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Tandem Mass Spectrometry-based Detection of C4'-Oxidized Abasic Sites at Specific Positions in DNA Fragments

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Abstract

Oxidative damage to DNA has been linked to aging, cancer, and other biological processes. Reactive oxygen species and various antitumor agents including bleomycin and ionizing radiation have been shown to cause oxidative DNA sugar damage. Detection of DNA lesions is important for understanding the toxicological or therapeutic consequences associated with such agents. C4'-oxidized abasic sites (C4'-AP) are produced by the antitumor drug bleomycin and ionizing radiation. The currently available methods for detection of C4'-AP cannot provide both structural and sequence information. We have developed an LC-ESI-MS based approach for specific detection and mapping of C4'-AP from a mixture of lesions. We show using Fe-bleomycin-damaged DNA, that C4'-AP can be detected at cytosine and thymine sites by direct mass spectrometric (MS) analysis. Our results reveal that collision-induced dissociation of C4'-AP-containing oligonucleotides results in preferential fragmentation at C4'-AP sites with the formation of the unique a* ions (18 a.m.u more than the a-B ions) that allow mapping of the C4'-AP sites. Various chemical modification strategies (e.g. reduction with NaBH₄ and NaBD₄ and derivatization with methoxyamine and hydrazine, followed by liquid-chromatography (LC)-MS analysis) were also used for unambiguous detection of C4'-AP sites. Finally, we show that the methods described here can detect the presence of C4'-AP at specific sites in a complex sample such as hydroxyl radical-damaged DNA. The LC-MS approach was also used for the simultaneous detection of the other C4'-oxidation end product, 3'-phosphoglycolate (3PG) at a specific site in hydroxyl radical-damaged DNA. Thus, LCMS provides a rapid and direct approach for the detection and mapping of oxidative DNA lesions.

Introduction

Oxidative damage to DNA has been linked to aging and various diseases including cancer (1–5). Although nucleobase oxidation has been extensively studied, there is growing evidence that oxidation of the deoxyribose can play a significant role in mutagenesis, carcinogenesis, aging, and cell death (1–9). About 10–20% of all DNA damage occurs in the

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Supporting Information Available. Figure S1, ESI-LC-MS/MS chromatogram and spectra of Fe-BLM-damaged ds DNA fragment 1 following reduction with NaBH₄. Figure S2, ESI-LC-MS/MS chromatogram and spectra of Fe-BLM-damaged ds DNA fragment 1 following reduction with NaBD₄. Figure S3, ESI-LC-MS/MS chromatogram and spectra of Fe-BLM damaged ds DNA fragment 1 following derivatization with methoxyamine. Figure S4, ESI-LC-MS/MS chromatogram and spectra of *m/z* 1016.2(–4) species formed in Fe-BLM-damaged DNA fragment 1 following hydrazine treatment. Figure S5, ESI-LC-MS/MS chromatogram and spectra of hydroxyl radical-damaged ds DNA fragment 1 following reduction with NaBD₄. Table S1, Calculated a-B and w ions of DNA fragment 1 and DNA fragment 1 having 6 or 7 in place of cytosine or thymine. Table S2, Calculated a-B and w ions of DNA fragment 1 and DNA fragment 1 having 8 in place of cytosine or thymine. This material is available free of charge via the Internet at <http://pubs.acs.org>.

deoxyribose backbone of DNA (10–12). Oxidative damage to the sugar results in the formation of various lesions including complex adducts, DNA and DNA-protein crosslinks, and various oxidized abasic sites including 2-deoxyribonolactone, C4 -oxidized abasic sites (C4-AP, **1**), and C2 -oxidized abasic sites (C2-AP) (13–23).

Antitumor agents (e.g. bleomycin and tirapazamine) (24–28), antibiotics (e.g. neocarzinostatin) (29, 30), metal complexes (e.g. Cu-phen (Copper-phenanthroline)) (31, 32), ionizing radiation (12, 16, 17), and reactive oxygen species (33) have been shown to cause oxidative DNA sugar damage. The study of oxidative DNA sugar damage is therefore important and often requires identification of the end products that are formed as a result of oxidative damage to DNA (16, 17, 34). Each end product is a signature of a particular hydrogen abstraction or oxidation event. For example, the 2-deoxyribonolactone lesion stems from C1 -H abstraction while C4 -oxidized abasic sites (C4-AP, **1**), 3 -phosphoglycolate (3PG, **3**), and base propenal are signature end products of C4 -H abstraction (16, 17). Abstraction of the C4 -hydrogen atom generates a carbon-centered radical which, if trapped by molecular oxygen, results in the formation of base propenal or malonaldehyde, 3PG, and 5 -phosphate-containing DNA fragments (35, 36). Alternatively, the C4 -carbon centered radical can be oxidized to the corresponding carbocation and ultimately to a C4-AP-containing DNA fragment (Scheme 1) (37, 38). The C4-AP sites account for about 40% of the products formed in DNA upon treatment with the antitumor agent bleomycin (BLM) (30, 39, 40). Other agents such as ionizing radiation, enedieynes, and hydroxyl radical are also known to produce C4-AP (12, 17, 20, 36, 41). C4-AP sites are mutagenic and cause DNA crosslinks (6, 7, 22, 23).

