hepatic carcinogen in humans.[24,25]

AαC and MeIQx also form a DNA adduct at the C8 atom of deoxyguanosine (Figure 1). *N*-(Deoxyguanosin-8-yl)-dG-C8-MeIQx and *N*-(Deoxyguanosin-8-yl)-dG-C8-AαC occur in

vitro by reaction of 2-hydroxyamino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (HONH-MeIQx) or 2-hydroxyamino-9*H*-pyrido[2,3-*b*]indole (HONH-AαC) with calf thymus DNA. These adducts are also formed in rat and human primary hepatocytes exposed to AαC and MeIQx[26–28] and have been detected in salivary DNA of smokers or meat-eaters.[29] Liver tissue of human donors catalyze the formation of AαC–DNA adducts, and a positive correlation was observed among the exogenously formed levels of AαC–DNA adducts, endogenous levels of hepatic DNA adducts, and P450 1A2 activities in microsomes prepared from the liver tissue.[30] The genotoxic potentials of AαC and MeIQx in humans are not known; however, both AαC and MeIQx are liver carcinogens in mice, transgene colon mutagens and inducers of aberrant crypt foci, early biomarkers of neoplasms, in the colon of mice.[31,32]

Epidemiologic studies conducted over the past two decades have consistently shown that tobacco smoking is a risk factor for cancers of the digestive tract,[33,34] and there is increasing evidence that tobacco smoke is an independent risk factor for hepatocellular carcinoma, the predominant form of human liver cancer.[35,36] However, the causal agents of these cancers in tobacco smoke are uncertain. The ability of chemical carcinogens to form DNA adducts is regarded as one important factor in their carcinogenic potential.[37,38] In vivo animal models have been employed to measure DNA adduct formation and the level of covalent binding of genotoxicants to DNA has been correlated to cancer risk.[39–41]

Freshly cultured human hepatocytes are also an excellent system to investigate different pathways of carcinogen metabolism and can be employed to measure DNA adduct formation within the cell, where cofactors are present at physiological concentrations and biotransformation pathways may closely simulate those which occur in vivo.[42] We recently reported that human hepatocytes in primary culture bioactivate AαC, 4-ABP, and the cooked meat carcinogens 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) and MeIQx to reactive species that bind to DNA.[28] The levels of AαC-DNA adducts were higher than the adduct levels formed with PhIP or MeIQx, and were comparable to the levels of adducts formed with 4-ABP in hepatocytes exposed to an elevated concentration (10 μM) of procarcinogens. However, the relationships between DNA adduct formation and procarcinogen exposure was not investigated. The objective of our current study was to examine DNA adduct formation of AαC in primary human hepatocytes as a function of exposure to AαC over wide concentration range (1 nM – 10 μM); the lowest concentration (1 nM) may approach the level of exposure to AαC in tobacco smokers.[11] The ability of AαC to form DNA adducts in different hepatocyte donors was compared to those levels of adducts formed by MeIQx, a rodent hepatocarcinogen, and to 4-ABP, a known human carcinogen. A second objective was to assess the role of cytochrome P450 1A2 in DNA adduct formation in hepatocytes. P450 1A2 is thought to be a major hepatic P450 isoform involved in the bioactivation and DNA adduct formation of HAAs and 4-ABP, on the basis of metabolism studies with human liver microsomes and recombinant human P450s.[43–45] As a third objective, we examined the capacity of human hepatocytes to repair the principal dG-C8 adducts of these structurally related genotoxicants (Figure 1). These data provide a first assessment of the DNA binding and genotoxic potential of AαC and MeIQx, in comparison to 4-ABP, in a human liver cell model where enzyme biotransformation and DNA repair pathways closely simulate those which occur in humans.[42,46,47]

MATERIALS AND METHODS

Caution

AαC, 4-ABP, and MeIQx are carcinogens, and they should only be handled in a well-ventilated fume hood with the appropriate protective clothing.

Chemicals

MeIQx, 3-[$^{2}H_3C$]-MeIQx, and AαC were purchased from Toronto Research Chemicals (Toronto, ON, Canada). [4b,5,6,7,8,8a-$^{13}C_6$]-AαC was a kind gift from Dr. Daniel Doerge, National Center for Toxicological Research (Jefferson, AR). The isotopic purity of all compounds exceeded 99.5% except for 3-[$^{2}H_3C$]-MeIQx, which was 96.5% isotopically pure. 4-ABP was purchased from Aldrich (Milwaukee, WI). 2-Nitro-9*H*-pyrido[4,5-*b*]indole was a kind gift from Dr. D. Miller, National Center for Toxicological Research (Jefferson, AR).. [$^{2}H_9$]-4-Nitrobiphenyl (99.3% isotopically pure) was purchased from C/D/N Isotopes, Inc. (Pointe-Claire, Quebec, Canada). [$^{2}H_9$]-4-ABP was prepared by reduction of [$^{2}H_9$]-4-nitrobiphenyl with Zn in C_2H_5OH/HCl 60 °C for 2 h as previously described.[48] [$^{13}C_{10}$]-dG (99.9% isotopically pure) was purchased from Cambridge Isotopes (Andover, MA). Alkaline phosphatase (from *E. coli*) and nuclease P1 (from *Penicillium citrinum*) were purchased from Sigma (St. Louis, MO). Phosphodiesterase I (from *Crotalus adamanteus* venom) was from Worthington Biochemical Corp. (Lakewood, NJ). All solvents used were high-purity B & J Brand from Honeywell Burdick and Jackson (Muskegon, MI). ACS reagent-grade formic acid (88%) was purchased from J.T. Baker (Phillipsburg, NJ).

Synthesis of the DNA Adducts

N-(Deoxyguanosin-8-yl)-AαC (dG-C8-AαC) and *N*-(deoxyguanosin-8-yl)-MeIQx (dG-C8-MeIQx) was prepared by a reaction of their *N*-acetoxy-HAA derivatives with dG or [$^{13}C_{10}$]-dG (5 mg) in 100 mM potassium phosphate buffer (pH 8.0). *N*-(Deoxyguanosin-8-yl)-4-aminobiphenyl (dG-C8-4-ABP) was prepared by the reaction of N-hydroxy-4-ABP with pyruvonitrile with dG or [$^{13}C_{10}$]-dG.[49] For the case of dG-C8-MeIQx, the internal standard was prepared by a reaction of its *N*-acetoxy derivative of 3-[$^{2}H_3C$]-MeIQx with dG.[26]

Cell Isolation, Culture and Carcinogen Treatment

Human liver samples were obtained from eight patients undergoing liver resection for primary or secondary hepatomas through the Biological Ressource Center (CHRU Pontchaillou, Rennes, France). The research protocol was conducted under French legal guidelines and fulfilled the requirements of the local institutional ethics committee. This study was approved by the Institutional Review Board at the Wadsworth Center. Due to limited number of cells, all experiments (DNA adducts and enzymatic activities) were performed in duplicate to quadruplicate in at least three different donors. Hepatocytes were isolated by a two-step collagenase perfusion procedure and parenchymal cells were seeded in Petri dishes at a density of 3×10^6 viable cells/19.5 cm^2 dish, in Williams' modified medium during the 36 h prior to incubation with AαC, 4-ABP, and MeIQx in DMSO (0.1% v/v) at the different (0 – 10 μM) concentrations and times, as previously described.[28] The media of the control and treated cells were renewed every 24 h.

