Chemical Research in Toxicology

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DNA-Polyamine Cross-Links Generated upon One Electron Oxidation of DNA

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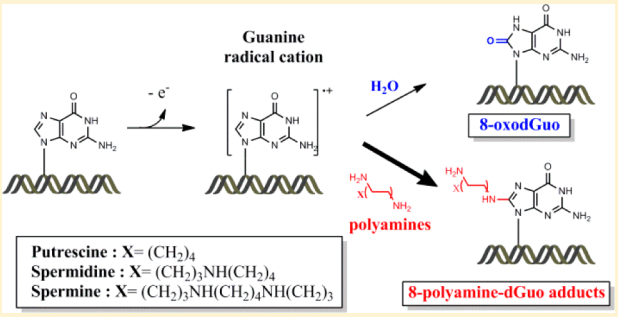
Supporting Information

ABSTRACT: The possibility to induce the formation of covalent cross-links between polyamines and guanine following one electron oxidation of double stranded DNA has been evaluated. For such a purpose, a strategy has been developed to chemically synthesize the polyamine-C8-guanine adducts, and efforts have been made to characterize them. Then, an analytical method, based on HPLC separation coupled through electrospray ionization to tandem mass spectrometry, has been setup for their detection and quantification. Using such a sensitive approach, we have demonstrated that polyamine-C8-guanine adducts could be produced with significant yields in double stranded DNA following a one-electron oxidation reaction induced by photosensitization. These adducts, involving either putrescine, spermine, or spermidine, are generated by the nucleophilic addition of primary amino groups of polyamines onto the C8 position of the guanine radical cation. Our data demonstrate that such a nucleophilic addition of polyamines is much more efficient than the addition of a water molecule that leads to 8-oxo-7,8-dihydroguanine formation.



INTRODUCTION

The DNA molecule is constantly exposed to various kinds of genotoxic agents, including among others reactive oxygen species (ROS) produced during oxidative stress.[1] It is well established that normal cellular metabolism is a source of ROS, which may interact with DNA, leading to chemical modifications including single and double strand breaks or base and sugar lesions.[2] These oxidative damages could also be generated after exposure to ionizing radiation, UV light, or to different exogenous chemical agents and are known to be involved in mutagenesis and carcinogenesis.[2,3]

For instance, photosensitization reactions are known to be able to induce oxidative DNA lesions through either the transient formation of singlet oxygen (Type II photosensitization reaction) or following a one electron oxidation reaction (Type I).[4] The latter mechanism is one of the main processes involved in the formation of oxidized base lesions.[3] Guanine is the preferential target of this reaction since it exhibits the lowest ionization potential among DNA components.[5] Various agents and free radicals are able to abstract one electron from guanine,[6] leading to the formation of a guanine radical cation $G^{\bullet+}$. The latter could also be produced following an electron transfer reaction from guanine to a radical cation initially generated on other DNA bases.[7]

Several nucleophilic addition reactions on the C8 position of the guanine radical cation have been already reported. The first described mechanism involves the formation of the well-known 8-oxo-7,8-dihydroguanine, through the addition of a water molecule onto C8 of guanine and subsequent oxidation of the latter generated radical.[8] This hydration reaction could also be followed by a competitive reduction, with an opening of the imidazole ring, producing 2,6-diamino-4-hydroxy-5-formamidopyrimidine.[6] Other guanine decomposition products have been identified and are certainly generated through an initial nucleophilic addition at C8, involving either C5′-OH group,[9] C5′-amino residues,[10−12] or even methanol as recently reviewed.[13]

Such a nucleophilic addition of amino or alcoholic residues onto C8 of guanine is of particular relevance in the issue of DNA−protein cross-links. In 2006, Perrier et al. described the formation of a covalent cross-link between a lysine moiety and 2′-deoxyguanosine (dGuo), after photosensitization of a TGT oligonucleotide in the presence of a trilysine peptide.[14] The cross-link involves the nucleophilic addition of the ε amino group of the central amino acid residue of the peptide onto the C8 position of deprotonated guanine radical cation $G(-H)^{\bullet}$. Such a reaction has also been implicated in the formation of intrastrand cross-links through the addition of a neighboring thymine to $G(-H)^{\bullet}$.[15,16] According to these observations, we hypothesized that other nucleophilic molecules would be able

Received: February 24, 2014
Published: May 5, 2014

   dx.doi.org/10.1021/tx500063d | *Chem. Res. Toxicol.* 2014, 27, 1011−1018

to add to C8 of guanine upon one electron oxidation of double stranded (ds) DNA.

Thus, in the present work, attempts have been made to determine if small polyamine molecules could also be involved in such a reaction. Polyamines are organic cations localized in the nucleus of all eukaryotic cells[17] at millimolar concentration ranges. They are biosynthesized by enzymatic decarboxylation of the amino acids ornithine and arginine by ornithine decarboxylase (ODC).[18] It has been proposed that due to their affinity for anionic sites of nucleic acids, the polyamines' main role is to stabilize and condense DNA.[19] They are also involved in several essential metabolic processes, such as regulation of RNA transcription, specific gene expression, and the progression of the cell cycle.[18] They play a role in cell growth and cancer, and an increase in their cellular concentration correlates with a loss of cell proliferation control and could consequently lead to neoplesia.[18] Furthermore, polyamines are present in a high concentration in several biological fluids in patients suffering from different types of cancer.[20] The assessment of their levels in urine, blood, or cerebrospinal liquid can be informative as a marker of tumor growth in treated patients. It has been used for the prediction of relapse as well as in the follow-up of chemotherapy efficiency.[21,22] For these reasons, polyamines are a potential target in cancer treatment, and various inhibitors of their biosynthesis have been developed. The most studied is α-difluoromethylornithine, which is an irreversible inhibitor of ODC. Polyamine oxidase, an enzyme involved in the catabolism of polyamines, is also considered as an interesting outlook in the development of antineoplasic drugs.[23] Finally, recent studies have highlighted the interest of using a low polyamine diet in addition to chemotherapy.[24] Therefore, the link between polyamines and cancer has been widely described. Nevertheless, the exact mechanism in which the upregulation of their metabolism could be implicated in carcinogenesis is still unknown.