Analytical methods that are used to detect and quantify C4-AP (**1**) include derivation with hydrazine (25, 42) or methoxyamine (43, 44), followed by enzymatic hydrolysis of the DNA to nucleosides and finally detection using gas chromatography coupled with MS (GC-MS) or accelerator MS. Although this method provides structural information about the degradation products, information about sequence selectivity of the modification is lost. Another method involves alkaline cleavage of the DNA at the sites of C4-AP formation followed by detection of the corresponding 3 -phosphate- or 5 -phosphate-containing DNA fragments using polyacrylamide gel electrophoresis (PAGE) (45). PAGE allows sequence specific detection and quantification of C4-AP sites. Because this method requires alkaline cleavage of the C4-AP containing DNA fragment, lesions that are susceptible to base mediated cleavage and produce 3 -phosphate- or 5 -phosphate-containing DNA fragments as end products may interfere with C4-AP detection. Moreover, identification of DNA modifications in PAGE are solely made based on the mobility of the bands relative to standards, and therefore no structural information can be obtained, especially for complex samples with multiple modifications. Recently Greenberg and coworkers have developed a fluorescent and biotin-tagged agent for the specific detection and quantitation of C4-AP sites in DNA (41).

Interestingly, the currently available methods do not always provide both structural and sequence information, and these methods often require the use of radioactivity, special reagents, authentic standards, and/or hydrolysis of the DNA to nucleosides. Therefore, for specific detection and mapping of C4-AP from a mixture of lesions, new methods are necessary that directly involve the C4-AP lesion. Accordingly, we used a combination of MS and chemical modification methods for the detection of C4-AP sites in oligonucleotides. Collision-induced dissociation (CID) of small oligonucleotides results in the formation of a-*B* and w ions as two major types of ions and thus can be used to sequence oligonucleotides (Scheme 2) (46, 47). Recently this approach (CID of oligonucleotide) has been used to detect normal abasic sites (AP), 3PG, N-7 alkylation, and other modifications in oligonucleotides (48–53). Here we present the use of an ultra performance (UP)-LC coupled

tandem MS based approach for the detection of C4-AP at specific sites in DNA fragments without the need for hydrolysis of the DNA fragment or the use of specialized reagents. The method allows the detection of various end products simultaneously.

Materials and Methods

Warning

Bleomycin is extremely toxic and a known carcinogen. It should be handled with extreme caution and protection should be worn at all times while handling it.

Materials—Unless otherwise mentioned all materials were obtained from Sigma-Aldrich (St. Louis, MO) and were of the highest purity available. Oligonucleotides were purchased from Midland Certified Reagent Co. (Midland, TX).

DNA Damage by Fe-BLM—Equimolar amounts of two complementary oligonucleotides having the sequence 5'-ACCCGCGTCCGCGCC (a region from exon 5 of the non-coding strand of the p53 gene) and 5'-GGCGCGGACGCGGGT (coding strand) were dissolved in 10 mM (sodium) 4-(2-hydroxyethyl)-1-piperazineethanesulfonate (HEPES) buffer (pH 7.5) and annealed by heating at 90 °C for 5 min followed by slow cooling to 23 °C. Activated BLM was prepared before use by mixing equimolar amounts of BLM (10 mM) and ferrous ammonium sulfate (10 mM) and was kept on ice before adding into the reaction mixture. All analyses were performed on the 5'-ACCCGCGTCCGCGCC strand.

In a typical reaction (50 µL total reaction volume), a double-stranded (ds) oligonucleotide (**1**, 20 µM, in 50 mM HEPES buffer, pH 7.5) was treated with activated BLM (50 µM) for 10 min at 23 °C under aerobic conditions. The reaction mixture was quenched by adding EDTA (1 mM), diluted to 100 µL with H₂O, passed through a spin column (Bio-Spin 6, Bio-Rad, Hercules, CA) and finally loaded into a UPLC (Waters Corporation, Milford, MA)-coupled LTQ MS system (Thermo Fisher, Waltham, MA).

For sodium borohydride reduction, the reaction mixture was either treated with NaBH₄ or NaBD₄ (100 mM) for 15 min at 23 °C. The stock solution of NaBH₄ or NaBD₄ (1 M) was prepared in C₂H₅OH. Following incubation, the reaction mixtures were again passed through a Bio-Spin Column and then loaded into the UPLC-coupled LTQ system.

For hydrazine or methoxyamine treatment, the reaction mixture was either treated with NH₂NH₂ (40 mM) or CH₃ONH₂ (20 mM) for 30 min at 23 °C in the presence of potassium phosphate buffer (50 mM, pH 7.4). Following incubation the reaction mixture were again passed through a Bio-Spin 6 column and then analyzed by LC-MS.