Dose Response Study of DNA Adduct Formation

Cells were treated with 4-ABP or AαC at a concentration of 0.001, 0.003, 0.010, 0.10, 1.0 or 10.0 μM for 8 h. Thereafter, the culture media were collected and immediately stored at −80 °C. The cells were then washed wish PBS, before being scraped from the Petri Dishes. The cells were centrifuged and the pellets containing DNA were also stored at −80°C. Cellular pellets were then assayed for DNA adducts. These concentrations did not produce toxicity on the basis of the methylthiazoltetrazolium test.[28]

DNA Adduct Repair Study

The cells were treated with AαC, MeIQx or 4-ABP at a concentration of 1 μM for 8 h. Thereafter, the medium containing the carcinogens was removed, cells were washed twice with PBS, and fresh medium was renewed without carcinogen. The medium was then

changed every 24 h. Cells and the media were collected every 12 h for 5 days and immediately stored at −80 °C. When the number of cells was limited, the time points investigated were 24, 48 or 120 h after removal of the carcinogens (donors 7 and 8). The incubation was terminated as described above. Cellular pellets were assayed for DNA adducts, and the media were assayed for the extent of metabolism of the carcinogens.

Measurement of P450 1A Activities

Ethoxyresorufin *O*-deethylase (EROD) and methoxyresorufin *O*-demethylase (MROD) activity associated with P450 1A2 in liver[50] were measured in all eight primary cultured hepatocytes used in this study as described previously.[51] The reaction rates were linear with time and proportional to protein concentration. Cellular protein content was estimated by the Bradford procedure.[52] Values (pmol/min/mg protein) are the mean ± SD of quadruplicate measurements.

Role of P450 1A2 in the formation of DNA adducts of AαC, MeIQx, and 4-ABP

The cells were pre-treated with furafylline (5 μM) or 0.1% DMSO (v/v) for 24 h. Then, the medium was renewed with or without furafylline, and the cells were incubated with 4-ABP or HAA (1 or 10 μM) for an additional 8 or 24 h. At the end of the treatment, the cells were washed with PBS, before being scraped from the Petri Dishes. Cellular pellets were assayed for DNA adducts.

Isolation and Digestion of DNA for Adduct Measurements

Cellular pellets were homogenized in 400 μL TE buffer pH 8.0 (50 mM Tris-HCl, 10 mM EDTA) and incubated with RNase T1 (318.75 U) and RNase A (2 μL of a 10 mg/mL solution) for 30 min at 37 °C. Thereafter, proteinase K (10 μL of a 20 mg/mL solution) and SDS (10 μL of a 20% solution) and the solutions were incubated at 37° C for 1 h. DNA was isolated by the phenol/chloroform method and precipitated with ethanol.[28] DNA was washed twice with 70% ethanol and dried at room temperature. The DNA was resuspended in 200 μL of sterile water and quantified with a NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific). DNA (5 μg) of each sample was spiked with isotopically labeled internal standards at a level of 1 adduct per 10^7 bases. DNA from the same concentrations of the individually treated 4-ABP and HAA hepatocyte samples were then pooled.

The enzymatic digestion of DNA was conducted in 5 mM Bis-Tris-HCl buffer (pH 7.1) with DNase I for 1.5 h, followed by incubation with nuclease P1 for 3 h, and then by digestion with alkaline phosphatase and phosphodiesterase for 18 h.[53] These enzyme digestion conditions were shown to be highly efficient in the recovery of the dG-C8 adducts of PhIP, MeIQx, and 4-ABP from calf thymus DNA modified by these carcinogens.[26,29] The samples were vacuum centrifuged to dryness and resuspended in 1:1 water:DMSO (30 μL). Following centrifugation (22,000 *g* for 5 min), the supernatant was transferred to capillary LC vials.

UPLC-ESI-MS/MS^3 Measurement of DNA Adducts

The DNA adduct analyses were conducted with a Waters NanoAcquity UPLC system (Waters Corp., New Milford, MA) equipped with a Waters Symmetry trap column (180 μm × 20 mm, 5 μm particle size), a Michrom C18 AQ column (0.3 × 150 mm, 3 μm particle size, Michrom Bioresources Inc., Auburn, CA) and a Michrom CaptiveSpray™ source interfaced to a linear quadrupole ion-trap mass spectrometer (LTQ Velos, Thermo Fisher, San Jose, CA). Samples (10 μL) were injected on to the trap column and washed with 100% A (solvent composition: 0.01% HCO_2H and 10% CH_3CN) for 3 min, to remove non-modified deoxynucleosides. Thereafter, the adducts were back-flushed on to the analytical

column and separated with a gradient. The solvent conditions were held at 100% A for 2 min, followed by a linear gradient to 100% B (solvent composition: 95% CH_3CN containing 0.01% HCO_2H) over 20 min at a flow rate of 5 μL/min.

Xcalibur version 2.07 software was used for data manipulations. Analyses were conducted in the positive ionization mode. Representative optimized instrument tuning parameters were as follows: capillary temperature, 270 °C; source spray voltage, 1.5 kV; source current, 0.3 μA; no sheath gas, sweep gas, or auxiliary gas was employed; capillary voltage, 32 V; tube lens voltage, 110 V. Helium was used as the collision damping gas in the ion trap and was set at a pressure of 1 mTorr. The LTQ MS was employed in the tandem MS/MS scan mode to monitor the loss of deoxyribose from the protonated molecules of the adducts ($[M + H - 116]^+$), followed by the consecutive reaction monitoring scan mode at the MS^3 scan stage, to characterize the product ions of the aglycone adducts $[BH_2]^+$. The ions monitored in MS → MS^2 → MS^3 scan modes were as follows: dG-C8-MeIQx (*m/z* 479.1 → 363.1 → 239.2, 318.4, 346.4); dG-C8-[$^2H_3$C]-C8-MeIQx (*m/z* 482.1 → 366.1 → 242.2, 321.5, 349.5); dG-AαC (*m/z* 449.1 → 333.1 → 209.2, 291.4, 316.4); [$^{13}C_{10}$]-dG-AαC (*m/z* 459.1 → 338.1 → 210.2, 295.5, 321.5); dG-C8-4-ABP (*m/z* 435.1 → 319.1 → 249.2, 277.3, 302.4); [$^{13}C_{10}$]-dG-C8-4-ABP (*m/z* 445.1 → 324.1 → 252.3, 281.4, 307.4). The reconstructed ion chromatograms containing these extracted ions produced at the MS^3 scan stage were used for quantitative measurements of the adducts and their respective internal standards.