The aim of our study is to investigate the possible formation of adducts between polyamines and 2′-deoxyguanosine in dsDNA following a one electron oxidation reaction. For such a purpose, we have designed a chemical synthesis of the potentially generated DNA adducts involving either putrescine, spermine, or spermidine. In addition, we have optimized a HPLC-MS/MS method to quantify these adducts in DNA subsequent to enzymatic digestion into corresponding free nucleosides. Using such a strategy, we have demonstrated that polyamine-C8-guanine adducts could be generated following a type I photosensitization reaction involving riboflavin and UVA, a well-known approach to generate guanine radical cations[25] in dsDNA.

EXPERIMENTAL PROCEDURES

Chemicals. *N*-Bromosuccinimide, 1,4-diaminobutane (putrescine), spermidine, spermine, triethylamine, and solvents for organic synthesis were purchased from Sigma-Aldrich (St. Louis, MO). 2′-Deoxyguanosine (dGuo) and 2′-deoxythymidine were obtained from Pharma Waldhof (Düsseldorf, Germany), HPLC grade acetonitrile was purchased from VWR BDH Prolabo (Fontenay-sous-Bois, France) and trifluoroacetic acid and heptafluorobutyric acid from Sigma-Aldrich. Enzymes for DNA digestion (nuclease P1, phospodiesterase I and II, and alkaline phosphatase) and calf thymus DNA were purchased from Sigma-Aldrich. NMR solvents were obtained from Euriso-top (Gif-sur-Yvette, France).

Instrumentation. The HPLC-MS/MS analyses were performed with a triple quadrupole mass spectrometer TSQ Quantum Ultra from Thermo Scientific equipped with a Thermo Accela Pump, an Accela autosampler, and an Accela photodiode array detector. The data were processed using Xcalibur software. HPLC purification was performed using an Agilent 1200 Series LC system. ^{1}H NMR spectra were recorded on Bruker Avance DMX 200 and Bruker Avance 500 spectrometers at 295 K in 400 μL of either CD_3OD (isotope purity >99.8%) or D_2O (isotope purity: 99.95%).

Chemical Synthesis. *8-Bromo-2′-deoxyguanosine (8-Br-dGuo, **1**).* dGuo (4 g, 15 mmol) was suspended in a mixture of water (40 mL) and acetonitrile (160 mL). *N*-Bromo-succinimide (4g, 22.5 mmol) was added at 0 °C in three portions. The reaction mixture was stirred for 30 min at room temperature and subsequently concentrated under reduced pressure. This material was then dissolved in acetone (80 mL) and stirred for 2 additional hours at room temperature. The resulting precipitate was collected by vacuum filtration with a Büchner funnel. The filtrate was rinsed twice with cold acetone and dried under vacuum to provide 3.85 g (11.1 mmol, 74%) of a slightly pink powder. This compound was used for the next reaction steps without further purification. ^{1}H NMR (D_2O, 200 MHz, δ): 2.13 (ddd, 1H, *J* = 2.3, 6.5, 13.6 Hz, H2′′), 3.07 (ddd, 1H, *J* = 6.3, 8.1, 13.6 Hz, H2′), 3.64 (dd, 1H, *J* = 4.2, 12.0 Hz, H5′′), 3.75 (dd, 1H, *J* = 3.4, 12.0 Hz, H5′), 3.89−3.95 (m, 1H, H4′), 4.48−4.54 (m, 1H, H3′), 6.30 (dd, 1H, *J* = 6.5, 8.1 Hz, H1′).

*8-((4-Aminobutyl)amino)-2′-deoxyguanosine (8-put-dGuo, **2**).* 8-Br-dGuo (**1**) (69 mg, 0,2 mmol) and 1,4 diaminobutane (100 μL, 1 mmol) were dissolved in DMSO (1.2 mL). Triethylamine (135 μL, 1 mmol) was added, and the reaction mixture was heated for 15 h at 110 °C. According to HPLC-UV monitoring, the substitution reaction afforded 25% of 8-put-dGuo. The crude material was diluted 10 times in water, neutralized with HCl, and then purified by C18 reverse phase HPLC. The elution was performed using a C18 column (Interchim uptisphere 5 μm ODB 250·4.6 mm), at a flow rate of 1 mL/min, with 0.1% trifluoroacetic acid as the mobile phase (eluent A) and a linear gradient of acetonitrile (eluent B, 0−14% over 21 min). The product which eluted at 15 min (*k*′ = 3.41) was collected, concentrated, and lyophilized. ^{1}H NMR (D_2O, 500 MHz, δ): 1.70−1.78 (m, 4H, $H_{putB,C}$), 2.26 (ddd, 1H, *J* = 1.8, 6.6, 14.5 Hz, H2′′), 2.77 (ddd, 1H, *J* = 6.6, 8.4, 14.5 Hz, H2′), 3.02−3.08 (m, 2H, H_{putD}), 3.34−3.42 (m, 2H, H_{putA}), 3.88 (dd, 1H, *J* = 2.3, 12.4 Hz, H5′′), 3.92 (dd, 1H, *J* = 2.1, 12.4 Hz, H5′), 4.12−4.14 (m, 1H, H4′), 4.63−4.65 (m, 1H, H3′), 6.31 (dd, 1H, *J* = 6.6, 8.4 Hz, H1′). MALDI-HRMS *m*/*z* for $C_{14}H_{24}N_7O_4^+$ ($[M + H]^+$): calcd 354.1890; found 354.1883.