DNA Damage by Hydroxyl Radical—Hydroxyl radical-damaged DNA was prepared by treating the ds oligonucleotide **1** with the well-known hydroxyl radical-generating system Fe/EDTA/H₂O₂/ascorbate following standard methods (54), with modifications. Briefly, ds oligonucleotide (40 µM) in potassium phosphate buffer (50 mM, pH 7.4) was treated with a mixture of ferrous ammonium sulfate (10 µM) and EDTA (20 µM), ascorbate (1.0 mM), and H₂O₂ (8.8 µM) for 5 min at 23 °C. Following the incubation, the reaction mixture was passed through a Bio-Spin 6 column and then subjected to further treatment or directly analyzed by LC-MS.

LC-MS and LC-MS/MS Analysis—LC-MS and LC-MS/MS were performed on a Waters Acquity UPLC system connected to an LTQ mass spectrometer using an Acquity UPLC BEH C18 column (1.7 µm, 1.0 mm × 100 mm). LC conditions were as follows: buffer A contained 10 mM NH₄CH₃CO₂ and 2% CH₃CN (v/v), and buffer B contained 10

mM $\text{NH}_4\text{CH}_3\text{CO}_2$ and 95% CH_3CN (v/v). The following gradient program was used, with a flow rate of $150 \mu\text{L min}^{-1}$: 0–3 min, linear gradient from 100% A to 97% A; 3–6 min, linear gradient to 75% A; 6–6.5 min, linear gradient to 100% B; 6.5–8.5 min, hold at 100% B; 8.5–9 min, linear gradient to 100% A; 9–12 min, hold at 100% A. The temperature of the column was maintained at 50°C . Samples ($10 \mu\text{L}$) were infused with an auto-sampler. MS analyses were performed in the negative ion mode. Electrospray ionization (ESI) conditions were as follows: source voltage 4 kV, source current $100 \mu\text{A}$, auxiliary gas flow rate setting 20, sweep gas flow rate setting 5, sheath gas flow setting 34, capillary voltage -49 V , capillary temperature 350°C , tube lens voltage -90 V . MSMS conditions were as follows: normalized collision energy 35%, activation Q 0.250, and activation time 30 ms. Product ion spectra were acquired over the range m/z 400–2000. The calculations of the CID of unmodified and some modified oligonucleotides were done using a program linked to the Mass Spectrometry Group of Medicinal Chemistry at the University of Utah (<http://library.med.utah.edu/masspec/mongo.htm>).

Results and Discussion

MS Analysis of C4-AP-Containing Oligonucleotide 1

The natural product BLM was used to generate C4-AP in DNA. BLM is a clinically used antitumor antibiotic that site-specifically abstracts the C4 -H atom from the deoxyribose backbone of DNA at pyrimidine sites in GT and GC sequences, resulting in the formation of C4-AP sites (**2** & **3**), 3PG (**4**), and base propenals (**5**) as end products (25, 26, 39, 40, 55). A ds oligonucleotide with the sequence 5 - $\text{A}_1\text{C}_2\text{C}_3\text{C}_4\text{G}_5\text{C}_6\text{G}_7\text{T}_8\text{C}_9\text{C}_{10}\text{G}_{11}\text{C}_{12}\text{G}_{13}\text{C}_{14}\text{C}_{15}$ (**1**) was treated with Fe-BLM under “single-hit” conditions (a significant amount of unmodified oligonucleotide remained as judged by LC-MS spectra) to generate C4-AP sites. The oligonucleotide **1** has four BLM target sites, at C_6 , T_8 , C_{12} , and C_{14} . LC-MS analysis of Fe-BLM damaged **1** resulted in the identification of two peaks at m/z 1090.0(−4) and 1093.9(−4), which correspond to oligonucleotide **1** having C4-AP (ring-opened unhydrated species) in place of thymine (T_8) and cytosine (C_6 , C_{12} , or C_{14}), respectively (Figure 1). Peaks corresponding to the ring-closed form of the C4 -oxidized abasic site (**3**) were also detected. This result is consistent with a previous report where the presence of the ring opened 4 -keto form (**2**) was detected in an oligonucleotide (32). CID of the quadruply-charged species at m/z 1090.0(−4), which corresponds to the presence of C4-AP in place of thymine, gave only the a_8^* [m/z 1120.3(−2)] and w_7 [m/z 1060.2(−2)] ions (Figure 2). Similarly, CID of the quadruply charged species at m/z 1093.9(−4) (which corresponds to the presence of C4-AP in place of cytosine) yielded three major peaks at m/z 1356.3 (−4, a_{14}^*), 1150.3 (−4, a_{12}^*), and 924.2 (−1, w_3). Interestingly, the observed fragments in the CID spectra were the a^* and w ions resulting from preferential cleavage at the C_6 , T_8 , C_{12} , and C_{14} sites. The a_6^* and w_9 ions resulting from cleavage at site C_6 [m/z 810.7(−2) and 1376.3(−2)] were observed, albeit with relatively low intensities compared to the other sites and probably due to reduced efficiency of C4 -H abstraction at site C_6 by Fe-BLM.

