Supporting Information

Materials and Methods

Chemicals

All chemicals were of the highest commercially available purity. Chromatography grade acetonitrile and methanol, formic acid, chloroform, ethanol and 2-choroacetaldehyde were acquired from Merck (Darmstadt, Germany). Fetal bovine serum was purchased from Cultilab (Campinas, Brazil). [$^{15}N_5$]-2'-Deoxyguanosine and [$^{13}C_2$]-acetaldehyde were acquired from Cambridge Isotope Laboratories (Andover, MA, USA). All other chemicals were from Sigma (St. Louis, MO, USA). Water was purified using a Milli-Q system (Millipore, Bedford, MA, USA).

Cell culture

Normal human lung fibroblasts IMR-90 were kindly provided from Dr. Mary Cleide Sogayar (Instituto de Química, Universidade de São Paulo, Brazil) and were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and 40 mg penicillin / L and 100 mg streptomycin / L, in an atmosphere of 5% CO_2 at 37ºC.

Cell treatment

The medium from confluent cell plates was changed, AA (1 or 10 mM) or [$^{13}C_2$]-AA (10 mM) was added to cells and DNA was extracted immediately (0 h) or after 3 h incubation. For lipid peroxidation experiments, the medium was replaced by Hank's solution prior to addition of acetaldehyde. After the incubation period, the medium from each plate was then transferred to test tubes and submitted for MDA determination. Protein concentration was determined via Bradford assay.

Determination of acetaldehyde and crotonaldehyde concentrations

2,4-Dinitrophenylhydrazine (20 mM) in 1 M HCl (1:1; % v/v)[21] was added to the IMR-90 cell culture medium containing AA. After incubation for 2 h at 37 °C the precipitate (hydrazone) was extracted with chloroform (1:1, % v: v) and dried under N_2. The dried product was dissolved in acetonitrile and analyzed by HPLC with UV detection (λ = 360 nm) coupled to mass spectrometer Quattro II (Micromass) using a C8 column (150 x 4.6 mm, 5 µm) (Phenomenex, Torrence, CA) at a flow rate of 0.6 mL / min. The following water/acetonitrile gradient method was used: from 0-30 min an isocratic mode (60% acetonitrile), 12-16 min from 60% to 90%, 16 to 25 min 90% acetonitrile, 25-27 min from 90% to 60% and 27-32 min to equilibrate the column with acetonitrile (60%). The mass spectrometer parameters were adjusted to the best ionization condition in the negative mode. The cone voltage was kept to 25 V, collision energy 10 eV, source temperature 100 ºC, desolvatation temperature 200 ºC. The analysis was performed in a MRM mode. A calibration curve for quantification was created using the reaction of acetaldehyde and a solution 1:1 (% v/v) of 20 mM 2,4-dinitrophenylhydrazine in 1M HCl.

DNA extraction

DNA was isolated using the chaotropic NaI method[22], with modifications. Briefly, the pelleted cells (3 x 10^8 cells were homogenized in 10 mL of solution A (320 mM sucrose, 5 mM $MgCl_2$, 10 mM Tris-HCl, 0.1 mM desferroxamine, and 1% (v/v) Triton X 100, pH 7.5). After centrifugation at 1500 *g* for 10 min, the pellets were suspended in another 10 mL lysis solution and centrifuged again at 1500 *g* for 10 min. The pellets were then suspended in 6 mL 10 mM Tris-HCl buffer, pH 8.0, containing 5 mM EDTA, 0.15 mM desferroxamine, and 350 µL SDS 10%. The enzymes RNase A (30 µL, 10 mg/mL) and RNase T1 (4 µL, 20 U/µL) in 10 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA and 2.5 mM desferroxamine, were added, and the reaction mixture was incubated at 37 ^{o}C. After 1 h, 300 µL proteinase K (20 mg/mL) was added, followed by incubation at 37^{o}C for 1 h. After centrifugation at 5000 *g* for 15 min, the liquid phase was collected and 1 mL of a solution containing 7.6 M NaI, 40 mM Tris-HCl (pH 8), 20 mM EDTA, and 0.3 mM desferroxamine were added, followed by the addition of 8 mL isopropanol. The reaction was mixed thoroughly by inversion until a whitish precipitate appeared. The precipitate was collected by centrifugation at 5000 *g* for 15 min and washed with 5 mL isopropanol 60% (5000 *g*, 15 min) followed by 5 mL ethanol 70% (5000 *g*, 15 min). The DNA pellet was solubilized in 100 µL desferroxamine (0.1 mM). DNA concentration was measured spectrophotometrically at 260 nm.