UPLC-ESI/MS^2 Measurements of Supernatants to Assess Biotransformation of AαC, MeIQx, and 4-ABP

The cell culture media (50 μL) from the same time points of individually treated carcinogens were pooled and then the equivalent of 1000 pg of [$^{13}C_6$]-AαC, [$^2H_9$]-4-ABP, and 3-[$^2H_3$C]-MeIQx was added, followed by CH_3OH (150 μL) and the mixtures were incubated on ice for 1 h, to promote the precipitation of salts and protein. The samples were then centrifuged at 15000 *g* for 3 min at 4 °C, and the supernatants were transferred to capLC recovery vials. The analyses were performed with a NanoAcquity UPLC system equipped with a Michrom C18 AQ column (0.3 × 150 mm, 3 μm particle size). The analytes were separated by a gradient: solvent A was 0.01% HCO_2H in H_2O, and solvent B contained 0.01% HCO_2H and 5% H_2O in CH_3CN. The flow rate was set at 5 μL/min, starting at 95% A and increased by a linear gradient to 99% B over 20 min, and then holding for 1 min.

The mass-spectral data were acquired on a Finnigan Quantum Ultra Triple Stage Quadrupole MS (Thermo Fisher, San Jose, CA) and processed with Xcalibur version 2.07 software. Analyses were conducted in the positive ionization mode and employed an Advance CaptiveSpray (Michrom Bioresources Inc, Auburn, CA). The spray voltage was set at 1400 V; the in-source fragmentation was 5 V; and the capillary temperature was 200 °C. There was no sheath or auxiliary gas. The peak widths (Q1 and Q3) were set at 0.7 Da. The measurement of the chemicals was done by selected reaction monitoring (SRM). The following transitions and collision energies were used for the quantification of MeIQx, AαC and 4-ABP: MeIQx and [$^2H_3$C]-MeIQx: *m/z* 214.1 → 199.1 and 217.1 → 199.1, at 32 eV; the transitions of AαC and [$^{13}C_6$]-AαC were *m/z* 184.1 → 167.1 and 190.1 → 173.1 at 27 eV; and the transitions employed for 4-ABP and [$^2H_9$]-4-ABP were *m/z* 170.1 → 151.9 and 179.1 → 160.1, 159.1, 158.1 at 25 eV. The dwell time for each transition was 5 ms. Argon was used as the collision gas and was set at 1.5 mTorr

Calibration Curves

Calibration curves were constructed with [$^{13}C_{10}$]-dG-C8-AαC, [$^{13}C_{10}$]-dG-C8-4-ABP and dG-C8-[$^2H_3$C]-MeIQx set at 10 adducts per 10^8 deoxynucleosides with unlabeled DNA

adducts added at a level of 0, 1 – 1000 adducts per 10^9 nucleotides (7 calibrant levels) in calf thymus DNA digest (5 μg). The calibration curves were done in triplicate at each level, and the data were fitted to a straight line (area of response of the adduct/internal standard versus the level of adduct per 10^8 nucleosides) using ordinary least-squares with equal weightings. The coefficient of determination (r^2) values of the slopes exceeded 0.998. The estimates of unmetabolized AαC, MeIQx and 4-ABP in the media were determined by a single point estimate of the area ratio of the unmetabolized carcinogen/internal standard.

RESULTS

P450 1A Activity in Human Hepatocytes

Metabolism studies with human liver microsomes or recombinant P450s reveal that P450 1A2 is a major P450 isoform involved in the bioactivation of HAAs and 4-ABP.[43–45] The basal activities of P450 1A were measured in human hepatocytes from eight donors (Table 1) using ethoxyresorufin and methoxyresorufin as substrates. These alkoxyresorufin homologues undergo dealkylation, by both P450s 1A and 1A2, at variable rates.[50] As we previously reported,[46] a wide interindividual variation in P450 1A enzyme activity was observed among the 8 donors. The variation of P450 activity was greater for MROD than for EROD activity: the EROD activity varied from 0.16 to 0.50 pmol/min/mg protein, whereas MROD activity ranged from 0.08 to 1.46 pmol/min/mg protein. These levels of enzyme activities overlap the ranges of activities measured in human hepatocytes reported in our previous studies.[54,55] The levels of expression of P450 1A1 and 1A2 protein were determined in hepatocytes from two donors with a sufficient amount of cells: Western blot analysis revealed that only the P450 1A2 protein was expressed (Unpublished observations, S. Langouët). The findings are consistent with previous studies showing that P450 1A1 protein is rarely detected in human liver.[56,57]

Furafylline, a selective mechanistic-based inhibitor of P450 1A2,[58] was more effective in diminution of MROD than EROD activity (Table 1). On the basis of MROD activity, P450 1A2 was inhibited by 80% and 91%, respectively, in hepatocytes of donors 4 and 5, and inhibition of P450 1A2 occurred by 38% in the hepatocytes of donor 6. The inhibition of EROD activity was significant but less pronounced than for MROD. Based on the strong inhibition of HAA- and 4-ABP-DNA adduct formation (vide infra), we surmise that a portion of the remaining *O*-dealkylase activities in hepatocytes is attributed to P450s other than P450 1A2.[59]

Metabolism and Bioactivation of AαC, MeIQx, and 4-ABP in Human Hepatocytes

The extent of metabolism of AαC, MeIQx, and 4-ABP in hepatocytes in primary culture treated with the carcinogens (1 μM) was estimated in donor 3, by measuring the amount of parent amine remaining in the media over time. The metabolism of AαC and MeIQx was more extensive than that of 4-ABP in this hepatocyte sample. After the 8 h treatment, 35% of the initial concentration of 4-ABP remained in the medium, whereas MeIQx and AαC were undetected (limit of detection was <0.1% of initial concentration) (Supporting Information, Figure S-1). These results are consistent with our previous findings showing that the metabolism of 4-ABP was slower than the rate of metabolism of HAAs.[28,46] The cell media were measured every 12 h after the 4-ABP and HAAs had been removed. The analyses showed that neither 4-ABP nor HAAs were stored within the cells and excreted into the media over time.

The Role of P4540 1A2 in DNA Adduct Formation of AαC, MeIQx, and 4-ABP in Human Hepatocytes

The contribution of P450 1A2 to activate AαC, MeIQx, and 4-ABP was assessed by measuring DNA adduct formation in cells pretreated with furafylline for 24 h in cultures of human hepatocytes from three different donors. Furafylline strongly inhibited the formation of DNA adducts in hepatocytes exposed to these procarcinogens (1 μM): DNA adduct formation was reduced by 70 to 90% (Figure 2). DNA adduct formation was also inhibited by furafylline, when the concentration of procarcinogens was increased to 10 μM, although the inhibition was less potent. The findings imply that P450 1A2 is a major P450 isoform involved in the bioactivation of AαC, MeIQx, and 4-ABP in human hepatocytes.

