Reaction of Malondialdehyde−DNA Adducts with Hydrazines—Development of a Facile Assay for Quantification of Malondialdehyde Equivalents in DNA

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Malondialdehyde is a ubiquitous product of lipid peroxidation that reacts with DNA to form premutagenic lesions. Principal among them is pyrimido-[1,2-α]purin-10(3*H*)-one (M_1G). M_1G has recently been found to be a reactive electrophile in DNA that couples with amines at basic pH or hydroxylamines at neutral pH. We explored the reaction of M_1G with hydrazines because of the possibility that the latter could act as bifunctional nucleophiles to strip the malondialdehyde equivalent from DNA. Pentafluorophenylhydrazine reacted rapidly with M_1G to form a hydrazone conjugate. This hydrazone was stable at room temperature and did not cyclize to form the corresponding pyrazole. In contrast, phenylhydrazine and benzylhydrazine reacted with M_1G to form phenylpyrazole and benzylpyrazole, respectively. Pentafluorobenzylhydrazine reacted rapidly with M_1G to form pentafluorobenzylpyrazole and dG in near quantitative yield. This reaction formed the basis for a quantitative assay for the presence of M_1G or M_1G equivalents in DNA or protein that utilized gas chromatography/negative chemical ionization mass spectrometry. The assay was extended to the oxopropenyl donors, M_1A, base propenal, and N^{ϵ}-3-oxopropenyl-lysine. Analysis of DNA treated with bleomycin demonstrated a linear increase in the level of oxopropenyl groups that plateaued at approximately 1 oxopropenyl group/100 bases at a bleomycin concentration of 200 μM. Parallel analysis of M_1G in the samples revealed that this adduct represents a small fraction of the total oxopropenyl units generated in DNA by treatment with bleomycin.

Introduction

Malondialdehyde (MDA)[1] is formed endogenously by both lipid peroxidation and prostaglandin biosynthesis (*1*, *2*). MDA is mutagenic in bacteria and mammalian cells (*3*−*5*) and is carcinogenic in rats (*6*). MDA reacts with the DNA bases guanine, adenine, and cytosine in vitro (*7*−*10*), but mainly with guanine and adenine in DNA (*11*) (Figure 1). The major DNA adducts are pyrimido[1,2-α]purin-10(3*H*)-one (M_1G) and N^6-(oxopropenyl)-adenine (OPA) (*11*). M_1G is present in DNA of healthy human beings making it likely that OPA is as well (*12*, *13*). Treatment of rodents with carbon tetrachloride to induce lipid peroxidation increases the levels of M_1G in liver DNA (*12*). M_1G is also formed in vitro by bleomycin treatment of DNA (*14*). Bleomycin initiates a free-radical oxidation of the deoxyribose moiety leading to a cascade of reactions that result in the formation of base propenals (*15*, *16*). The latter react with guanine residues to produce M_1G (*17*).

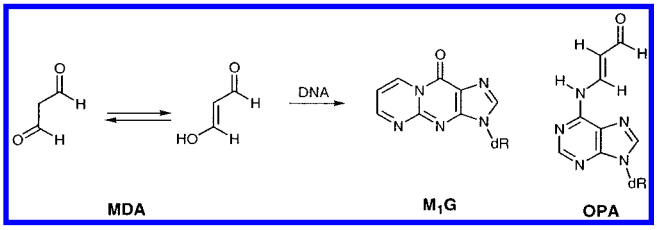

Figure 1. Formation of M_1G and OPA.

M_1G reacts as an aldehyde equivalent in DNA (*18*). Water or hydroxide adds to C-8 of M_1G to form N^2-(oxopropenyl)-guanine (OPG). (*19*, *20*). Hydrolysis also occurs when M_1G is placed in duplex DNA opposite cytosine residues (*21*, *22*). Furthermore, both M_1G and OPG react with amines to form imines and with hydroxylamines to form oximes (*18*, *20*). We hypothesized that bifunctional nucleophiles such as hydrazines would react with both reactive centers of M_1G (C-8 and C-6) to strip the MDA unit from the nucleoside and generate a pyrazole (Figure 2). Indeed Giloni et al. reported some 20 years ago that phenylhydrazine reacts with base propenals to form 1-phenylpyrazole (*15*). Therefore, we investigated the chemistry of reaction of M_1G and OPG with a series of hydrazines, and based on these studies, we developed a facile analytical procedure for the determination of total MDA units in DNA that should be applicable in a range of in vitro and in vivo experiments.

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[1] Abbreviations: MDA, malondialdehyde; M_1G, pyrimido[1,2-α]-purin-10(3*H*)-one; OPA, N^6-(oxopropenyl)-adenine; OPG, N^2-(oxopropenyl)-guanine; M_1GdR, 3-(2′-deoxy-β-D-erythro-pentofuranosyl)-pyrimido-[1,2-α]purin-10(3*H*)-one; OPAdR, 3-(2′-deoxy-β-D-erythro-pentofuranosyl)-N^6-(3-oxo-1-propenyl)-adenine; PFPH, 2,3,4,5,6-pentafluorophenylhydrazine; PFBH, 2,3,4,5,6-pentafluorobenzylhydrazine; NaMDA, sodium salt of malondialdehyde; PFPP, pentafluorophenylpyrazole; PFBP, pentafluorobenzylpyrazole; $[^2H_2]M_1GdR$, 6,8-dideutero-M_1GdR.