The a^* ion, which results from preferential fragmentation at C4-AP sites, has a characteristic mass of 16 a.m.u. more than normal a-B ions. The proposed structure of the a^* ion is shown in Figure 2A and the fragmentation at C4-AP is consistent with that seen in AP-containing oligonucleotides (51). Based on the preferential fragmentation at C4-AP sites and the characteristic mass of the a^* ion (18 a. m. u. more than the corresponding a-B ion), the position of the C4-AP sites in an oligonucleotide can be easily determined. Thus, the presence of C4-AP in an oligonucleotide can be detected and mapped by direct MS analysis of the reaction mixture. However, other abasic sites with similar mass and fragmentation properties (e.g. 2-deoxyribonolactone (mass same as C4-AP) and normal abasic sites (AP, mass 2 a.m.u. more than C4-AP)) may interfere, especially in case of multiply charged species.

LCMS and LC-MS/MS Analysis of C4-AP-Containing Oligonucleotide **1** after Reduction with NaBH₄ or NaBD₄

Because oligonucleotide with C4-AP sites fragments selectively at the site of the modification resulting in a* and w ions, it is not possible to obtain sequence information of the oligonucleotide using the MS/MS data. We hypothesized that this preferential cleavage at the site of C4-AP formation is due to the increased acidity of the C2 proton. Accordingly, we reduced the C4-AP to the corresponding alcohol **6**. Reduction of C4-AP should in theory reduce the C1 -aldehyde and C4 -ketone to the corresponding alcohol, thereby increasing the mass of the DNA fragments by 4 mass units and decreasing the acidity of the C2 proton (Scheme 3). The decrease in the acidity of the C2 proton should prevent selective fragmentation occurring at the site of C4-AP formation and consequently, the CID should produce all the theoretical a-B and w ions pertaining to the oligonucleotide. Reduction should also increase the sensitivity of the detection method. C4-AP in DNA exists in equilibrium between the 4 -keto form (**2**) and its ring-closed hydrated form (**3**), and reduction of C4-AP should convert both species to a single ring-opened diol species **6** (Scheme 1) (10).

It has been previously shown that C4-AP can be reduced by treatment with an alcoholic solution of NaBH₄ (10). Accordingly, Fe-BLM damaged DNA was treated with an ethanolic solution of NaBH₄ or NaBD₄. LC-MS spectra (Figure 3A & B) of Fe-BLM-damaged DNA clearly showed the presence of species at *m/z* 1091.2(−4) and 1094.9(−4) for NaBH₄ treatment and *m/z* 1091.7(−4) and 1095.4(−4) for NaBD₄ treatment. The increase of 4 and 6 mass units (1 and 1.5 *m/z* units) for NaBH₄ and NaBD₄ reduction, respectively, clearly indicated the presence of two reducible carbonyl groups and is consistent with the presence of a C4-AP.

CID of the *m/z* 1091.2(−4) gave a single peak at 3.10 min in the TIC (total ion chromatogram) trace that fragments to give a-B and w ions consistent with the presence of **6** in oligonucleotide **1** (Figure 3C and Supporting Information, Table S1). The masses of all ions from a₂-B₂ to a₇-B₇ and w₁ to w₇ are similar to those of the unmodified oligonucleotide **1**, while the masses of ions from a₉-B₉ to a₁₄-B₁₄ and w₈ to w₁₄ (w₁₁ was not detected) are 106 units (corresponding to presence of **6** in place of thymine) less than that of the unmodified oligonucleotide (Figure 3C and Supporting Information, Table S1). Because **6** does not have a base, no a₈-B₈ ion was observed in the CID spectrum. This result clearly indicated that **6** is not present between nucleotides 1–7 and 9–15 of the oligonucleotide. Together these data indicate that the oligonucleotide corresponding to *m/z* 1091.2(−4) has the sequence 5'-ACCCGCGTCCGCGCC and contains **6** in place of T₈. LC-MS/MS analysis of the *m/z* 1094.9 species gave two peaks at *t_R* 3.02 and 3.13 min in the TIC (Supporting Information, Figure S1). Analysis of the CID spectra of the *t_R* 3.02 min peak indicated that it corresponds to oligonucleotide **1** having **6** in place of C₁₄, whereas the *t_R* 3.13 min peak corresponds to oligonucleotide **1**, having **6** in place of C₆ or C₁₂ (Supporting Information, Figure S1). For experiments in which NaBD₄ was used as a reducing agent, similar results were obtained with the exception that the masses of all detected fragments that contain **7** increased by 2 a.m.u, consistent with the addition of two deuterium atoms (Figure 3 and Supporting Information, Figure S2 and Table S1). For example, in case of the *m/z* 1094.9(−4) species eluting at *t_R* 3.01–3.02 min, the w₂ ion in the CID spectra had a mass of 504 and 506 for NaBH₄ and NaBD₄ treatment, respectively, providing clear evidence for the presence of **6** and **7** at C₁₄ (Supporting Information, Figure S1 and S2). This data indicated the presence of C4-AP in oligonucleotide **1**. Thus, this method of reduction with NaBH₄ and NaBD₄ allowed sequencing and detection of oligonucleotides containing C4-AP sites. However this method provided evidence for the presence of C4-AP

only in the absence of 2 -deoxyribonolactone, because these lesions may be reduced by NaBH₄ to form **6**.