*8-((3-((4-Aminobutyl)amino)propyl)amino)-2′-deoxyguanosine and 8-((4-((3-aminopropyl)amino)butyl)amino)-2′-deoxyguanosine (8-spd-dGuo **3a** and **3b**).* The synthesis was performed following the same procedure as for compound **2**, using 1 equiv of 8-Br-dGuo, 5 equiv of spermidine, and 5 equiv of triethylamine. The crude material was purified by HPLC, as described for compound **2**, with a gradient of eluent B from 0 to 12% over 20 min. The two products **3a** and **3b** which eluted at 14.3 min (*k*′ = 3.21) and 15.7 min (*k*′ = 3.62), respectively, were collected. The two isomers exhibit identical ^{1}H NMR spectral features: ^{1}H NMR (D_2O, 500 MHz, δ): 1.76−1.80 (m, 3H, H_{spd}), 2.06−2.12 (m, 3H, H_{spd}), 2.34 (ddd, 1H, *J* = 2.2, 6.1, 14.3 Hz, H2′′), 2.74 (ddd, 1H, *J* = 6.5, 9.1, 14.3 Hz, H2′), 3.09−3.12 (m, 4H, H_{spd}), 3.14−3.18 (m, 2H, H_{spdG}), 3.44−3.47 (m, 2H, H_{spdA}), 3.88 (dd, 1H, *J* = 2.8, 12.4 Hz, H5′′), 3.94 (dd, 1H, *J* = 2.1, 12.4 Hz, H5′), 4.19−4.20 (m, 1H, H4′), 4.63−4.66 (m, 1H, H3′), 6.42 (dd, 1H, *J* = 6.1, 9.1 Hz, H1′). MALDI-HRMS *m*/*z* for $C_{17}H_{31}N_8O_4^+$ ($[M + H]^+$): calcd 411.2468; found 411.2534.

*8-((3-((4-((3-Aminopropyl)amino)butyl)amino)propyl)amino)-2′-deoxyguanosine (8-spm-dGuo, **4**).* The synthesis was performed following the same procedure as that for compound **2**, using 1 equiv of 8-Br-dGuo, 5 equiv of spermine, and 5 equiv of triethylamine. The crude material was purified by HPLC, as described for compound **2**, with a gradient of eluent B from 0 to 14% over 21 min. The product which eluted at 13.8 min (*k*′ = 3.06) was collected. ^{1}H NMR (D_2O, 500 MHz, δ): 1.36−1.39 (m, 4H, $H_{spmE,F}$), 1.64−1.72 (m, 4H, $H_{spmB,I}$), 1.94 (ddd, 1H, *J* = 2.0, 6.2, 14.3 Hz, H2′′), 2.35 (ddd, 4H, *J* = 6.3, 8.6, 14.3 Hz, H2′), 2.68−2.72 (m, 8H, $H_{spmC,D,G,H}$), 2.74−2.77 (m, 2H, H_{spmJ}), 3.09−3.13 (m, 2H, H_{spmA}), 3.48 (dd, *J* = 2.6, 12.4 Hz, H5′′),

3.53 (dd, J = 2.4, 12.4 Hz, H5′), 3.77−3.79 (m, 1H, H4′), 4.23−4.25 (m, 1H, H3′), 5.99 (dd, J = 6.2, 8.6 Hz, H1′). MALDI-HRMS m/z for $C_{20}H_{38}N_9O_4^+$ ([M + H]$^+$): calcd 468.3047; found 468.3094.

UV Characterization of Polyamine-dGuo Adducts. The concentration of the polyamine solutions was determined by quantitative ^{1}H NMR and used for the determination of the molar extinction coefficients of the different adducts. For the ^{1}H NMR quantification, the relaxation time t1 of the anomeric proton of C8 polyamine adducts was measured between 2 and 3 s. Thus, acquisition was performed during 512 scans in D_2O; the relaxation delay was set at 12 s. The concentration was calculated by comparing the integrals of the anomeric protons of the polyamine adducts to those of 2′-deoxythymidine in a calibrated solution (obtained by UV 3,0068 mM, ε = 9318 L·mol^{-1}·cm^{-1}). A capillary containing a reference solution of 3-(trimethylsilyl)propionic acid sodium (Merck, Darmstadt, Germany) was inserted into each NMR tube in order to normalize integrations. The molar extinction coefficient was determined by measuring UV absorbance of the calibrated polyamine-dGuo adducts and applying the Beer−Lambert law.

UVA Irradiation and Digestion of Isolated DNA Samples. An aqueous solution of calf thymus DNA (0.1 mg/mL) containing NaCl (0.2 M), riboflavin (40 μM), and 500 μM of each of the three polyamines was irradiated at room temperature with a 500 W halogen lamp placed at a distance of 20 cm. The solution was kept saturated with oxygen by continuous air bubbling and maintained at room temperature by circulating water. Samples of 50 μL (5 μg of DNA) were collected at 0, 1, 2, 5, and 10 min. DNA was precipitated by adding 125 μL of 100% cold ethanol and 10 μL of NaCl (4 M), the DNA pellet was rinsed 3 times with 70% ethanol, dissolved in 50 μL of water, and digested as follows:[26] to each sample of irradiated DNA (0.1 μg/μL, 50 μL), 0.025 U of phosphodiesterase II, 2.5 U of DNase II, 0.5 U of nuclease P1 in 2.5 μL of buffer P1 (300 mM ammonium acetate and 1 mM $ZnSO_4$, pH 5.3), and 2.5 μL of buffer MNSPDE (200 mM succinic acid, 100 mM $CaCl_2$, pH 6) were added. The samples were incubated for 2 h at 37 °C. Then, 0.015 U of phosphodiesterase I was added, together with 6 μL of buffer Palk 10× (500 mM Tris and 1 mM EDTA, pH 8) and 2 U of alkaline phosphatase. The given solutions were incubated again for 2 h at 37 °C. Then, 3.5 μL of hydrochloric acid 0.1 N was added. The resulting nucleoside mixture was then analyzed by HPLC-MS/MS.