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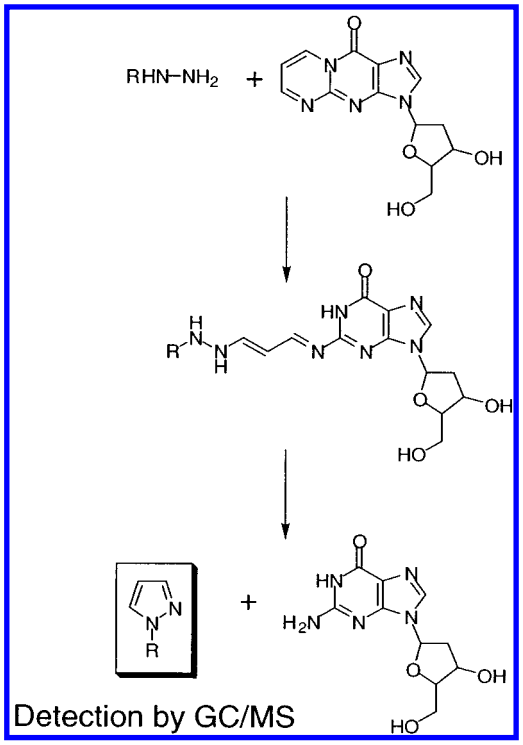

Figure 2. Reaction of M_1GdR with hydrazines. R = aryl or aralkyl.

Materials and Methods

Reagents and Enzymes. All chemicals were purchased from Aldrich (Milwaukee, WI), Fluka (Milwaukee, WI), Sigma (Milwaukee, WI), or Acros (Pittsburgh, PA) and were used as received. Adenine propenal was purchased from Salford Ultrafine Chemicals, U.K. M_1GdR, OPAdR, and ϵ-propenal-*N*-acetyl-lysine-methyl-ester (lysine propenal) were synthesized according to published procedures (*11*, *23*, *24*) and characterized by ^{1}H NMR and MS. [$^2H_2$]M_1GdR was synthesized as described (*12*).

Instrumental Analysis. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AM-300, AM-400 or AM-500 spectrometer; chemical shifts are expressed in ppm relative to tetramethylsilane as internal standard. Spin multiplicities are given as s (singlet), d (doublet), dd (doublet of doublets), t (triplet), q (quartet), or m (multiplet). Coupling constants are given in Hz.

2,3,4,5,6-Pentafluorophenylpyrazole. 1,1,3,3-Tetramethoxypropane (5.8 mL, 35 mmol) was added dropwise to a solution of 5.0 g (25 mmol) of 2,3,4,5,6-pentafluorophenylhydrazine (PFPH) in 100 mL of ethanol containing 4 mL of concentrated HCl. The mixture was heated under reflux for 2 h. Solvent was removed and the residue was distilled under reduced pressure. The fraction boiling at 76 °C (less than 1 mmHg) was collected, yielding 3.0 g of pyrazole (52%). ^{1}H NMR ($CDCl_3$) δ 8.43 (d, 1H, J = 2 Hz, H_3), 7.68 (d, 1H, J = 1 Hz, H_5), 6.56 (dd, 1H, J = 1, 2 Hz, H_4). GC/EI-MS: M^+, *m*/*z* 234; $[M\text{-}NH_2]^+$, *m*/*z* 194; $[C_6F_5]^+$, *m*/*z* 167.

Identification of the PFPH-M_1GdR−Conjugate. M_1GdR and PFPH were dissolved in d_3-MeOH and added to an NMR tube. Spectra were recorded, then the sample was analyzed by LC/MS as described below. ^{1}H NMR (d_3-MeOH), δ 8.09 (s, 1H, H_{2trans}), 7.94 (s, 1H, H_{2cis}), 7.73 (d 1H, J = 9 Hz, H_{8trans}), 7.64 (d, 1H, J = 5 Hz, H_{8cis}), 7.45 (d, J = 14 Hz, 1H, H_{6trans}), 7.27 (d, J = 9 Hz, 1H, H_{6cis}), 6.40 (m, 1H, H'_{1trans}), 6.25 (m, 1H, H'_{1cis}), 5.98 (dd, 1H, J = 9 Hz, H_{7trans}), 5.22 (dd, 1H, J = 5 Hz, H_{7cis}), 4.55 (m, 1H, H'_{3trans}), 4.00 (m, 1H, H'_{4trans}), 3.76 (m, 2H, H'_{5trans}, H''_{5trans}), 3.51 (m, 2H, H'_{5cis}, H''_{5cis}), 2.7−2.43 (m, 2H, H'_{2trans}, H''_{2trans}), 2.61−2.35 (m, 2H, H'_{2cis}, H''_{2cis}).