Concentration-Dependent Formation of AαC- and 4-ABP-DNA Adducts in Human Hepatocytes

We examined the capacity of hepatocytes to metabolize AαC and 4-ABP to form DNA adducts as a function of procarcinogen concentration. The levels of AαC- and 4-ABP-DNA adducts were determined in hepatocyte primary culture from 3 different donors with concentrations of carcinogens ranging from 1 nM to 10 μM. An exposure period of 8 h was chosen to investigate the concentration-response relationship of DNA adduct formation, because the highest levels of adducts were formed at this time point in our earlier study with primary cultured human hepatocytes.[28] Representative UPLC-ESI/MS^3 chromatograms of DNA adducts obtained from untreated, and cells exposed to AαC- and 4-ABP at concentrations of 1 nM or 1 μM are presented in Figure 3. The major DNA adducts were identified as the dG-C8 adducts. The treatment of hepatocytes with MeIQx was only conducted at a concentration of 1 μM, and the level of adducts formed were ~5-fold lower than for AαC and 4-ABP. A UPLC-ESI/MS^3 chromatogram for dG-C8-MeIQx formation is shown in Supporting Information, Figure S-2. The product ion spectra for all three dG-C8 adducts at the MS^3 scan stage are shown in Supporting Information, Figure S-3.

The formation of dG-C8-AαC and dG-C8-4-ABP adducts occurred in a concentration-dependent manner, and DNA adducts were detected at the lowest concentration of exposure (1 nM). The levels of adducts formed in hepatocytes of donor 3 as a function of concentration of AαC or 4-ABP are depicted Figure 4, and the levels of adduct formation as a function of concentration for two other donors are reported in Supporting Information (Table S-1). The level of dG-C8 adduct formation began to reach a plateau at the highest concentration of exposure to AαC and 4-ABP (10 μM), and a power function was a better fit of the data than a linear regression curve. However, the coefficient of determination values (r^2) for the linear regression analysis for both dG-C8-AαC and dG-C8-4-ABP formation were >0.999, when the highest concentration of procarcinogen treatment was excluded from the analysis of the data (AαC: $Y = 4.559(X) + 1 \times 10^{-8}$ and 4-ABP: $Y = 3.768(X) + 3 \times 10^{-10}$, where X = concentration in molarity and Y = adduct level per DNA base; the 95% confidence intervals of the Y intercepts include the origin when $X = 0$).

Persistence of dG-C8 Adducts of AαC, MeIQx, and 4-ABP in Human Hepatocytes

The persistence of DNA adducts was investigated in primary hepatocytes from three donors (Figure 5). For donor 3, there was a sufficient number of hepatocytes to measure the level of DNA adducts over multiple time points. The amount of hepatocytes for donor 7 and 8 were limiting, and the levels of adducts were measured at 2 or 3 time points up to 120 h after treatment with the carcinogens. For donor 3, the initial levels of adducts after 8h of treatment were: dG-C8-AαC 530 ± 34; dG-C8-4-ABP 476 ± 17; and dG-C8-MeIQx 137 ± 23 per 10^8 bases. The amounts of adducts remaining in the cells were then measured every 12 h over 5 days (Figure 5). The adduct levels for each carcinogen decreased to about half of the initial levels 12 – 24 h after the exposure to the compounds had ceased. Thereafter, the

remaining adducts persisted during the next 4 – 5 days. The rates of adduct removal displayed appreciable interindividual variation for donors 7 and 8. The dG-C8 adduct of 4-ABP was the most persistent lesion, followed by dG-C8-AαC and then dG-C8-MeIQx in the hepatocytes of the three donors used in this study.

DISCUSSION

Liver is by far the most metabolically active tissue in the biotransformation of HAAs.[17] We showed previously that human hepatocytes in primary culture closely reflect the metabolism of HAAs in vivo; the metabolite profile of MeIQx and PhIP in primary human hepatocytes closely matched the urinary metabolite profile of both HAAs in healthy humans.[46,60,61] In this study, we have examined the capacity of human hepatocytes to bioactivate HAAs and 4-ABP to DNA damaging agents and the ensuing repair of the resultant DNA lesions. In vitro studies conducted with human liver microsomes or recombinant human P450s have implicated P450 1A2 as the primary P450 isoform involved in N-oxidation and bioactivation of 4-ABP and HAAs.[43–45] The treatment of hepatocytes with furafylline, a selective, mechanism-based inhibitor of P450 1A2,[58] show that P450 1A2 is also the primary P450 isoform in human hepatocytes involved in N-oxidation of HAAs and 4-ABP, and the catalytic activity of P450 1A2 is critical for DNA adduct formation (Figure 2).

Human hepatocytes are highly efficient in the bioactivation of AαC, MeIQx, and 4-ABP into reactive intermediates that bind to DNA. In fact, the levels of adduct formation are comparable to or greater than the levels of dG-C8 adducts of all three genotoxicants formed in Chinese hamster ovary (CHO) cells stably transfected with P450 1A1 or P450 1A2 co-expressed with N-acetyltransferase (NAT2*4).[62–64] The global DNA adduct formation and the genotoxicity of AαC, MeIQx, and 4-ABP in the hypoxanthine phosphoribosyl transferase (*hprt*) gene of these CHO cell lines revealed that the potential of the dG-C8 adducts of AαC and MeIQx to induce *hprt* mutations was greater than that of dG-C8-4-ABP, when the mutation frequency was normalized to the levels of the dG-C8 adducts.

DNA adduct formation of AαC and 4-ABP in human hepatocytes occurred in a linear, concentration-dependent manner over a thousand-fold exposure range (Figure 4). The lowest concentration of procarcinogen assayed (1 nM) may approach the exposure levels of AαC in tobacco smokers.[11] DNA adducts are regarded as one important factor in the carcinogenic potential of a genotoxicant, and DNA adducts have been used for interspecies extrapolation of toxicity data for human risk assessment.[37,38] The carcinogenic potential of genotoxic chemicals in rodents has been assessed by the carcinogen DNA binding index and bench mark dose values.[39–41] The doses of chemicals and the levels of their DNA adducts required at the steady-state to induce liver tumors in rodents vary greatly.[39–41] The steady-state levels of some types of DNA adducts in rats chronically exposed to a single carcinogen, including HAAs or arylamines, generally must be greater than 100 adducts per 10^8 DNA bases to increase the frequencies of tumors by 10% or greater than the spontaneous background levels.[41] The levels of dG-C8-AαC and dG-C8-4-ABP formed in human hepatocytes are ~5 adducts per 10^9 DNA bases, following a single exposure to AαC or 4-ABP at the lowest concentration assayed (1 nM); however, because of their persistence, the levels of adduct are likely to increase during repeated exposures. The development of human cancer is complex and requires a number of steps, including multiple mutations, genetic alterations, and cell proliferation.[65] AαC is one of many genotoxicants present in tobacco smoke. AαC may act as an initiating agent in the development of human cancer during chronic smoking, in which many other mutagens, carcinogens, tumor promoters, and factors stimulating tumor progression exist.[7]