HPLC-MS/MS Analysis. The separation of hydrolyzed nucleosides was achieved using a ODB uptisphere 3 μm 150 × 2.1 mm column from Interchim under linear gradient conditions as previously reported for measuring other DNA lesions.[27] The flow rate was set at 0.2 mL/min with 0.1% heptafluorobutyric acid in water as the mobile phase (eluent A) and acetonitrile from 0 to 45% (eluent B) over 30 min. Unmodified nucleosides were detected using a UV detector set at 280 nm. The multiple reactions monitoring mode (MRM) was used in order to detect two specific transitions for each adduct: 354.3 → 221.2 and 354.3 → 238.5 for 8-put-dGuo; 468.0 → 207.2 and 468.0 → 352.4 for 8-spm-dGuo; 411.5 → 207.2 and 411.5 → 221.2 for 8-spd-dGuo. Collision energies and ionization parameters have been optimized by infusing standard solutions of each adduct as reported for 8-oxodGuo.[28] For each series of samples, calibrated solutions of a mixture of normal nucleosides, 8-oxodGuo, or polyamine-dGuo adducts were first injected to check the sensitivity of the MS/MS system. Then, after an analysis of a maximum 12 samples, these standards were reinjected to make sure that no variation in the sensitivity of the detector occurred with time. The levels of normal nucleosides and lesions were calculated by external calibration for both UV and MS/MS detection, respectively, and results were expressed as the number of DNA lesions (8-oxodGuo or polyamine-dGuo adducts) per million normal nucleosides.

RESULTS

Synthesis of C8 Polyamine-2′-deoxyguanosine Adducts. Adducts between dGuo and polyamines (putrescine, spermidine, and spermine) have been prepared according to the strategy described in Scheme S1 (Supporting Information). The synthesis consists first in the bromination of dGuo onto the C8 position using N-bromosuccinimide,[29] followed by the nucleophilic substitution of generated 8-Br-dGuo by the three polyamines under alkaline conditions as described previously for aromatic amines.[30] According to HPLC analysis, the estimated yield for the reaction was about 25%, whatever the nature of the polyamine.

The three adducts (8-put-dGuo, 8-spd-dGuo, and 8-spm-dGuo, Figure 1) were purified using C18 reverse phase HPLC.

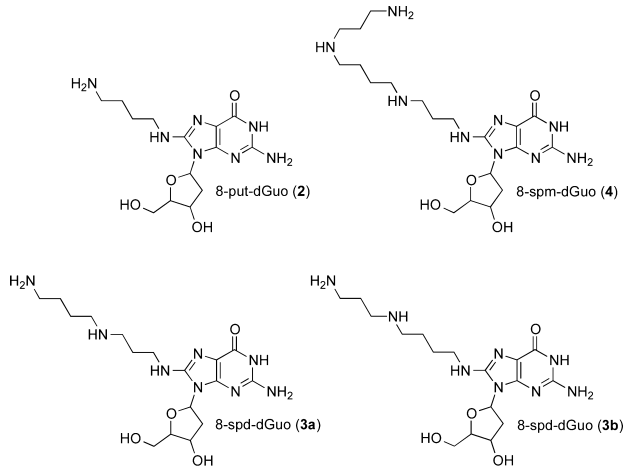


Figure 1. Chemical structures of C8 polyamine-dGuo adducts.

Interestingly, the substitution of 8-Br-dGuo by spermidine led to two possible isomers of 8-spd-dGuo (**3a** and **3b**) with similar yields. The two isomers were isolated by HPLC purification and identified according to their fragmentation pattern (vide infra). Solutions of the three adducts were calibrated by NMR and were used as external standards for the HPLC-MS/MS determination of the amounts of adducts generated in dsDNA upon one electron mediated oxidation.

Characterization of Polyamine-dGuo Adducts. Structures of the three synthesized adducts have first been confirmed by ^{1}H NMR. Besides hydrogen atoms of 2′-deoxyribose, on ^{1}H NMR spectra one can observe additional signals attributed to methylene protons of the polyamine moiety. The lack of the signal corresponding to the H8 proton of the guanine base confirmed that the polyamine residues are bound to the C8 atom of guanine. Furthermore, the hydrogen atoms in the α position of the NH-guanine bond and those at the end of the polyamine chain are not equivalent confirming that one primary amino group is involved in the cross-link with C8 of guanine.

UV spectra exhibit a maximum absorption at around 260 nm (Figure S1, Supporting Information) and a shoulder at around 300 nm. A bathochromic shift is observed for the adducts compared to dGuo as was already observed for 8-lysine-2′-deoxyguanosine[14] and for 8-oxodGuo. Molar extinction coefficients, determined by NMR quantification, have been found to be higher than that of the nonsubstituted 2′-deoxyguanosine and to slightly depend on the length of the polyamine chain, as shown in Table 1.

Mass spectra of the adducts in positive ionization mode (Figure 2) exhibit intense molecular ions at m/z 354, 411, and 468 for 8-put-dGuo, 8-spd-dGuo, and 8-spm-dGuo, respectively. These ions correspond to the protonated molecules [M + H$^+$] (molecular weight = 353, 410, and 467, respectively).

dx.doi.org/10.1021/tx500063d | Chem. Res. Toxicol. 2014, 27, 1011−1018

Table 1. UV Spectral Features of 8-Polyamine-dGuo Adducts

compd name	λ_{max} (nm)	ε (λ_{max}) ($M^{-1}\cdot cm^{-1}$)
8-put-dGuo (**2**)	260	16 930
8-spd-dGuo (**3a**, **3b**)	258	19 590
8-spm-dGuo (**4**)	258	20 820