Benzylpyrazole. 1,1,3,3-Tetramethoxypropane (5.8 mL, 35 mmol) was added dropwise to a solution of benzylhydrazine (3.0 g, 15 mmol) in 100 mL of ethanol containing 4 mL of concentrated HCl, and the mixture was heated under reflux for 2 h. Solvent was removed and the residue was distilled under reduced pressure. The fraction boiling at 110 °C (less than 1 mmHg) was collected yielding 1.4 g of benzylpyrazole (60%). ^{1}H NMR (d_6-DMSO) δ 7.81 (d, 1H, J = 2 Hz, H_3), 7.46 (d, 1H, J = 2 Hz, H_5), 7.34−7.18 (m, 5H, aryl) 6.27 (dd, 1H, J = 2, 2 Hz, H_4) 5.38 (s, 2H, CH_2). GC/EI-MS: M^+, *m*/*z* 157; $[benzyl]^+$, *m*/*z* 91.

2,3,4,5,6-Pentafluorobenzylhydrazine (PFBH). To an ice cold solution of 5 g (19 mmol) pentafluorobenzyl chloride in 20 mL of dry methanol, 5.8 g (180 mmol) of hydrazine (6 mL in 10 mL of MeOH) was added dropwise. After additional stirring at room temperature for 2 h, the methanol was evaporated. The residue was dissolved in 200 mL of water, and the solution was extracted twice with diethyl ether. The organic phase was dried with $MgSO_4$ and evaporated. The residue was distilled under reduced pressure. The fraction boiling at 85 °C (less than 1 mmHg) was collected, yielding 2.8 g of the hydrazine (70%). ^{1}H NMR (d_6-DMSO) δ 3.83 (s, 2H, CH_2), 3.64 (bs, 3H, $NH-NH_2$). ESI-MS: $[MH]^+$, *m*/*z* 213, $[benzyl]^+$, *m*/*z* 181.

2,3,4,5,6-Pentafluorobenzylpyrazole. NaMDA (1.3 g, 14 nmol) was added to a solution of 3.0 g (14 nmol) of PFBH in 100 mL of ethanol. The solution as heated under reflux for 1 h, then the solvent was evaporated. The residue was dissolved in 200 mL of water, and the solution was extracted twice with diethyl ether. The organic phase was dried with $MgSO_4$ and evaporated. The residue was distilled under reduced pressure. The fraction boiling at 95 °C (less than 1 mmHg) was collected yielding 0.7 g of the pyrazole (21%). ^{1}H NMR (d_6-DMSO) δ 7.84 (d, 1H, J = 2 Hz, H_3), 7.43 (d, 1H, J = 2 Hz, H_5), 6.25 (dd, 1H, J = 2, 2 Hz, H_4), 5.47 (s, 2H, CH_2). GC/EI-MS: $[M]^+$, *m*/*z* 248; $[M\text{-}HF]^+$, *m*/*z* 229; $[pentafluorobenzyl]^+$, *m*/*z* 181; [pentafluorobenzyl-HF]$^+$, *m*/*z* 161.

ϵ-Propenal-N-Acetyl-Lysine-Methyl-Ester. NaMDA (0.4 g, 4 nmol) was added to a solution of *N*-acetyl-L-lysine-methyl-ester (1 g, 4 nmol) in 20 mL of ammonium acetate buffer (pH 4.5). The reaction was stirred for 15 h at room temperature. The reaction mixture was lyophilized and the residue purified by column chromatography on silica gel (CH_2Cl_2:MeOH, 80:20). ^{1}H NMR of *trans*-isomer (d_6-DMSO) δ 8.95 (d, 1H, J = 9 Hz, H_1), 8.22 (d, 1H, J = 8 Hz, H_3), 7.59 (d, 1H, H_{10}), 7.27 (m, 1H, H_4), 5.05 (dd, 1H, J = 13, 13 Hz, H_2), 4.20 (m, 1H, H_9), 3.62 (s, 3H, H_{14}), 2.98 (q, 2H, J = 13 Hz, H_5), 1.84 (s, 3H, H_{12}), 1.74−1.29 (m, 6H, H_6-H_8). ESI/MS: $[MH]^+$, *m*/*z* 258; [MH^+-propenal], *m*/*z* 203.

Derivatization with Various Hydrazines. The sample (50 μL, containing, e.g., M_1GdR) was added to an Eppendorf tube. The pH was adjusted by adding 10 μL of buffer [borate-phosphate-citrate universal buffer, pH 2−12 (*25*)] and 10 μL of PFBH solution (150 nmol/μL in MeCN) was added. The optimum conditions for derivatization were pH 7 at room temperature for 15 h using a 25-fold molar excess of PFBH. The total reaction volume was 120 μL. After incubation, the sample was extracted with 200 μL of hexane. The hexane layer was transferred to a vial and analyzed by GC/MS. The aqueous phase was lyophilized and the residue dissolved in 100 μL of water. The solution was analyzed by HPLC/UV.