MS Detection of C4-AP in Oligonucleotide **1** after Derivatization with Methoxyamine

Although we were able to detect the presence of C4-AP by measuring the increases in mass of 4 and 6 a.m.u. (1 and 1.5 *m/z* units) following reduction using an LTQ instrument, this approach is not always reliable unless a high-resolution instrument is used. Moreover, the presence of 2 -deoxyribonolactone lesions may interfere. Thus, for detection of C4-AP in the presence of 2 -deoxyribonolactone we used a methoxyamine derivatization method. Methoxyamine is known to react with aldehydes and ketones (C4-AP and AP sites) in DNA to form the corresponding oximes but does not react with 2 -deoxyribonolactone or 3PG (43, 44). For C4-AP containing DNA, two molecules of methoxyamine react with each C4-AP site (owing to the presence of two carbonyl groups in C4-AP, Scheme 2) whereas for AP sites there is addition of one molecule of methoxyamine. Therefore the mass difference between C4-AP and a normal abasic site following addition of methoxyamine will be 27 a.m.u., sufficiently large to be detected using a normal mass spectrometer.

Derivatization of Fe-BLM-treated DNA with methoxyamine followed by LC-MS analysis yielded species at *m/z* 1104.9(−4) and 1108.4(−4), which correspond to oligonucleotide **1** having **8** in place of thymine and cytosine, respectively (Figure 4 and Supporting Information, Figure S3). CID of the two quadruply-charged species gave the expected a-B and w ions (Figure 4 and Supporting Information, Figure S3 and Table S2). This approach provided a method for the detection of C4-AP in the presence of 2 -deoxyribonolactone and AP sites and does not require high-resolution MS.

MS Detection and Mapping of C4-AP in Oligonucleotide **1** after Derivatization with Hydrazine

Hydrazine is known to react with C4-AP to form a pyridazine and cause DNA cleavage at the site of the modification (Scheme 3) (25, 42). We exploited this reaction with C4-AP for the detection and mapping of multiple C4-AP sites in an oligonucleotide. The reaction of hydrazine with C4-AP results in the formation of a 5'-phosphate and a 3'-phosphomethylpyridazine (PMP)-containing DNA fragment (**9**). Both of these fragments can be readily detected and sequenced using LC-MS/MS. The presence and position of C4-AP in an oligonucleotide can be confirmed on the basis of the sequence of **9** and the unmodified DNA fragment.

Accordingly, Fe-BLM-damaged oligonucleotide was treated with hydrazine and the reaction mixture was subjected to MS analysis. LC-MS analysis of Fe-BLM-damaged oligonucleotide that had been treated with hydrazine led to the detection of two peaks at *m/z* 1148.8(−3) and 1016.2(−4) (Figure 5 and Supporting Information, Figure S4). Each of the two peaks corresponds to the expected mass of PMPs resulting from hydrazine-mediated cleavage of oligonucleotide **1** having C4-AP at C₁₂ and C₁₄, respectively (Figure 5 and Supporting Information, Figure S4). The expected mass of the doubly-charged PMP resulting from modification at T₈ is similar to that of the quadruply-charged unmodified DNA fragment **1** (*m/z* 1118). Consequently, its presence cannot be confirmed by this method. Consistent with the 3PG data, the PMP resulting from C4-AP formation at C₆ was not detected, either due to its loss in the spin column (owing to its small size) or due to reduced modification by Fe-BLM at that site.

To confirm the sequence and positions of the detected PMPs, LC-MS/MS analysis was performed on the peaks with *m/z* 1148.8(−3) and 1016.2(−4). In theory, the masses of all a-B ions of a PMP should be similar, while mass of all w ions should be 172 a.m.u. higher

than that of an oligonucleotide with 3'-OH and the same sequence (the increase corresponding to the terminal phosphopyridazine moiety). CID spectra of the m/z 1148.8(−3) and 1016.2(−4) peaks gave a-B ions that are similar to unmodified oligonucleotides of the sequence 5'-ACCCGCGTCCG and 5'-ACCCGCGTCCGCG, respectively. The w ions were found to have an extra mass of 172 a.m.u, consistent with the presence of a pyridazine moiety at the 3' terminal (Figure 5 and Supporting Information, Figure S4). To gain further evidence for the presence of the pyridazine moiety, the terminal w ion [w_1 , m/z 518(−1)] was subjected to CID. Fragmentation of the w_1 ion (Figure 5) was consistent with the structure **9**. Because the PMP moiety stems from a C4-AP group, this data provides additional direct evidence for the presence of a C4-AP. Together this method allows for the unambiguous detection and mapping of C4-AP sites in an oligonucleotide.