Enzymatic hydrolysis of DNA

12 µL 1M sodium acetate buffer (pH 5) and 100 fmol of [$^{15}N_5$]-1,N^2-εdGuo and [$^{15}N_5$]-1,N^2-propanodGuo or [$^{13}C_4$, $^{15}N_5$]-1,N^2-propanodGuo were added to an aliquot of 0.1 mM desferroxamine solution containing 200 µg DNA, which was then digested with 5 units of nuclease P1 at 37°C for 30 min. Then, 12 µL 1M Tris-HCl buffer (pH 7.4), 12 µL phosphatase buffer, and 24 units alkaline phosphatase were added for an additional 1 h incubation at 37°C. The final volume of the solution was adjusted to 200 µL with water. The resulting aqueous layer was subjected to HPLC/ESI/MS-MS analysis (100 µL of the DNA solution/injection). The amounts of reagents and labeled internal standards were proportionally adjusted for hydrolysis and analysis of other DNA quantities. In case of [$^{13}C_2$] N^2-ethyldGuo analysis the addition of 100 fmol of [$^{13}C_2$, $^{15}N_5$] N^2-ethyldGuo was performed after 3 subsequent incubations with 2.6 mg of sodium ciano-borohydreto for 30 min (the first two at room temperature and the last one at 37ºC).

[$^{13}C_4$]1,N^2–propanodGuo and [$^{13}C_2$] N^2ethyldGuo were quantified by high-performance liquid chromatography/electrospray ionization tandem mass spectrometry (HPLC-ESI/MS-MS) with SPE pre-purification. The nucleosides, after hydrolysis, were added in a SPE Strata X column (1 mL, 30 mg – Phenomenex, Torrence, CA). The columns were equilibrate with methanol (1 mL) and water (1 mL) before samples loading and washed with methanol 5 % (1 mL) and methanol 10% (1 mL). The adducts were collected with methanol 100% (1 mL). An aliquot of each sample was put aside for HPLC/UV dGuo quantification.

Synthesis of 1,N^2–εdGuo and [$^{15}N_5$]1,N^2–εdGuo standards

The 1,N^2–εdGuo standard was obtained from the reaction of dGuo (40 mM) with 2M chloroacetaldehyde. The [$^{15}N_5$]1,N^2–εdGuo standard was obtained from the reaction of [$^{15}N_5$]dGuo with 2M chloroacetaldehyde with subsequent purification by HPLC as described by Loureiro *et al* [23] .

Synthesis of 1,N^2-propanodGuo, [$^{15}N_5$]1,N^2–propanodGuo and [$^{13}C_4$,$^{15}N_5$]1,N^2–propanodGuo standards

25 μmoles dGuo was dissolved in 2 mL phosphate buffer (100 mM pH 8), with 1mmol acetaldehyde and 0.05 mmol lysine. The reaction was shaken at 500 rpm and 37 ºC for 24 h. The products were purified by HPLC as described by Garcia *et al* [19]. Synthesis of internal standards were performed with isotopic labeled dGuo and AA.

Synthesis of N^2-ethyldGuo and [$^{13}C_2$,$^{15}N_5$]N^2–ethyldGuo standards

The N^2–ethyldGuo standard was obtained as described previously[24] from the reaction of dGuo (0.05 mmol) with 100 μL of acetaldehyde, 0.30 mmol of $NaBH_3CN$ in H_2O/MeOH 1:1 (3 mL). The [$^{13}C_2$,$^{15}N_5$]N^2–ethyldGuo standard was obtained from the reaction of [$^{15}N_5$]dGuo and [$^{13}C_2$]acetaldehyde with subsequent purification by HPLC using the same method to 1,N^2-propanodGuo purification.