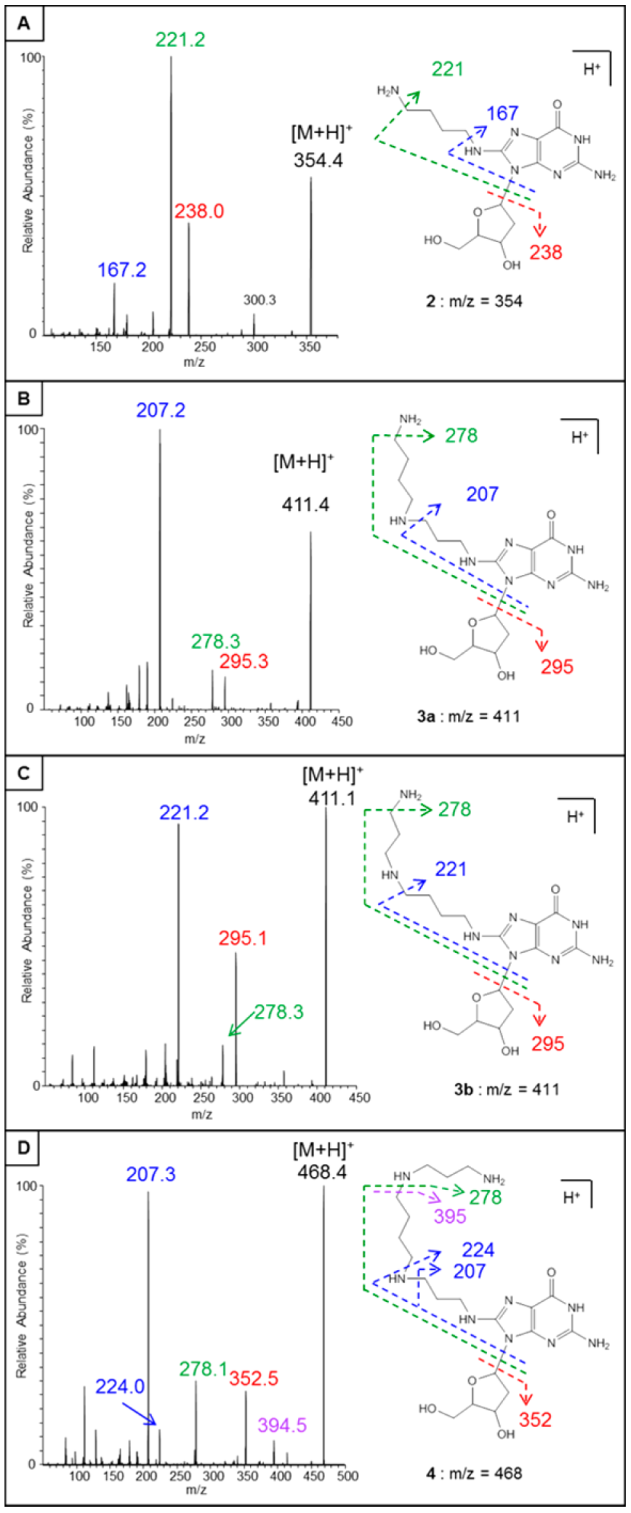


Figure 2. Collision-induced dissociation mass spectra of the $[M + H]^+$ pseudomolecular ion of the different 8-polyamine-dGuo adducts (positive ionization mode). A, 8-put-dGuo (**2**); B, 8−spd-dGuo **3a**; C, 8-spd-dGuo **3b**; and D, 8-spm-dGuo (**4**).

Fragmentation spectra obtained following collision induced dissociation of the pseudomolecular ion exhibit intense ions resulting from the loss of the sugar moiety (m/z 238, 295, and 352) as classically observed for 2′-deoxyribonucleosides.[31] In addition, other ions resulting from fragmentations occurring along the polyamine chain in the α position of amino groups (m/z 221 for 8-put-dGuo; 278 and 207 for 8-spd-dGuo **3a**; 278 and 221 for 8-spd-dGuo **3b**; and 278, 224, and 207 for 8-spm-dGuo) are also observed. Interestingly, the two adducts **3a** and **3b** isolated after the substitution of 8-Br-dGuo with spermidine exhibit a different fragmentation pattern of the spermidine moiety, which is explained by the lack of symmetry of the polyamine residue, that, following addition of one or the other amino residue, gives rise to two distinct isomers.

Development of an HPLC-MS/MS Method of Quantification. An analytical method to detect the three adducts was then developed, using HPLC coupled through electrospray ionization to tandem mass spectrometry. We used the so-called MRM mode in order to have a sensitive, specific, and quantitative detection method. Optimization of collision energies was performed for specific fragmentations of each adduct, as reported in Table 2. For the simultaneous

Table 2. Main Transitions Used for the Detection of the Different 8-Polyamine-dGuo Adducts in the Multiple Reaction Monitoring (MRM) Mode (Positive Ionization)

		main transitions		
adduct	molecular weight	precursor ion (m/z)	fragment ions (m/z)	relative intensity (%)
8-put-dGuo (**2**)	353.4	354.3	221.2	100
		354.3	238.5	6
8-spd-dGuo (**3a**, **3b**)	410.5	411.5	207.2 (**3a**)	50
		411.5	221.3 (**3b**)	88
		411.5	295.2 (**3a** and **3b**)	100
8-sp-dGuo (**4**)	467.6	468.0	207.2	100
		468.0	352.4	23

measurement of the three adducts we have defined common ionization parameters and optimized HPLC conditions for their separation. Strong acids, such as trifluoroacetic acid (TFA) or heptafluorobutyric acid (HBFA), were tested as the mobile phase in order to improve the chromatographic behavior of these molecules which contain primary and secondary amino groups.