There are two reports on the genotoxicity of AαC in human cells. One study reported an induction of DNA double strand breaks and micronucleus formation in human lymphoblastoid cell line MCL-5 cells treated with AαC at concentrations above 50 nM.[66] Another study reported that AαC induced double strand breaks in peripheral blood lymphocytes and micronuclei in the human hepatoma HepG2 cell line, when employing even higher concentrations of AαC.[67] Our data show that human hepatocytes in primary culture are far more susceptible to the genotoxicity of AαC than MCL-5 and HepG2 cell lines, certainly because of the superior capacity of hepatocytes to bioactivate AαC. A possible explanation for the high levels of AαC-DNA adducts formed in hepatocytes may be attributed to UDP-glucuronosyltransferase (UGTs) enzymes. Usually UGTs are important enzymes involved in the detoxication of procarcinogens; however, we discovered that several UGTs bioactivate HONH-AαC and catalyze the formation of an *O*-glucuronide conjugate of HONH-AαC, a reactive intermediate that binds covalently to DNA.[68] In addition, NAT2 and SULT1A1, which are highly expressed in liver,[69,70] catalyze the binding of HONH-AαC to DNA.[71]

Approximately half of the dG-C8-AαC, dG-C8-MeIQx, and dG-C8-4-ABP adducts underwent repair within the 12 –24 h following the removal of the carcinogens from the media for two of the hepatocyte donors. Thereafter, the remaining adducts persisted over the next 120 h. In the case of donor 8, dG-C8-4-ABP was stable over the entire study. NMR solution structural studies have shown that several dG-C8-HAA and dG-C8-arylamine adducts in some oligonucleotide sequence contexts adopt the glycosidic torsion angle in the *syn* conformation instead of the normally occurring *anti* conformation.[72–74] These conformational changes in the DNA helix induced by HAA–purine or arylamine-purine adducts are important determinants of the adducts' biological effects and miscoding properties during translesional syntheses with polymerases,[75–77] and also influence the adducts' persistence and rate of adduct removal in vivo,[78–80] by the nucleotide excision repair (NER) complex.[75] NER is the primary pathway responsible for the removal of bulky DNA lesions, including those formed by aromatic amines and HAAs.[81,82] There are two subpathways of NER: global-genome repair (GG-NER) and transcription-coupled repair (TC-NER). These enzyme complexes only differ by the manner in which the substrate lesions are recognized, thereafter, the two enzyme complexes share a common set of steps to complete the repair process.[81] Persistent adducts are viewed as most biologically relevant, because the unrepaired DNA adducts can be bypassed by error-prone DNA polymerases, leading to the accumulation of mutations.[65,77] We surmise that the initial rapid removal of dG-C8 adducts of AαC, MeIQx, and 4-ABP adducts occurred either by GG-NER or TC-NER of actively transcribed genes in hepatocytes, and the persistent adducts resided in sequence contexts that minimally perturbed DNA conformation and escaped GG-NER,[81,83] or the remaining adducts were situated in transcriptionally silent portions of the genome. Our findings show that a significant proportion of the dG-C8-AαC, dG-C8-MeIQx, and dG-4-ABP adducts in genomic DNA persist in human hepatocytes.

Tobacco smoking is a recognized risk factor for cancers of the digestive tract [33,34] and an independent risk factor for hepatocellular carcinoma.[35,36] Several epidemiologic studies have reported that frequent consumption of well-done meat containing HAAs increase the risk of colorectal cancer, particularly in individuals who harbored rapid phenotype for both P450 1A2 and NAT2, which bioactivate AαC and other HAAs, and the risk was greatly increased in smokers.[6,84] The high level of adduct formation and the relative persistence of the dG-C8-AαC in hepatocytes suggest that dG-C8-AαC can serve as a biomarker to assess the exposure and DNA damage induced by AαC in liver and possibly extrahepatic tissues of tobacco smokers. The considerably higher levels of AαC present in tobacco smoke in comparison to 4-ABP, other aromatic amines and HAAs, combined with the propensity of AαC to undergo bioactivation by phase I and II enzymes expressed in the liver and

extrahepatic tissues provide a biochemical mechanism for a role for AαC in the etiology of tobacco-associated cancers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of abbreviations

AαC	2-amino-9*H*-pyrido[2,3-*b*]indole
HONH-AαC	2-hydroxyamino-9*H*-pyrido[2,3-*b*]indole
PhIP	2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine
MeIQx	2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline
HONH-MeIQx	2-hydroxyamino-3,8-dimethylimidazo[4,5-*f*]quinoxaline
4-ABP	4-aminobiphenyl
HONH-4-ABP	4-hydroxyaminobiphenyl
dG-C8-4-ABP	*N*-(deoxyguanosin-8-yl)-4-ABP
dG-C8-AαC	*N*-(deoxyguanosin-8-yl)-AαC
dG-C8-MeIQx	*N*-(deoxyguanosin-8-yl)-MeIQx
CHO	Chinese hamster ovary
HAA	heterocyclic aromatic amine
hprt	hypoxanthine phosphoribosyl transferase
EROD	ethoxyresorufin O-deethylase
GG-NER	global-genome repair
MROD	methoxyresorufin O-demethylase
NAT	*N*-acetyltransferase
NER	nucleotide excision repair
ppb	part-per-billion
SULT	Sulfotransferase
TC-NER	transcription-coupled nucleotide excision repair
UGT	UDP-glucuronosyltransferase
UPLC-ESI/MS^n	ultra performance liquid chromatography−electrospray ionization/ multistage scan mass spectrometry