Analysis of 1,N^2-εdGuo and 1,N^2-propanodGuo by high-performance liquid chromatography/electrospray ionization tandem mass spectrometry (HPLC-ESI/MS-MS)

Analyses in the positive mode were carried out on an API 4000 QTRAP mass spectrometer (Applied Biosystems), as described by Garcia *et. al.*[19], with minor modifications . An Agilent HPLC system consisting of an autosampler (Agilent 1200 High performance), an automated switching valve, pumps (Agilent 1200 Binary pump SL and 1200 Isocratic pump SL) and a detector (Agilent 1200 DAD G1315C) were controlled by Analyst 1.4.2 software. The analytical column Luna C18 (150 mm x 2 mm i.d., 3 μm) (Phenomenex) kept at 18ºC in an Agilent 1200 G1216B column oven. The adducts in the DNA samples were detected by multiple reaction monitoring (MRM) as described before. Briefly, the DNA hydrolysates containing 100 fmol of the internal standards were injected into the HPLC/ESI/MS-MS system. The m/z 292→176 (1,N^2–εdGuo), 297→181 ([$^{15}N_5$]1,N^2–εdGuo), 338→222 (1,N^2–propanodGuo), 342→226 ([$^{13}C_4$]1,N^2–propanodGuo) and 343→227 ([$^{15}N_5$]1,N^2–propanodGuo) transitions were monitored with a dwell time of 280 ms. All other parameters of the mass spectrometer were adjusted for acquisition of the best $[M+H]^+$/$[M+H-2\text{-}D\text{-}erythro\text{-pentose}]^+$ transition. The Turbo ion spray voltage was kept at 5500 V, the curtain gas at 25 psi and the nebulizer and auxiliary gas at 60 psi. The temperature was set to 450 ºC, and the collision cell nitrogen pressure was adjusted to high.

Analysis [$^{13}C_4$]1,N^2–propanodGuo and [$^{13}C_2$] N^2ethyldGuo by high-performance liquid chromatography/electrospray ionization tandem mass spectrometry (HPLC-ESI/MS-MS) with SPE pre-purification

Analyses in the positive mode were carried out on an API 4000 QTRAP mass spectrometer (Applied Biosystems). An Agilent HPLC system consisting of an autosampler (Agilent 1200 High performance), an automated switching valve, pumps (Agilent 1200 Binary pump SL and 1200 Isocratic pump SL) and a detector (Agilent 1200 DAD G1315C) were controlled by Analyst 1.4.2 software. The analytical colum was a Luna C18 (150 mm x 2 mm i.d., 3 μm) (Phenomenex) kept at 25ºC in an Agilent 1200 G1216B column oven. The gradient used for analyte elution started with 0% MeOH and increased to 15% in 25 min, then the MeOH increased to 80% in 10 min and kept at this % for 5 min then returned to 0% in 1 min. The adducts in the DNA samples were detected by multiple reaction monitoring (MRM). Briefly, the DNA hydrolysates containing 100 fmol of the internal standards were injected into the HPLC/ESI/MS-MS system. The 342→226 ([$^{13}C_4$]1,N^2–propanodGuo), 347→231 ([$^{13}C_4$,$^{15}N_5$]1,N^2–propanodGuo), 298→182 (N^2ethyldGuo) and 303→187 ([$^{13}C_4$,$^{15}N_5$] N^2ethyldGuo) transitions were monitored with a dwell time of 150 ms. All of the parameters of the mass spectrometer were adjusted for acquisition of the best $[M + H]^+$/$[M + H - 2\text{-}d\text{-}erythro\text{-pentose}]^+$ transition. The curtain gas was adjusted to 10 psi, the source temperature was held at 600 °C, the nebulizer and auxiliary gas were maintained at 55 psi, the Turbo Ion Spray voltage was 5500 V. For the 342/226 and 343/227 transitions, the following parameters were selected: collision energy, 29 V; collision cell exit, 16 V; and declastering potential, 66 V. For the 298/182 and 303/186 transitions were selected 19 V of collision energy, 10 V of collision cell exit, and 36 V of declastering potential. The data were processed using Analyst 1.4.2 software.

Malondialdehyde determination

MDA was measured as previously described[25]. After treatment, 7 mL of a 0.4% (w/v) solution of TBA in 0.2 N HCl / H_2O (2:1) and 1 mL of a 0.2% (w/v) solution of BHT in 95 % ethanol were added to the cell samples, and the mixture was heated to 90 ºC for 45 min, cooled on ice and extracted with isobutanol. The isobutanolic phase was injected through a Shimadzu auto injector model SIL-10AD/VP (Shimadzu, Kyoto, Japan) in a Shimadzu HPLC system, consisting of two LC-6AD pumps connected to a Lichrosorb 10 RP-18 (Phenomenex, Torrance, CA) reversed-phase column (250 mm x 4.6 mm i.d. particle size 10 μm). The flow rate of the isocratic eluent (25 mM potassium phosphate buffer pH 7 with 40% methanol) was 1 mL/min. An RF-10A/XL fluorescence detector was set at an excitation wavelength of 515 nm and an emission wavelength of 550 nm. The data were processed using the Shimadzu Class-VP 5.03 software. Malonaldehyde-bisdiethylacetal was used for calibration of the fluorescence data, yielding a quantitative adduct of the malonaldehyde-TBA product. The data were expressed as μmol of MDA normalized to the protein content, determined by the Bradford method [26].