To improve the specificity of detection, two main transitions were monitored simultaneously for each lesion (Figure 3), whereas normal nucleosides were quantified on line by UV detection set at 280 nm. The quantification was performed by considering the most intense transition for each adducts, and results were normalized compared to the amount of unmodified nucleosides. The second transition served as a qualifier (Table 2). For 8-spd-dGuo, the relative amounts of the two isomers were similar, and thus, the reported amounts of generated 8-spd-dGuo adducts were the sum of the two **3a** and **3b** isomers. The limit of detection of the assay was close to 1 pmol injected for each adduct. In parallel, 8-oxodGuo was also measured using the 284 → 168 MRM transition as already reported.[28]

8-Polyamine-dGuo Adduct Formation in Isolated DNA. In order to investigate the formation of 8-polyamine-2′-dGuo adducts, calf thymus dsDNA was exposed to UVA

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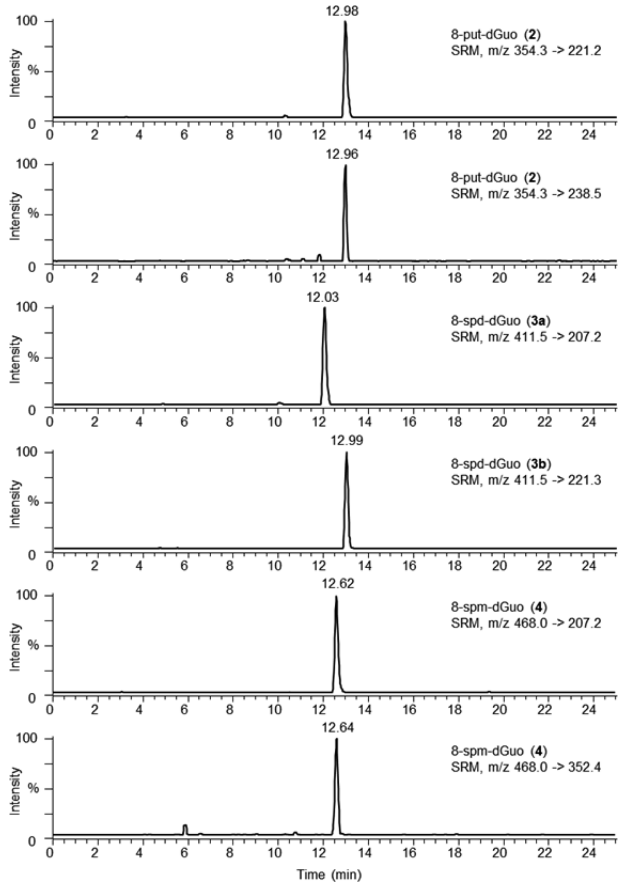

Figure 3. Typical HPLC chromatograms obtained for detection by HPLC-MS/MS of 8-polyamine-dGuo adducts. Chromatograms represent the analysis of a mixture of the 4 polyamine dGuo adducts, corresponding to the injection of 1 pmol of each adduct.

light in the presence of riboflavin. Such a photosensitization reaction is known to produce a high amount of 8-oxodGuo through the transient formation of a guanine radical cation.[8] Thus, as expected under these conditions an increase in the level of 8-oxodGuo was observed when increasing the irradiation time. Levels as high as 5000 8-oxodGuo per million nucleosides were obtained following 10 min of irradiation (Figure 4). This photosensitization reaction was then

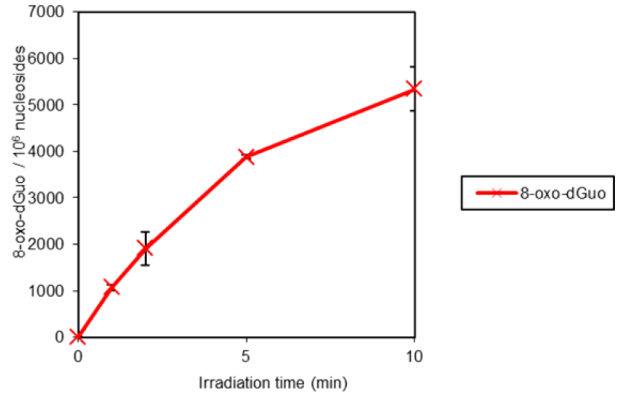

Figure 4. Kinetics of riboflavin and UVA-mediated formation of 8-oxodGuo in dsDNA. Data represent the average and standard deviation of at least three independent analyses and are expressed as the number of 8-oxodGuo per million normal nucleosides.

performed in the presence of the different polyamines at a concentration of 500 μM, which is below the concentration found in the nucleus of eukaryotic cells. Kinetics of formation of the different adducts are presented in Figure 5, for 8-put-

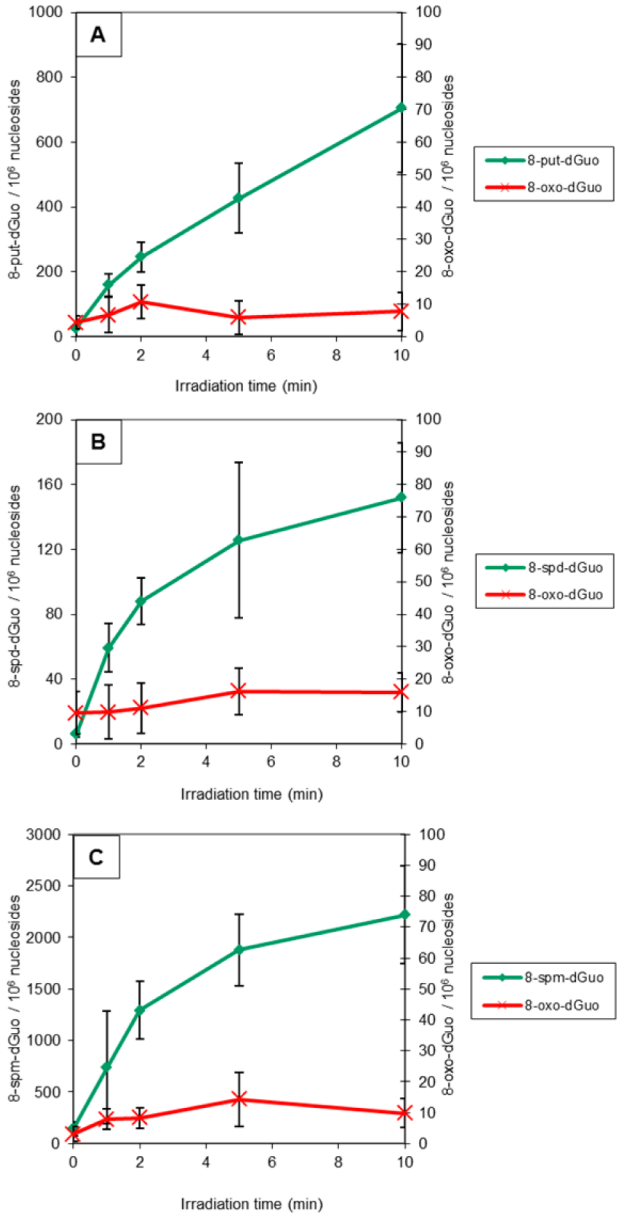

Figure 5. Kinetics of formation of 8-put-dGuo, 8-spd-dGuo, and 8-spm-dGuo in dsDNA exposed to riboflavin and UVA-mediated one electron oxidation in the presence of the corresponding polyamine at 500 μM. Data represent the average and standard deviation of at least three independent determinations and are expressed as the number of lesions per million normal nucleosides.