HPLC/UV Analysis of the Reaction of M_1GdR or OPA with PFPH. A Varian 9010 HPLC pump equipped with an HP 1040A diode array detector was used. The reaction mixture was analyzed by analytical HPLC on a reversed-phase column (C_{18}, 250 mm length, 4.6 mm i.d., 5 μm particle size). Separation was effected by a linear gradient of (A) 10 mM ammonium formate (pH 5.0) and (B) acetonitrile from 0 to 70% B over the course of 25 min (1.5 μL/min flow rate).

HPLC/ESI-MS Analysis of the Reaction of M_1GdR and PFPH. Analysis was performed on a Waters HPLC system interfaced to a ThermoFinnigan (San Jose, CA) TSQ 7000 triple quadrupole mass spectrometer. Chromatography was carried out on a Zorbax RX C18 column (2.1 × 150 mm, 5 μm, Hewlett-Packard, Wilmington, DE) at a flow rate of 300 μL/min. The eluting solvents included solvent A (10 mM NH_4Ac, pH 4.5) and solvent B (100% MeCN), the elution was carried out by a linear

gradient from 0 to 70% B in 30 min. The elution time of M_1GdR was 7.3 min and the hydrazine derivative was 16.6 min. A voltage of 3.5 kV was applied to the electrospray needle, and 70 psi nebulizer gas (N_2) was employed to stabilize the spray. The MS was operated in full scan mode (CID offset) from *m*/*z* 150 to *m*/*z* 600.

PFBH Derivatization. The sample (100 μL, containing, e.g., M_1GdR or DNA) was added to a silanized glass vial. Sodium phosphate buffer (10 μL, 500 mM, pH 7.0) was added followed by 10 μL of PFBH (50 nmol/μL in MeCN). The total reaction volume was 100 μL. After incubation, the sample was extracted with 200−500 μL of hexane. The hexane layer was transferred to a vial and analyzed by GC/MS.

SPE Separation. Samples were applied to columns of 100 mg of silica (Waters, Sep-Pak) that had been washed with 2 mL of hexane. After the samples were loaded, the columns were washed with 1 mL of hexane, and the pyrazole PFBP was eluted with acetonitrile. The fractions were collected in glass vials and subjected to GC/MS analysis as described below.

GC/ECNCI-MS Analysis of PFBP. A CE Instruments GC8000Top gas chromatograph interfaced to a Finnigan Voyager mass spectrometer equipped with a SPD-50 (Supelco, Bellefonte, PA) capillary column (15 m × 0.25 mm i.d., 0.25 μm film thickness) was used for analysis. Helium was used as the carrier gas at a flow rate of 1.5 mL/min. The GC oven was held at 50 °C for 1 min, programmed at 20 °C/min to 300 °C, and held for 1 min at 300 °C. The temperature of the injector and the transfer line was 250 °C. Analysis was performed in the splitless mode, and 1 μL of sample was injected into the GC. The MS was operated in the negative chemical ionization mode with electron energy of 230 eV, source temperature of 150 °C, and a source pressure for methane of 2.3 Torr. The signals were monitored in the full scan mode from *m*/*z* 50 to *m*/*z* 400 or by SIM at *m*/*z* 67 and *m*/*z* 228.

Treatment of DNA with Bleomycin. The reaction was performed by mixing an aqueous solution of bleomycin sulfate (Sigma) with ferrous ammonium sulfate at a molar ratio of 4:5, then adding the mixture to a solution of calf thymus DNA in 50 mM sodium phosphate buffer (pH 7). The reaction proceeded for 1 h at 37 °C.

Immunoslotblot Assay. Bleomycin-treated DNA samples were diluted with PBS to 3.5 μg/150 μL. For quantification standards, MDA-modified calf thymus DNA was diluted with unmodified calf thymus DNA to give 7 μg of DNA in 300 μL of PBS containing a range of modification levels. DNA samples were denatured by sonication for 15 min in a water bath, boiling for 10 min, and incubation on ice for 10 min. Ammonium acetate (2 M, 1 vol.) was added to each sample and DNA (1 μg/well) was blotted in triplicate onto a BA79 nitrocellulose membrane (Schleicher and Schuell, Keene, NH) using a Minifold II blotting apparatus (Schleicher and Schuell). The membrane was baked for 90 min at 80 °C then blocked with nonfat milk for 1 h and incubated with a 1:50000 dilution of D10A1 anti-M_1G monoclonal antibody (0.3 mg/mL stock) (*26*). Membranes were incubated with goat anti-mouse IgG horseradish peroxidase conjugate (1:3000), and the peroxidase activity was visualized by enhanced chemiluminescence with Super Signal West Dura (Pierce, Rockford, IL) and exposure to Hyperfilm ECL (Amersham Pharmacia Biotech, Buckinghamshire, England). Bands were quantified by densitometry using a Fluor-S MAX Multimager and quantity One software (BioRad, Hercules, CA). For normalization, the amount of immobilized DNA was determined by propidium iodide staining followed by fluorescence quantitation (*17*). Standard curves were produced by plotting the adduct level against the quotient of the chemiluminescence signal divided by the propidium iodide fluorescence intensity. M_1G levels in bleomycin-treated samples were based on standard curves analyzed in parallel on the same blot.