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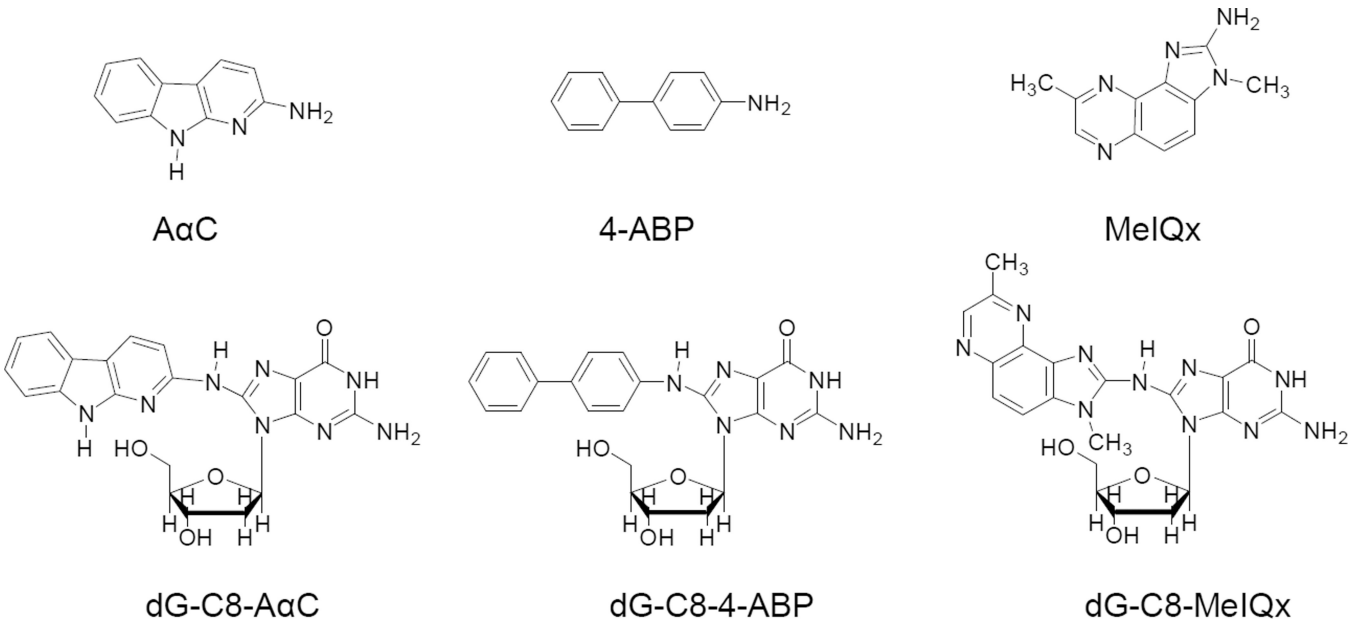

Figure 1.
Chemical structures of AαC, 4-ABP, MeIQx, and their dG-C8 adducts.

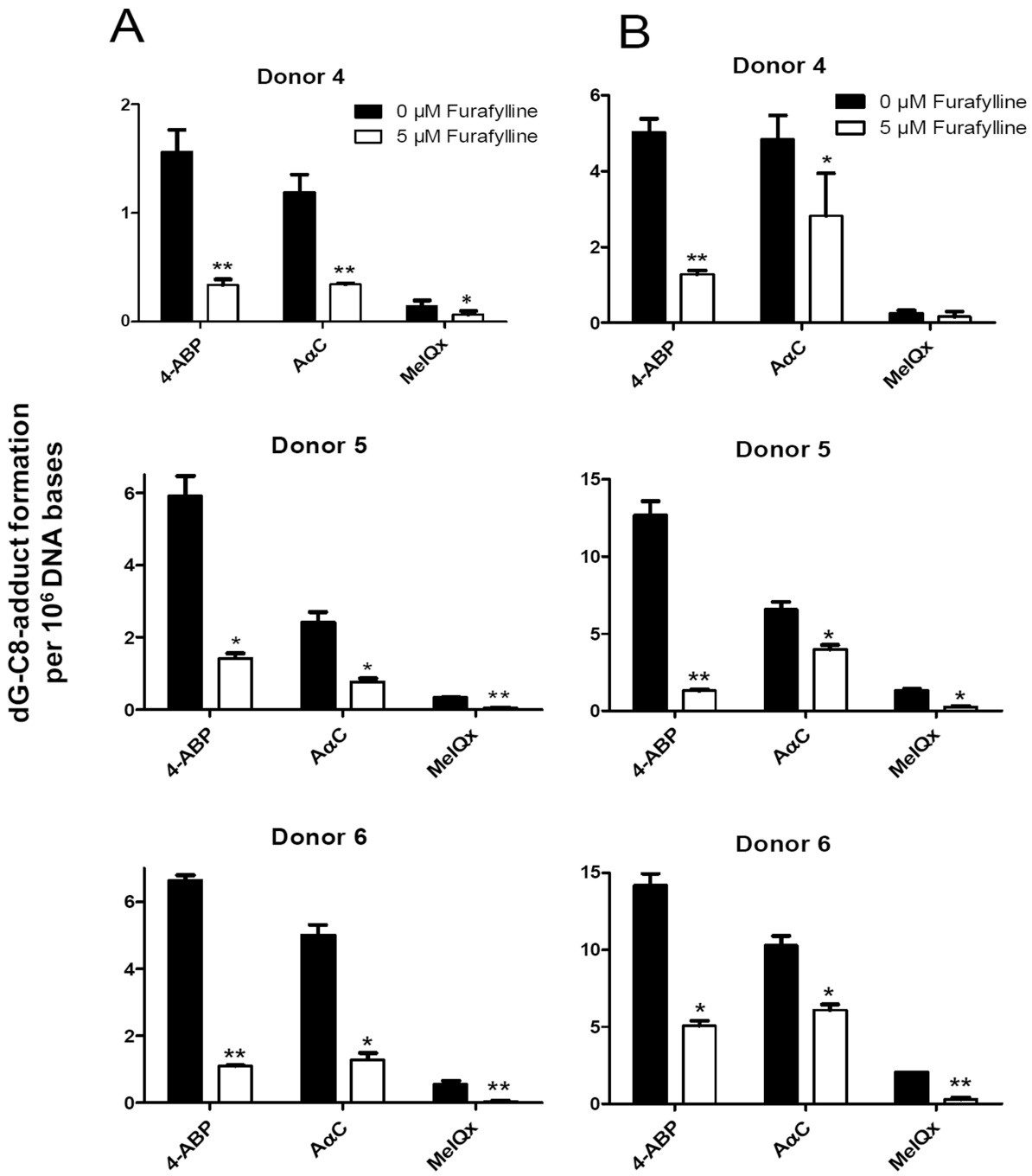

Figure 2.

DNA adduct levels formed in human hepatocytes treated with AαC, 4-ABP or MeIQx at concentrations (A) 1 µM or (B) 10 µM for 24 h without or with pretreatment of cells with furafylline (5 µM). DNA adducts were measured independently in duplicate or triplicate, and the data are plotted as the mean and standard deviation of the adduct levels donors 4, 5 and 6). Treatment with furafylline resulted in a significant decrease in adduct levels for all treatments (unpaired Student *t* test, *$p < 0.02$, **$p < 0.002$) except for MeIQx adduct formation at 10 µM for donor 4.