Results

Determination of acetaldehyde concentration

AA is a highly volatile compound and has a boiling point of 21°C at 1 atm. All the concentration values of treatments correspond to the AA added at the beginning of the 3 h incubation period. However, for better accuracy, it is extremely important to know the actual concentration of AA to which the cells were exposed. Therefore, AA concentration was evaluated by conjugation with DNPH and detected the resulting hydrazones. Using this method, we observed that high amounts of AA had either evaporated or reacted with compounds in the culture medium only a few seconds after AA addition. The concentration of AA showed a decrease of ca 65% in the water stock solution, before cell treatment. An aliquot of the cell medium was also incubated with DNPH before and after cell treatment. The concentrations of AA at the end of exposure were 155 μM and 703 μM (ca 10 times less than added solution). These results were assessed using a calibration curve at different AA concentrations and with an excess of DNPH in the culture medium in order to minimize matrix interference. It is important to emphasize that AA

concentration were estimated in all experiments, and the presented levels correspond to the media of the analyzed samples.

Malondialdehyde determination

The treatment of normal human lung fibroblasts (IMR-90) with different concentrations of AA resulted in a dose-dependent increase in lipid peroxidation measured as MDA production (figure 1).

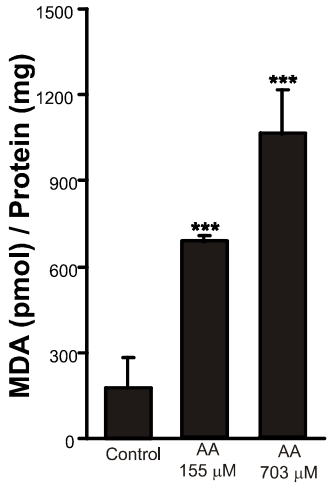

Figure 1. MDA (pmol) / protein (mg) levels in cells incubated with acetaldehyde for 3 h (155 and 703 μ M). *** P <0.001 compared to control and acetaldehyde concentrations.

Assessment of [$^{13}C_4$]1,N^2–propanodGuo generated by AA carryover during DNA extraction

A possible artefactual adduct formation due to reaction of naked DNA with AA liberated from reversible Schiff bases from cell and medium proteins during the process of DNA extraction was assessed. [$^{13}C_4$] AA was added to the cell culture media and immediately after adding the compound, DNA extraction was performed. For the detection of the adduct a new protocol of analysis was used as described in material and methods. This new methodology consist of a pre-purification step with an SPE column, enable the analysis of 250 μg of DNA. The detection limit for this methodology was 10 fmol. Figure 3 shows that no [$^{13}C_4$]1,N^2–propanodGuo was detected, on the other hand, incubating the cells for 3 h in media containing [$^{13}C_4$] AA, 1,N^2–propanodGuo was detected confirming the data already obtained using the previous method. It's important to emphasize that we cannot exclude small artefactual formation, since this is related to the sensibility of the methodology used.

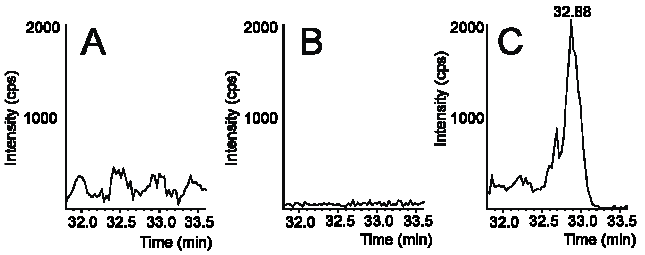

Figure 3. Chromatogram presenting the peak corresponding to [$^{13}C_4$]1,N^2–propanodGuo in (A) control, (B) immediately and (C) three hours after AA exposure.