Results

Reaction of PFPH with M_1GdR. PFPH has been reported to react with MDA−protein conjugates to produce pentafluorophenylpyrazole (PFPP) (*27*). Therefore, we investigated its chemistry with M_1G residues. M_1GdR was incubated with a 50-fold molar excess of PFPH, and the progress of the reaction was assessed by HPLC (Figure 3). Although M_1GdR disappeared with time, neither dG nor PFPP was detected by HPLC or by GC/EI-MS. Rather, an additional peak was detected in the HPLC chromatogram at 26.9 min with an absorbance maximum at 330 nm (inset B). This product was analyzed by HPLC/MS and exhibited an intense peak at *m*/*z* 502, corresponding to the mass of an M_1GdR conjugate with PFPH. This was confirmed by ^{1}H NMR. The low-field region of the spectrum (Figure 4) suggested the presence of both *cis*- and *trans*-isomers of the enamino-imine conjugate of M_1GdR in a ratio of approximately 1:3. The protons were assigned by comparison to conjugates of M_1G to amines and hydroxylamines as well as by ^{1}H^{1}H-COSY-NMR (data not shown) (*20*).

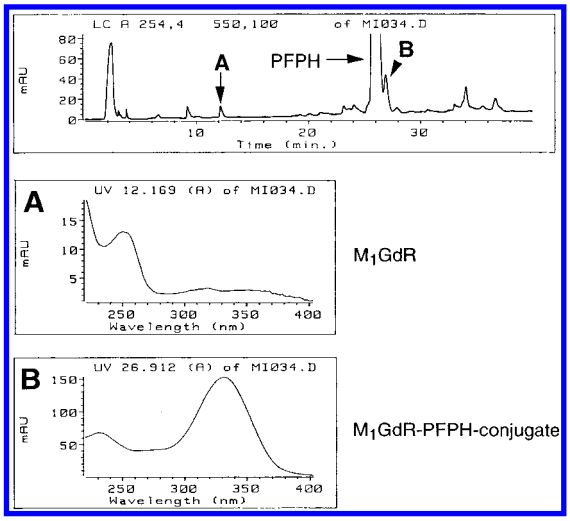

Figure 3. HPLC chromatogram of the reaction mixture of M_1GdR and PFPH at 254 nm. The profile displayed was from a reaction performed at pH 8 for 20 h. The UV spectra of peak A and B. M_1GdR elutes at position A and the major product elutes at position B.

Reaction of Phenylhydrazine and Benzylhydrazine with M_1GdR. The reaction of phenylhydrazine and benzylhydrazine with M_1GdR was performed under the identical conditions used for PFPH. Reactions were monitored by HPLC and by GC/EI-MS. In contrast to the results with PFPH, conjugates of phenylhydrazine or benzylhydrazine with M_1GdR were not detected. Rather, products were detected that cochromatographed with the pyrazole derivatives and exhibited the appropriate mass spectra (data not shown).

Reaction of PFBH with M_1GdR. On the basis of the ability of phenylhydrazine and benzylhydrazine to form pyrazoles on reaction with M_1GdR, we explored the reaction of PFBH with M_1GdR. Incorporation of the pentafluorobenzyl group provides a powerful electrophore for ECNCI mass spectroscopic detection (*28*). PFBH and M_1GdR were incubated in aqueous solutions over a range of pHs. Reaction was monitored by HPLC and GC/EI-MS (Figure 5). The expected product PFBP was synthesized, characterized and used as a reference compound for mass spectrometry. In reactions containing 60 nmol of M_1GdR and a 25-fold excess of PFBH, the yield of pyrazole exceeded 90%. No substantial differences in yield were observed between pH 3 and pH 9 (Figure 6). A decrease in yield was noted at pH 10.