Detection of C4-AP in Hydroxyl Radical-Damaged oligonucleotide

The methods reported for detecting the presence of C4-AP at a particular site were used for a more complex system, i.e. hydroxyl radical-damaged oligonucleotide. Hydroxyl radical is known to produce a plethora of damage in DNA including C4-AP, 3PG, and other abasic sites and therefore presents a challenging opportunity to test the LC-MS based methods developed here (54, 56–58). Hydroxyl radicals were generated in situ using the well-known Fe-EDTA/H₂O₂/ascorbate system (54, 59).

The ds oligonucleotide **1** was treated with the Fe-EDTA/H₂O₂/ascorbate system and the reaction mixture was subsequently analyzed using the methods described here. LC-MS analysis of the hydroxyl radical-damaged oligonucleotide **1** revealed the presence of quadruply-charged species at m/z 1093.8 that is consistent with the formation of an oxidized abasic site (C4-AP and/or 2'-deoxyribonolactone) in place of cytosine in oligonucleotide **1**. CID of the m/z 1093.8 peak gave a* ions at positions where cytosine bases are present, indicating the formation of oxidized abasic site (C4-AP and/or 2'-deoxyribonolactone) (Figure 6). This result is consistent with our earlier observation with Fe-BLM damaged oligonucleotide that a* ions were observed at the site of C4-AP formation. The sequence-independent formation of oxidized abasic sites is also consistent with the fact that DNA damage by hydroxyl radical is usually sequence independent (54, 56, 59).

Reduction of hydroxyl radical-damaged oligonucleotide **1** with NaBH₄ or NaBD₄ resulted in the formation of peaks at m/z 1094.8 and 1095.4, respectively. However the peaks may result from a C4-AP, 2'-deoxyribonolactone or normal abasic sites. CID of the peaks clearly indicated the species to be oligonucleotide **1** with one of the cytosines replaced by **6** (Figure 6). The singly charged w_2 ions (m/z of 504 and 506 for NaBH₄ and NaBD₄, respectively) with a mass difference of 2 for NaBH₄ and NaBD₄ treatment, suggested the presence of **6** either at the C₁₅ or C₁₄ (Figure 6 and Supporting Information, Figure S5). The mass difference of 2 indicates the presence of either C4-AP or 2'-deoxyribonolactone.

Finally, we used the hydrazine derivatization method for the detection of C4-AP in hydroxyl radical-damaged oligonucleotide **1**. Because damage caused by hydroxyl radicals is usually sequence independent, we decided to focus on only two sites, C₁₄ and G₁₁. LC-MS analysis of hydroxyl radical-damaged oligonucleotide **1** treated with hydrazine led to the detection of peaks at m/z 1016 (−4) and 1039(−3) (Figure 7). LC-MS/MS of the m/z 1016(−4) species gave two peaks at t_R 4.32 and t_R 4.37 min (TIC). CID of the t_R 4.47 min peak is consistent with an oligonucleotide of the sequence 5'-ACCCGCGTCCGCG and having a PMP (**9**) moiety at the 3'-terminal (Figure 7A and B). Similarly, LC-MS/MS of the m/z 1039(−3) species gave a peak at t_R 4.44 min in the TIC. CID of the t_R 4.44 min peak is consistent with an oligonucleotide of the sequence 5'-ACCCGCGTCC and having a PMP (**9**) moiety at the 3'-terminal (Figure 7C and D). These results clearly indicate the formation of C4-AP at C₁₄

and G₁₁ in hydroxyl radical-damaged oligonucleotide **1**. Together these results clearly indicate that C4-AP can be easily detected at specific sites in oligonucleotides using the methods described here.

Detection of 3PG in Hydroxyl Radical-Damaged DNA

LC-MS analysis of hydroxyl radical-damaged oligonucleotide samples also revealed the presence of a quadruply-charged species at m/z 1007.5(−4) that corresponds to 3PG resulting from C4 -H abstraction at C₁₄ in oligonucleotide **1** (Figure 8). LC-MS/MS of the m/z 1007.5(−4) species gave two peaks at t_R 2.97 and t_R 3.05 min (TIC). The CID spectrum of the peak at t_R 2.97 min is consistent with an oligonucleotide of the sequence 5 - ACCCGCGTCCGCG and having a 3PG moiety at the 3'-terminal (Figure 8), consistent with previous reports (52).