dGuo, 8-spd-dGuo, and 8-spm-dGuo. After 10 min of irradiation, yields of formation reach around 700, 150, and 2000 lesions per million normal nucleosides for 8-put-dGuo, 8-spd-dGuo, and 8-spm-dGuo, respectively. The kinetics of formation of adducts was almost linear with the irradiation time at least for short irradiation periods; however, a saturation occurred for long irradiation periods, as reported already for 8-oxodGuo and 8-lys-dGuo. Interestingly, when irradiation was performed in the presence of the polyamines, the yield of

dx.doi.org/10.1021/tx500063d | Chem. Res. Toxicol. 2014, 27, 1011−1018

formation of 8-oxodGuo was dramatically reduced (Figure 4 compared to Figure 5). Under these conditions, 8-oxodGuo formation never exceeded 20 lesions per million nucleosides. Thus, in the presence of the polyamine, the yield of formation of 8-oxodGuo was reduced by about 2 orders of magnitude, and the yield of polyamine-dGuo adducts was significantly higher than that of 8-oxodGuo, regardless of the polyamine's identity. It is notable that spermine was more prone to generate adducts compared to putrescine and spermidine.

DISCUSSION

The one electron induced oxidation of DNA is a complex process leading to the formation of numerous oxidative base damages, especially involving the guanine moiety. In this work, attempts have been made to determine the ability of polyamines, ubiquitous molecules found in contact with DNA in all eukaryotic cells, to react with the guanine radical cation, and thus to induce the formation of polyamine−guanine cross-links.

According to the literature, we anticipated that nucleophilic addition of polyamines could occur at the C8 position of $G^{\bullet+}$ or rather its deprotonated G(-H)$^{\bullet}$ form that should predominate at neutral pH to generate C8-guanine adducts. Thus, we developed a chemical synthesis of the different possible adducts, involving a nucleophilic addition of the amine onto 8-bromo-2′-deoxyguanosine, as reported previously for aromatic amines. The structure of the generated adducts was confirmed by the mean of mass spectrometry, UV, and NMR. NMR was also used to prepare calibrated solutions in order to be able to obtain quantitative data. Interestingly, spermidine being an asymmetric molecule, reaction of either the aminopropyl or aminobutyl moiety with Br-dGuo could produce the two different isomers **3a** and **3b**, respectively (Figure 1). These two isomers were undistinguishable by ^{1}H NMR but gave different fragmentation patterns. Indeed, when the polyamine is linked by the aminobutyl moiety, which is the case for putrescine, an intense ion at m/z = 221 is observed. When the cross-link involves the aminopropyl moiety, as observed for 8-spm-dGuo, an ion at 207 is detected. These ions which arose from the fragmentation of the glycosidic bond of the nucleoside and of the polyamine residue as described in Figure 3 allowed us to characterize the 8-spd-dGuo (**3a**) adduct as 8-((3-((4-aminobutyl)amino)propyl)amino)-2′-deoxyguanosine and **3b** as 8-((4-((3-aminopropyl)amino)butyl)amino)-2′-deoxyguanosine. However, since these two adducts are generated in a similar yield (not shown), reported data represent the sum of the two adducts **3a** and **3b**, named 8-spd-dGuo (**3**).

The quantification of C8 polyamine-dGuo was performed by HPLC coupled to tandem mass sepectrometry in the classical MRM mode[32] that provides a very low limit of detection (about 20 fmol injected for 8-oxodGuo[33]). To improve specificity, two transitions were used for each lesion (Table 2). Quantification was performed by external calibration, and results were expressed as the number of lesions per million normal nucleosides quantified by UV detection set at 260 nm.

We performed type I photooxidation of dsDNA in the presence of polyamines, in order to determine if 8-polyamine-dGuo adducts are generated upon a one electron oxidation reaction. Under these conditions, we detected a significant formation of 8-put-dGuo, 8-spd-dGuo, and 8-spm-dGuo using the developed analytical HPLC-MS/MS method. Our results indicate that the three polyamines, putrescine, spermine, and spermidine, are able to react with the guanine radical cation. We also noticed that spermine is more reactive than the other polyamines. Interestingly, we observed that formation of adducts is accompanied by a decrease in 8-oxodGuo formation. Indeed, the irradiation in the presence of a high concentration of polyamines (500 μM) was found to generate more polyamine adducts than 8-oxodGuo, especially in the case of spermine. These results highlight the competition between water and the polyamines as nucleophiles. However, the decrease in 8-oxodGuo formation is not completely compensated for by polyamine adduct formation, and thus the sum of damages (polyamine adducts + 8-oxodGuo, Figure 5) that is produced in the presence of the polyamines is lower than the yield of 8-oxodGuo (Figure 4) produced in the absence of any polyamine. This observation adds evidence to the radioprotective ability of polyamines in inhibiting the formation of 8-oxodGuo, as reported by Douki et al.[34]