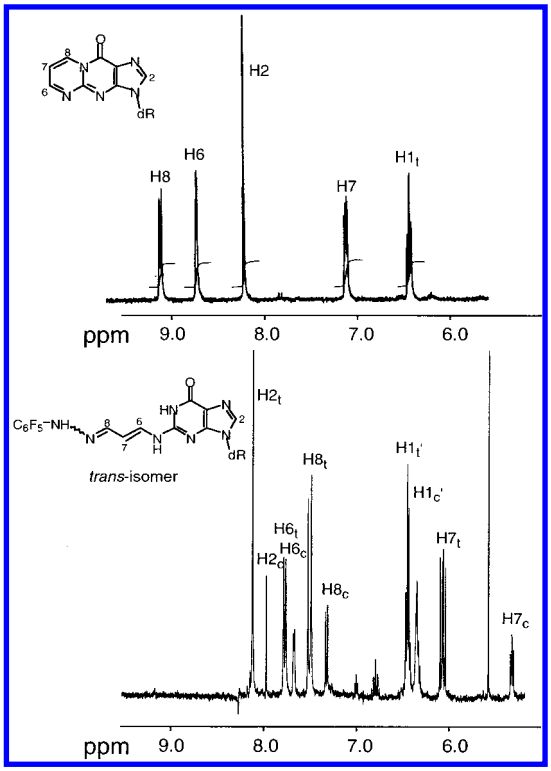

Figure 4. ^{1}H NMR spectrum (400 MHz) of the M_1GdR−PFPH derivative (*trans*- and *cis*-isomer) in comparison to the spectrum of M_1GdR. The protons of the M_1GdR−PFPH derivative were assigned by comparison to the spectrum of the M_1GdR-Tris conjugate (*18*).

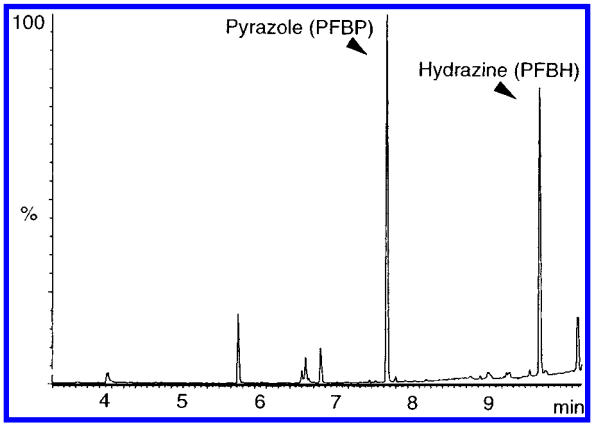

Figure 5. Total ion chromatogram (GC/EI-MS) of the reaction mixture of M_1GdR and PFBH after hexane extraction.

To optimize reaction conditions, the time and temperature dependence of pyrazole yield as well as the optimal amount of PFBH were investigated. Figure 6 shows that the reaction with a 25-fold excess of PFBH at pH 7 was essentially complete after 5 h although there was a slight increase in yield up to 15 h. Attempts to shorten the reaction time by increasing the temperature were unsuccessful.

6,8-Dideuterated M_1GdR ([$^2H_2$]M_1GdR) was synthesized for use as an internal standard for mass spectral analysis of PFBP. A mixture of M_1GdR and [$^2H_2$]M_1GdR was reacted with PFBH and subjected to LC/MS analysis. Figure 7 displays the EI and NCI mass spectra of the undeuterated pyrazole that was present in the mixture of undeuterated and deuterated products. The high mass peak in the EI spectrum at *m*/*z* 248 is the molecular ion, whereas the high mass peak at *m*/*z* 228 in the NCI

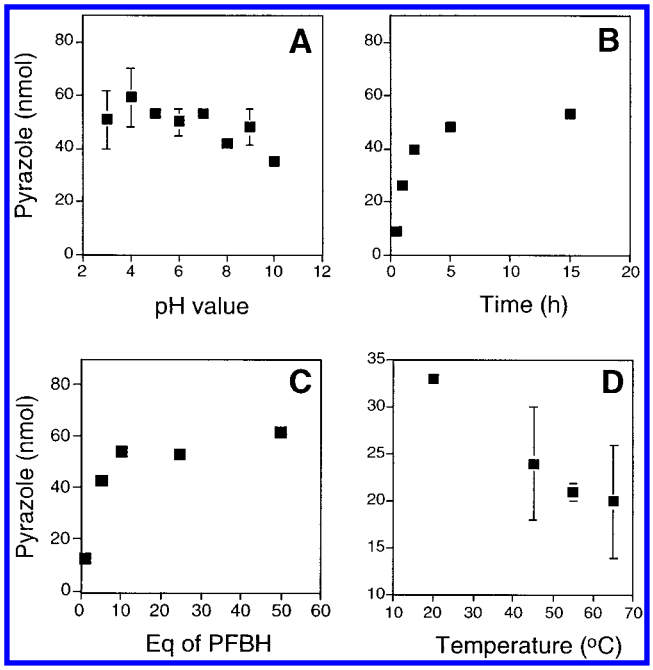

Figure 6. Parameters in the reaction of M_1GdR and PFBH. Different conditions were varied (A) pH of buffer; (B) reaction time; (C) amount of PFBH; and (D) reaction temperature. Reactions were performed using the conditions described under Methods except for the varied parameter. For experiments summarized in panels A and C, the aqueous phase was extracted with hexane after a 15 h reaction and the organic phase was analyzed by GC/MS. For experiments in panel D, the reaction time was 2 h.