Limitations of the MS-based approach

Although the MS based approaches allow unambiguous detection and mapping of C4-AP sites, it is limited to oligonucleotides. With larger DNA fragments the spectra will not only become complex owing to multiply charged species but the difference in m/z values between various modified and unmodified species will be small, resulting in the potential overlap of peaks. In addition, with increasing length the CID spectra too will become complicated. Another drawback of the use of large DNA fragments is sensitivity. With increasing DNA length the yield of modification at a particular site will decrease thereby making sensitivity of detection a limiting factor. However both complexity of the spectra and sensitivity are related to the mass spectrometer. With the development of mass spectrometers having better sensitivity and higher resolution the size of DNA fragment can be increased to a certain length. The method is developed for the fast and unambiguous detection of C4-AP and possibly other lesions produced by an agent in oligonucleotides *in vitro* with the goal to understand the spectra of modification caused by a DNA damaging agent.

Conclusions

Detection of DNA lesions is important for understanding the toxicological consequences associated with the agent that produced them and to elucidate the mechanism of formation of the lesions. With the identification of various complex lesions and the development of more sensitive mass spectrometers, MS has become the method of choice for the detection of DNA lesions. Mass spectrometric-based approaches not only allow unambiguous detection of the lesions but can also be rapid and high throughput. To this end we have presented an ESI-LC-MS/MS based approach for the detection and mapping of C4-AP sites in intact DNA fragments. We show using Fe-BLM-damaged DNA that C4-AP sites can be detected by direct MS analysis. Our experiments reveal that CID of C4-AP containing DNA results in preferential fragmentation at the sites of C4-AP formation resulting in the formation of a unique a^* ion. Based on this property of C4-AP sites, its positions in DNA were mapped. C4-AP sites in DNA were unambiguously detected using various chemical modification strategies such as reduction with NaBH₄ and NaBD₄ and derivatization with methoxyamine and hydrazine followed by LC-MS analysis. Interestingly, we found that reduction of the C4-AP sites to the corresponding alcohol abrogates preferential fragmentation of DNA at sites of C4-AP formation and produced expected a -B and w ions in the CID spectra that allowed sequencing of the DNA fragment. Finally, we showed that we could detect the presence of C4-AP sites in a complex sample, e.g. hydroxyl radical-damaged oligonucleotide, using the methods described here. Our methods allow the simultaneous detection of both the C4-oxidation end products C4-AP and 3PG at a specific site. The method described here is fast and does not require hydrolysis of the DNA to

nucleosides. Further, it does not require radioactive or any specialized reagents, and it allows detection of C4-AP in the presence of other abasic sites such as 2 -deoxyribonolactone and AP and also simultaneous detection of multiple end products.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AP	abasic site(s)
BLM	bleomycin
C2-AP	C2 -oxidized abasic sites
C4-AP	C4 -oxidized abasic site(s)
CID	collision-induced dissociation
Cu-phen	copper-phenanthroline
ds	double-stranded
EDTA	ethylenediaminetetraacetic acid
EIC	extracted ion chromatogram
ESI	electrospray ionization
GC-MS	gas chromatography mass spectrometry
HEPES	(sodium) 4-(2-hydroxyethyl)-1-piperazineethanesulfonate
LC-MS	liquid chromatography mass spectrometry
PAGE	polyacrylamide gel electrophoresis
3PG	3 -phosphoglycolate
PMP	3 -phosphomethylpyridazine
TIC	total ion chromatogram
UPLC	ultra performance liquid chromatography.

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Scheme 1.

Proposed mechanism of formation of C4-AP and 3PG in DNA.

Scheme 2.

Fragments resulting from collision-induced dissociation of DNA

Scheme 3.

Products formed as a result of reaction of C4-AP-containing DNA fragments with NaBH_4 , NaBD_4 , methoxyamine, and hydrazine.

Figure 1.

ESI-LC-MS chromatogram and spectra of Fe-BLM-damaged ds DNA fragment **1**. The DNA fragment **1** (20 μ M) in 50 mM HEPES buffer (pH 7.5), was treated with activated BLM (50 μ M) for 10 min at 23 $^{\circ}$ C under aerobic conditions and analyzed by LC-MS. A) TIC of activated BLM damaged ds DNA fragment **1**. B) LCMS spectra of the peaks eluting near t_R 3.10 min (TIC).

Figure 2.

ESI-LC-MS/MS chromatogram and spectra of two species formed in Fe-BLM-damaged DNA fragment **1**. The species with m/z 1090.0(-4) and 1093.9(-4) correspond to DNA fragment **1** having C4-AP (ring-opened unhydrated species) in place of thymine (T₈) and cytosine, respectively. A) Proposed structure of the a^* ion resulting from preferential fragmentation at the C4-AP site. B) Calculated a^* and w ions resulting from fragmentation at BLM target sites. C) LC-MS/MS chromatogram of m/z 1090.0(-4). D) CID spectra of m/z 1090.0(-4) species showing the presence of two peaks corresponding to selective cleavage at T₈. E) LC-MS/MS chromatogram of m/z 1093.9(-4) species. F) CID spectra of the m/z 1093.9(-4) species showing the presence of peaks corresponding to selective cleavage at C₆, C₁₂, and C₁₄.