As a result, we hypothesize that the decrease in 8-oxodGuo formation could be explained by two processes: first, by the ability of polyamines to condense DNA[35] and in this way to protect it from oxidation; and second by the competitive nucleophilic addition of polyamines onto the guanine radical cation that reduces the yield of 8-oxodGuo formation but in the meantime produces other DNA adducts. Regarding this latter point, our data indicate that the addition of the amino group of polyamines is much more efficient than that of water. Indeed, in the presence of spermine at submillimolar concentration, the yield of 8-spm-dGuo formation is about 2 orders of magnitude higher than that of 8-oxodGuo. Interestingly, it has been demonstrated[35] that the B-structure of dsDNA is preserved even in the presence of high concentrations of polyamines, and thus, the decrease in 8-oxodGuo formation could not be attributed to a change in DNA structure but rather due to condensation. Moreover in the above-mentioned work, it has been shown that spermine and spermidine undergo hydrogen bonding interactions (or at least alter the existing interaction) with guanine N7, and this may explain why nucleophilic addition of one amino group of the polyamines with C8 of $G^{+\bullet}$ in dsDNA is more efficient than the addition of a water molecule to produce 8-oxodGuo. Extrapolating these observations to cells and taking into consideration that concentration of polyamines in the nuclei of eukaryotic cells is within the millimolar range, DNA-polyamine adducts might be generated in higher yield than 8-oxodGuo and thus may represent suitable biomarkers of oxidative stress. In addition, the high proportion of generated polyamine adducts compared to 8-oxodGuo could also be partly explained by the fact that these polyamines are positively charged at neutral pH and that this may favor their interaction with negatively charged DNA, promoting the formation of polyamine adducts. In that respect, we noticed that when dsDNA is photosensitized in 10 mM phosphate buffer, the yield of polyamine adduct formation is significantly reduced (data not shown) but is still higher than that of 8-oxodGuo. We have also noticed that the formation of polyamine-dGuo adducts is linear for short irradiation times but then reaches a plateau when a high amount of adduct is present in dsDNA (Figure 5). This could be certainly explained by the sensitivity of such C8 adducts to undergo secondary decomposition reactions as reported previously for 8-oxodGuo and also for the lysine−guanine adduct. Secondary decomposition reactions of the C8-polyamine-dGuo adducts probably generate spiro-related derivatives as reported for 8-oxodGuo[36,37] through a second nucleophilic addition of polyamine at

C5. Additional work has to be performed to confirm that hypothesis.

Additional work will be performed to search for the formation of these new DNA lesions in irradiated cells. However, the limit of detection of our developed method for 8-polyamine-dGuo detection is around 1 pmol injected. Such a sensitivity is good enough to monitor the formation of these lesions in isolated irradiated DNA because the yield of cross-link formation with polyamines is relatively high. Nevertheless, in biological environments, the yield of formation of DNA lesions is generally much lower, and thus the investigation of polyamine adducts at the cellular level would require a higher sensitivity, with a limit of quantification of only a few fmol. It should be noted that the observed limit of detection for the polyamine−guanine adducts studied here is mostly explained by the nonclassical chromatographic behavior of this kind of molecule. The presence of a primary amino group in these adducts causes the compounds to elute as a broad peak, this effect being particularly important for low injected amounts (below 1 pmol under our conditions). Thus, efforts will be made to improve the sensitivity of the method, and for such a purpose, attempts to derivatize the primary free amino group are in progress.

CONCLUSIONS

This work demonstrates that polyamines are able to react onto the C8 position of guanine following one electron oxidation and that such a process generates polyamine−guanine cross-links. In the meantime, we demonstrated that the presence of polyamines inhibits the formation of 8-oxodGuo. This study suggests that besides the ability of polyamines to protect DNA from oxidation by condensation, the observed decrease of 8-oxodGuo formation in the presence of these molecules could be partially explained by the competitive formation of polyamine adducts. Thus, the observed protection effect of polyamine might be considered with caution.

Further work is in progress to improve the sensitivity of the HPLC-MS/MS method for the detection of such polyamine adducts, in order to be able to investigate their formation within cells. In addition, attempts will be made to determine their biological consequences and to identify repair mechanisms involved in the elimination of these specific DNA lesions.

The investigation of DNA-polyamine cross-links also represents a convenient model for the study of other more complex lesions involving nucleophilic addition onto C8 of the guanine radical cation or its deprotonated form. A similar strategy will be applied to search for cross-links between guanine and several amino acids, such as lysine, arginine, and serine. This outlook could be promising in the understanding of structural aspects of DNA−protein cross-links.[38]

ASSOCIATED CONTENT

Supporting Information

Conditions for exact mass determination, general procedure for the chemical synthesis of the polyamine-C8 guanine adducts, UV absorption spectra of 8-put-dGuo compared to dGuo, and ^{1}H NMR spectra of 8-spm-dGuo. This material is available free of charge via the Internet at http://pubs.acs.org.

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Funding

This work was partly supported by Labex PRIMES (ANR-11-LABX-0063), COST action CM1201 "Biomimetic Radical Chemistry," and TODNAL project (ANR-09-PIRI-0022-01).

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The contributions of Pierre-Alain Bayle (LRM/SCIB/CEA-Grenoble) to the NMR analysis and Hassan Kanso to the preliminary experiments are gratefully acknowledged.

ABBREVIATIONS

ROS, reactive oxygen species; dGuo, 2′deoxyguanosine; $G^{+\bullet}$, guanine radical cation; $G(-H)^{\bullet}$, deprotonated guanine radical cation; 8-Br-dGuo, 8-bromo-2′-deoxyguanosine; 8-oxodGuo, 8-oxo-7,8-dihydro-2′-deoxyguanosine; 8-put-dGuo (**2**), 8-((4-aminobutyl)amino)-2′-deoxyguanosine; 8-spd-dGuo, 8-((3-((4-aminobutyl)amino)propyl)amino)-2′-deoxyguanosine (**3a**) and 8-((4-((3-aminopropyl)amino)butyl)amino)-2′-deoxyguanosine (**3b**); 8-spm-dGuo (**4**), 8-((3-((4-((3-aminopropyl)-amino)butyl)amino)propyl)amino)-2′-deoxyguanosine; ODC, ornithine decarboxylase; dsDNA, double stranded DNA; HPLC-MS/MS, HPLC coupled through electrospray ionization to tandem mass spectrometry

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