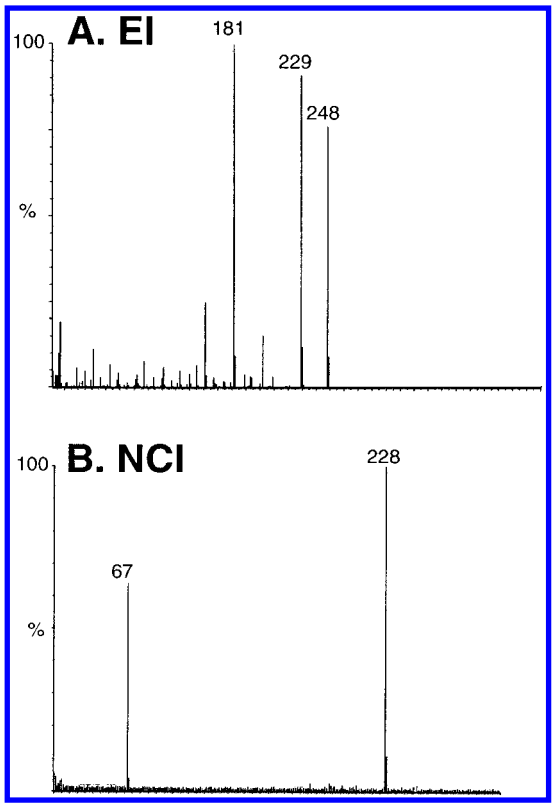

Figure 7. Mass spectra of PFBP. The upper spectrum was obtained by electron impact (EI) and the lower spectrum was obtained by negative chemical ionization (NCI).

spectrum corresponds to a fragment ion corresponding to the loss of hydrogen fluoride. The abundance of the $[M - H]^-$ ion relative to the $[M - PFB]^-$ ion was surprisingly high considering that M − PFB ions are

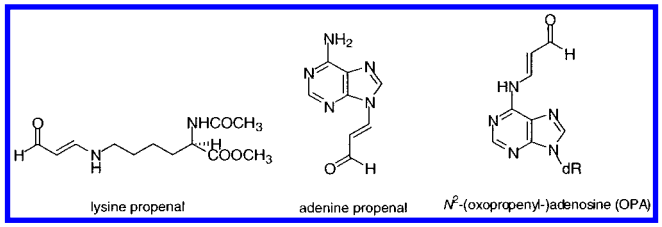

Figure 8. MDA equivalents in protein and DNA.

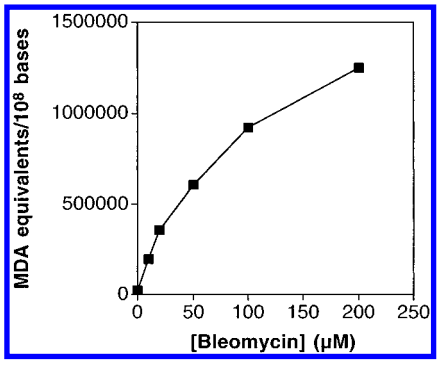

Figure 9. Determination of MDA equivalents induced by bleomycin treatment of DNA. Calf thymus DNA was incubated for 1 h at 37 °C. The reaction mixture was subjected to PFBH treatment without further purification. The values represent the means of two independent experiments.

commonly observed as base peaks in ECNCI mass spectra. Using different amounts of the chemically synthesized PFBP standard, a detection limit of 500 amol (signal-to-noise ratio = 3) was achieved in the ECNCI mode. No exchange of deuterium occurred during the reaction of the [$^2H_2$]M_1GdR internal standard with PFBH. Full-scan analysis revealed less than 0.1% nondeuterated PFBP after reaction and workup.

Reaction of PFBH with OPA, Adenine Propenal and Lysine Propenal. To assess the ability of PFBH derivatization to detect other MDA equivalents covalently attached to biomolecules, we subjected OPA and adenine propenal to the reaction conditions developed for M_1GdR (Figure 8). OPA and adenine propenal reacted quantitatively with PFBH to form the corresponding pyrazole, whereas the yields with M_1GdR and lysine propenal were 92 and 74%, respectively. These results demonstrate that PFBH is a suitable derivatization agent for the removal and quantitation of MDA moieties covalently attached to an array of biological macromolecules.

Measurement of MDA Equivalents in DNA Treated with Bleomycin. Bleomycin, chelated with iron, reacts with DNA in the presence of molecular oxygen to form *N*-propenal-substituted derivatives of thymine, guanine, adenine, and cytosine (*15*). The individual base propenals react with phenylhydrazine to form 1-phenylpyrazole (*15*). Therefore, the PFBH stripping reaction was used for the determination of MDA equivalents in bleomycin-treated DNA. A concentration-dependent increase in MDA equivalents was observed (Figure 9). This result demonstrates the potential applicability of the derivatization method for assaying base propenals. In parallel to the stripping reaction, the concentration of M_1G formed during bleomycin treatment was quantitated by an immunoslotblot assay (Figure 10) (*29*). Comparing the amount of M_1G detected by immunoslotblot analysis to the total level of MDA (or oxopropenyl) equivalents detected by PFBH stripping provided an estimate of the efficiency of M_1G generation during

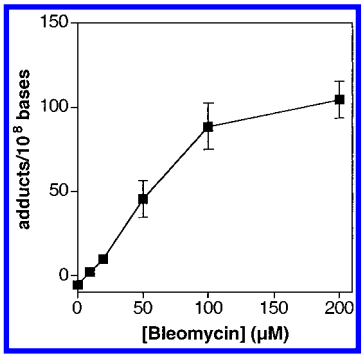

Figure 10. Level of M_1G in DNA after bleomycin treatment.

bleomycin oxidation of DNA. For example, at 200 μM bleomycin, the yield of M_1G was 1 in 12 500 (0.008%) that of total oxopropenyl units (e.g., base propenals, M_1A, etc.).