Figure 3.

ESI-LC-MS and ESI-LC-MS/MS spectra of Fe-BLM-damaged ds DNA fragment **1** following reduction with NaBH₄ or NaBD₄. An aqueous solution of the Fe-BLM (50 μM)-damaged DNA fragment **1** (20 μM) was treated with either NaBH₄ or NaBD₄ (100 mM) for 15 min at 23 °C and analyzed by LC-MS. A) LC-MS spectra of a region of the chromatogram of NaBH₄-reduced Fe-BLM-damaged DNA showing the presence of peaks at *m/z* 1091.2(−4) and 1094.9(−4). B) LC-MS spectra of a region of the chromatogram of NaBD₄-reduced Fe-BLM-damaged DNA showing the presence of peaks at *m/z* 1091.7(−4) and 1095.4(−4). C) CID spectra of *m/z* 1091.2(−4) species from NaBH₄-reduced Fe-BLM-damaged DNA. D) CID spectra of *m/z* 1091.7(−4) species from NaBD₄-reduced Fe-BLM-damaged DNA.

Figure 4.

ESI-LC-MS/MS chromatogram and spectra of Fe-BLM damaged ds DNA fragment **1** following derivatization with methoxyamine. The Fe-BLM (50 μ M) damaged DNA fragment (20 μ M) was treated with CH_3ONH_2 (20 mM) for 30 min at 23 $^\circ\text{C}$ in the presence of potassium phosphate buffer (50 mM, pH 7.4). A) LC-MS/MS chromatogram of m/z 1108.4. B) CID spectra of m/z 1108.4(-4) species.

Figure 5.

ESI-LC-MS/MS and ESI-LC-MS³ chromatograms and spectra of m/z 1148.8(-3) species formed in Fe-BLM-damaged DNA fragment **1** following hydrazine treatment. A Fe-BLM (50 μ M) damaged DNA fragment (20 μ M) was treated with NH_2NH_2 (40 mM) for 30 min at 23 $^\circ\text{C}$ in the presence of potassium phosphate buffer (50 mM, pH 7.4) and analyzed using tandem MS. A) LC-MS/MS chromatogram of m/z 1148.8(-3). B) CID spectrum of m/z 1148.8(-3) species. C) LC-MS³ chromatogram of m/z 1148.8(-3), 518(-1) species. D) CID spectrum of the m/z 1148.8(-3), 518(-1) species.

Figure 6.

ESI-LC-MS/MS chromatogram and spectra of two products formed in hydroxyl radical-damaged ds DNA fragment **1** in the absence of treatment and after reduction with NaBH₄. The products with m/z 1093.8(-4) correspond to DNA fragment **1** having C4-AP (ring open unhydrated species) in place of cytosine; m/z 1095(-4) corresponds to DNA fragment **1** having C4-AP reduced to **6** by NaBH₄. ds DNA fragment **1** (40 μ M), in potassium phosphate buffer (50 mM, pH 7.4), was treated with a mixture of ferrous ammonium sulfate (10 μ M) and EDTA (20 μ M), ascorbate (1 mM) and H₂O₂ (8.8 μ M) for 5 min at 23 °C. For reduction, hydroxyl radical-damaged DNA was treated with NaBH₄ (100 mM) for 15 min at 23 °C and analyzed by LC-MS. A) LC-MS/MS chromatogram of m/z 1093.8(-4). B) CID spectra of the m/z 1093.8(-4) species showing the presence of peaks corresponding to selective cleavage at cytosine bases. C) LC-MS/MS chromatogram of m/z 1095(-4) species in hydroxyl radical-damaged DNA after NaBH₄ reduction. D) CID spectrum of m/z 1095(-4) species from NaBH₄-reduced hydroxyl radical-damaged DNA.

Figure 7. ESI-LC-MS/MS chromatograms and spectra of the m/z 1016(–4) and 1039(–3) species formed in hydroxyl radical damaged DNA fragment **1** following hydrazine treatment. The species with m/z 1016(–4) and 1039(–3) corresponds to 3PMP resulting from C4 -H abstraction at C₁₄ and G₁₁, respectively. A) LC-MS/MS chromatogram of m/z 1016(–4) species. B) CID spectra of m/z 1016(–4) species. C) LC-MS/MS chromatogram of m/z 1039(–3) species. D) CID spectra of m/z 1039(–3) species.

Figure 8.

ESI-LC-MS/MS chromatogram and spectrum of m/z 1007.5(-4) species formed in hydroxyl radical damaged DNA fragment **1**. The species with m/z 1007.5(-4) corresponds to 3PG resulting from C4 -H abstraction at C₁₄. A) LC-MS/MS chromatogram of m/z 1007.5(-4) species. B) CID spectra of m/z 1007.5(-4) species.