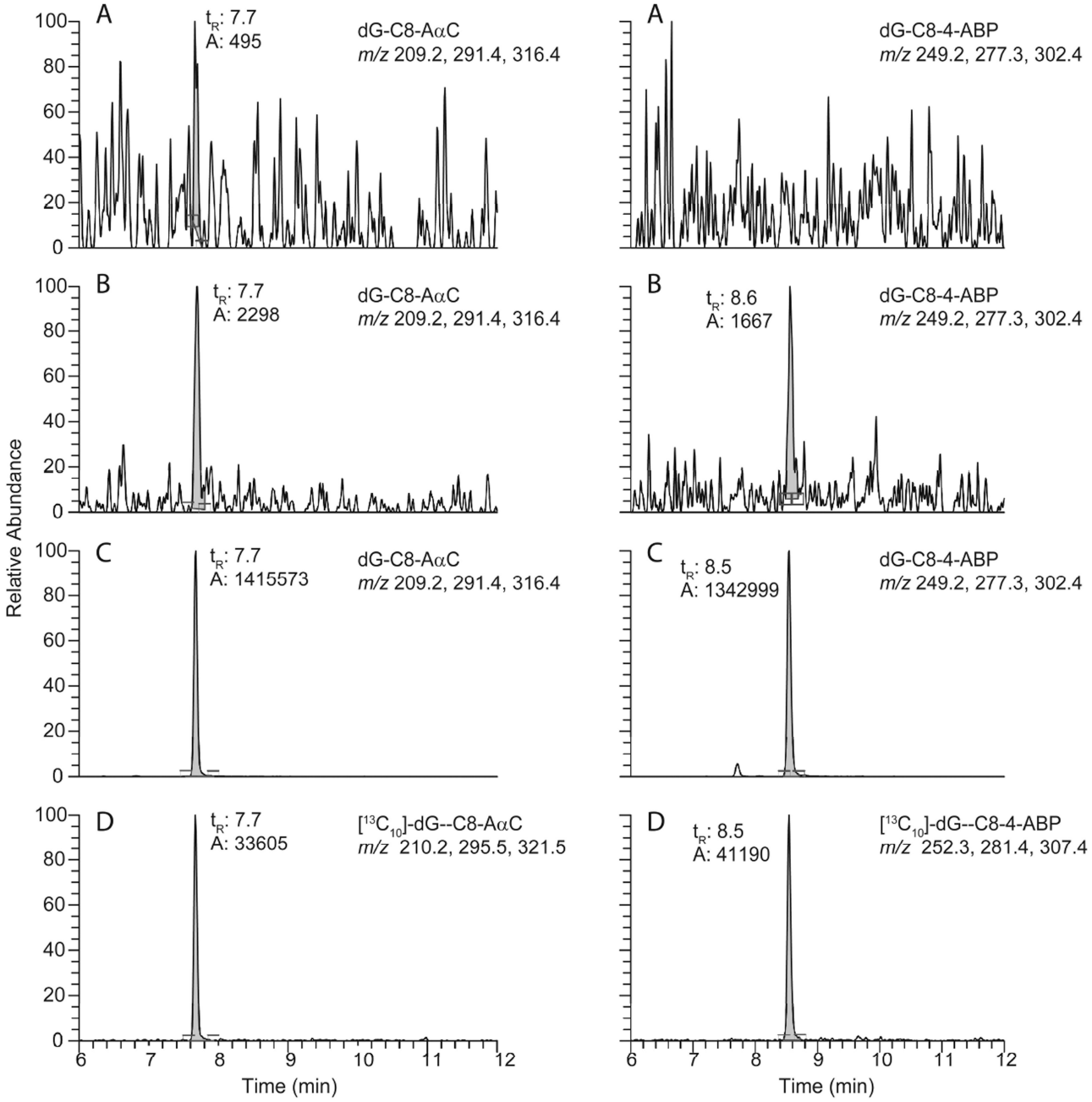

Figure 3.

UPLC-ESI/MS^3 chromatograms of dG-C8-AαC and dG-C8-4-ABP in (A) untreated hepatocytes, (B) hepatocytes treated with 0.001 μM carcinogen, (C) hepatocytes exposed to 1 μM carcinogen for 8 h, and (D) internal standards [$^{13}C_{10}$]-dG-C8-AαC and [$^{13}C_{10}$]-dG-C8-4-ABP were added at a level of 1 adduct per 10^7 DNA bases.

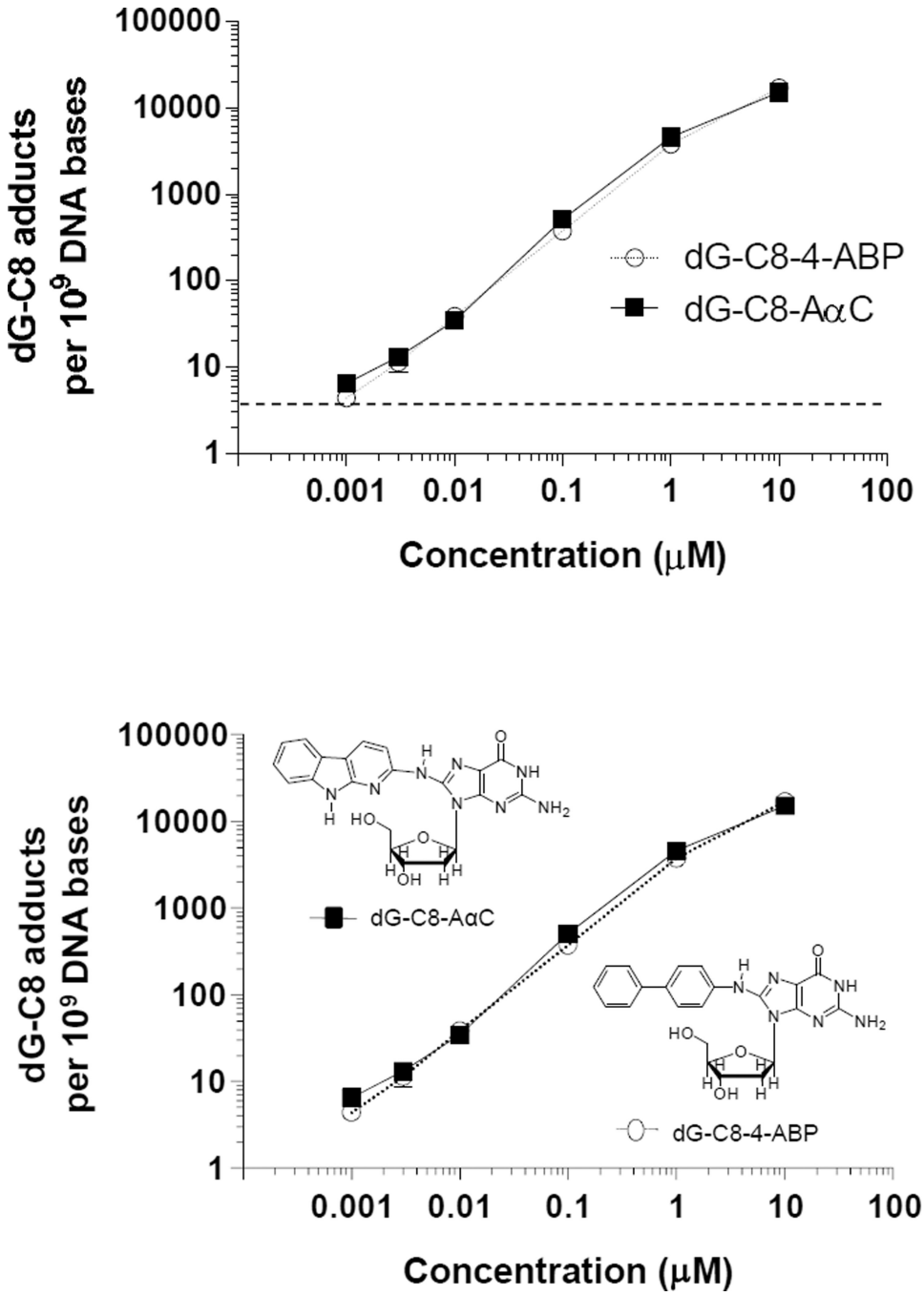

Figure 4.