Discussion

M_1G is a reactive electrophile both in solution and in DNA (*20*). It reacts with amines to form unstable enaminoimine conjugates, with hydroxylamines to form oximes, and with hydroxide to form N^2-(3-oxopropenyl)-G (*18, 20*). The latter reaction also occurs in duplex DNA where it is catalyzed by the N^4-amino group of the C residue in the complementary strand (*21*). Hydrolysis of M_1G in solution requires pHs above 9, whereas the DNA-catalyzed ring-opening occurs at neutral pH (*19*). The present work extends the reactivity of M_1G to hydrazines, which have the potential to form pyrazoles with release of dG.

Our initial studies were conducted with PFPH, which has been reported to strip MDA equivalents from protein conjugate (*27*). We found no evidence for such a reaction with M_1G but isolated a stable hydrazone conjugate that did not cyclize to pentafluoropyrazole. Apparently, the hydrazone nitrogen is not sufficiently nucleophilic to attack the β carbon of the propenyl unit of the hydrazone. The hydrazone conjugate is analogous to the enaminoimine conjugate formed by reaction of M_1G with Tris, but it is more stable. The hydrazone is isolable at room temperature and can be subjected to chromatography with no decomposition. In contrast, the Tris conjugate is only detectable in frozen aqueous solutions and rapidly reverts to M_1G and Tris on warming to room temperature (*18*).

Phenylhydrazine and benzylhydrazine both reacted with M_1G to form the corresponding phenyl and benzyl pyrazoles. This highlights the negative impact of the pentafluorophenyl group on the reactivity of the α-nitrogen of PFPH. Interposing a methylene group between the α-nitrogen and the pentafluorophenyl ring permitted cyclization of the intermediate hydrazone to occur following its formation from M_1G. High yields of the PFBP were produced under mild reaction conditions.

The presence of the pentafluorobenzyl group enabled highly sensitive detection by GC/MS with negative ion chemical ionization detection (limit of detection ~500 amol) (*28*). Theoretically, one should be able to use the procedure described to quantify M_1G in DNA at levels of 1 adduct in 10^8 bases from small amounts of DNA. However, we have observed an artifactual formation of small amounts of the PFBP in the absence of M_1G that limits the sensitivity of the assay to ~1 adduct in 10^7

bases. Exhaustive efforts to eliminate this artifact including purification of PFBH as well as the use of ultrapure organic solvents and water were unsuccessful. The origin of the low levels of pyrazole is not clear, but it is important to note that PFBP is formed in the complete absence of DNA constituents in the reaction with PFBH. Despite this complication, the sensitivity obtainable with the procedure described is adequate for most routine applications. Given that the method is suitable for automated operation, the assay may be useful for analysis of a broad range of DNA samples. Indeed, this single analytical method can be used for the analysis of nucleic acid and protein conjugates.

We demonstrated the utility of the PFBH assay by reacting bleomycin with DNA then analyzing for M_1G/oxopropenyl/MDA equivalents by reaction with PFBH. The DNA was not purified or dialyzed after reaction with bleomyin to increase the probability of detecting reactive equivalents present on DNA or released following strand scission (e.g., base propenals). A direct relation was observed between bleomycin concentration and the extent of generation of MDA equivalents (MDA, base propenals, M_1G, M_1A, etc.). At the highest concentration of bleomycin used (200 μM), the level of MDA/oxopropenyl equivalents was $1.25/10^2$ bases or approximately 1 equiv/100 bases. Parallel analyses of these samples for M_1G by immunoslotblot indicated that the content of this adduct represented only 0.008% of the total MDA/oxopropenyl equivalents generated. Thus, most of the reactive moieties detected by the PFBH assay must be base propenals, MDA, or related molecules that are unable to form additional M_1G. This inefficiency of M_1G production from in situ generated MDA or base propenals may reflect the low reactivity of the conjugate base of MDA at neutral pH or conformational restrictions on the reaction of base propenals with DNA.

The present report expands the repertoire of reactions of M_1G in DNA toward nucleophiles and utilizes the findings to provide a robust and straightforward assay for the presence of oxopropenyl equivalents in DNA. Since a number of drugs generate free radicals capable of degrading DNA to products with oxopropenyl equivalents, this assay should find broad use as a convenient method for quantifying oxidative DNA damage.

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