DNA adduct formation in human hepatocyte donor 3 treated with AαC or 4-ABP at concentrations of 0.001, 0.003, 0.010, 0.10, 0.50, 1.0, or 10.0 μM. DNA adducts were measured in duplicate or triplicate, and the adduct levels were plotted as the mean and standard deviation. The horizontal dashed line delineates the adduct level for the limit of quantification of DNA adducts.

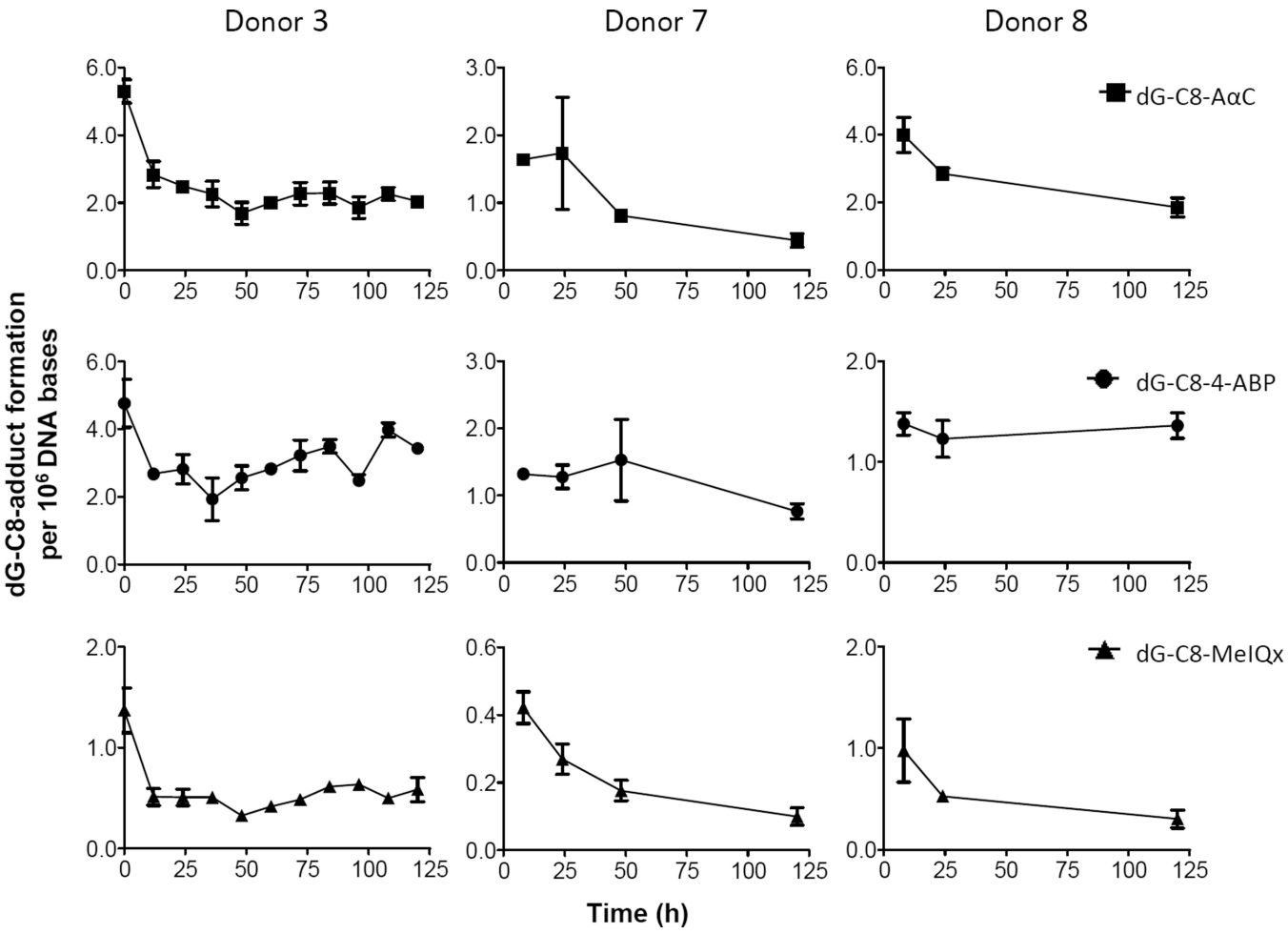

Figure 5.

Kinetics of DNA adduct removal. Time 0 h corresponds to the level of adducts present at the end of the 8 h treatment with AαC, MeIQx, or 4-ABP (1 μM), when the carcinogens were removed from the media. Adduct measurements were made with either 2 or 3 independent cell cultures (donors 3, 7 and 8). Data are expressed as the mean and the standard deviation.

Table 1

P450 1A basal activities of EROD and MROD measured in human hepatocytes

	EROD[a] 24 h	EROD[a] + Furafylline	MROD[a] 24 h	MROD[a] + Furafylline
Donor 1	0.16 ± 0.02	-	0.08 ± 0.07	-
Donor 2	0.44 ± 0.04	-	0.31 ± 0.07	-
Donor 3	0.50 ± 0.07	-	0.37 ± 0.03	-
Donor 4	0.27 ± 0.07	0.16 ± 0.02**	1.04 ± 0.03	0.21 ± 0.09**
Donor 5	0.22 ± 0.01	0.16 ± 0.01**	1.46 ± 0.18	0.15 ± 0.01**
Donor 6	0.33 ± 0.04	0.28 ± 0.09	0.48 ± 0.01	0.30 ± 0.05*
Donor 7	0.21 ± 0.01	-	0.58 ± 0.11	-
Donor 8	0.27 ± 0.02	-	0.77 ± 0.02	-

[a]activities are expressed in pmol/min/mg protein, mean ± SD (N = 4 measurements), after 60 h of culture Due to limited number of cells, the effect of furafylline was studied only with donors 4, 5 and 6.

(Unpaired Sudent *t*- test: *$p < 0.004$; **$p < 0.001$)