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Traceless Tandem Lesion Formation in DNA From a Nitrogen-Centered Purine Radical

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Abstract

Nitrogen-centered nucleoside radicals are commonly produced reactive intermediates in DNA exposed to γ -radiolysis and oxidants, but their reactivity is not well understood. Examination of the reactivity of independently generated 2'-deoxyadenosin-N6-yl radical (dA $\bullet$) reveals that it is an initiator of tandem lesions, an important form of DNA damage that is a hallmark of γ -radiolysis. dA $\bullet$ yields O₂ dependent tandem lesions by abstracting a hydrogen atom from the C5-methyl group of a 5'-adjacent thymidine to form 5-(2'-deoxyuridinyl)methyl radical (T $\bullet$). The subsequently formed thymidine peroxy radical adds to the 5'-adjacent dG, ultimately producing a 5'-OxodGuo-fdU tandem lesion. Importantly, the initial hydrogen abstraction repairs dA $\bullet$ to form dA. Thus, the involvement of dA $\bullet$ in tandem lesion formation is traceless by product analysis. The tandem lesion structure, as well as the proposed mechanism, are supported by LC-MS/MS, isotopic labeling, chemical reactivity experiments, and independent generation of T $\bullet$. Tandem lesion formation efficiency is dependent on the ease of ionization of the 5'-flanking sequence, and the yields are >27% in the 5'-d(GGGT) flanking sequence. The traceless involvement of dA $\bullet$ in tandem lesion formation may be general for nitrogen centered radicals in nucleic acids, and presents a new pathway for forming a deleterious form of DNA damage.

Graphical Abstract

INTRODUCTION

DNA damage is deleterious to cells and can potentially lead to cell death or cancer. The cytotoxicity of DNA damage is exploited by cancer treatments such as ionizing radiation, which oxidizes DNA yielding modified DNA lesions.^{1,2} Advances in LC tandem mass

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Supporting Information

Representative autoradiograms, expanded LC-MS/MS data analysis, MS characterization of oligonucleotides containing modified nucleotides. The Supporting Information is available free of charge on the ACS Publications website.

spectrometry have provided insight on the structure of these DNA lesions and mechanism of their formation.^{3–5} In this product-based approach, the DNA lesions detected are considered to be the direct consequences of the corresponding nucleotides reacting with exogenous oxidizing agents. We recently reported tandem lesion formation, an important type of DNA damage produced by ionizing radiation, from a neutral purine radical, 2'-deoxyadenosin-*N*6-yl radical (dA•).⁶ dA• is produced via the direct effect of ionizing radiation via deprotonation of the radical cation (dA•+, Scheme 1). The indirect effect of ionizing radiation produces dA• via dehydration of the corresponding hydroxyl adduct, a process that is competitive with O₂ trapping.⁷ Herein, we report the characterization and mechanism of formation of this tandem lesion. Interestingly, dA• is repaired during tandem lesion formation. Thus, the involvement of dA• is traceless by product analyses. The role of dA• in the chemical process is brought to light by its independent generation within chemically synthesized oligonucleotides, and reveals a possible general role of nucleobase nitrogen-centered radicals in DNA damage that was previously unrecognized.

Clustered lesions are defined as two or more damaged nucleotides within ~1.5-turns of duplex DNA.^{8–10} Although some antitumor agents (e.g. neocarzinostatin) produce clustered lesions, they are a hallmark of γ -radiolysis.^{11–13} Their formation by γ -radiolysis is significant because clustered lesions pose a more significant obstacle to base excision repair (BER) than isolated modified nucleotides, result in increased promutagenic events upon replication in cells, and are potential sources of double-strand breaks.^{14–16} Tandem lesions, which consist of contiguously damaged nucleotides, are a subset of clustered lesions that are unique to ionizing radiation.¹⁷ Some tandem lesions have been identified in cellular DNA following irradiation.^{18–20} In addition to posing a greater challenge to BER, tandem lesions can be more potent replication blocks and more highly mutagenic than the respective isolated lesions.^{18,20–22}

Most tandem lesions have been attributed to pyrimidine nucleobase radicals,^{21,23–32} and have been investigated by product analysis. Independent generation of pyrimidine nucleobase radicals has provided a number of examples in which the radical and/or corresponding peroxy radical adds to the adjacent nucleobases.^{23–25,32} Addition to the π -system is typically favored over hydrogen atom abstraction from the 2'-deoxyribose component of the 5'-adjacent nucleotide.^{27,28,33,34} Importantly, the involvement of pyrimidine radicals leads to formation of modified nucleotides at the site which the radical is generated. However, in one example, independent generation of a C3'-radical under aerobic conditions in single-stranded DNA resulted in C5'-hydrogen atom abstraction from the 3'-adjacent nucleotide by of the corresponding peroxy radical.³⁰

Neutral purine radicals are commonly observed in ionizing radiation, but there is a dearth of information concerning their role in tandem lesion formation. For instance, dA• produced by the direct and indirect effects of γ -radiolysis (Scheme 1).^{2,35} However, prior to our preliminary report on dA•, there was only one other example of a tandem lesion from a purine radical.²⁹ Formation of dA• from **1** upon photolysis in **2** produced a tandem lesion in which damage at dG₁₁ and T₁₂ was detected by denaturing PAGE. That damage at these nucleotides was part of a tandem lesion, and arose from dependent chemical events, was based upon the variance of the relative amounts of cleavage depending on which terminus of

the oligonucleotide was ^{32}P -labeled (Figure 1). Cleavage was induced either by alkali (piperidine) treatment or incubation with the BER enzyme, formamidopyrimidine glycosylase (Fpg), which cleaves DNA at many purine lesions.^{6,36} 8-Oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodGuo) was believed to be the major product at dG₁₁, based upon its lability to Fpg and the ability of β -mercaptoethanol (BME) to prevent piperidine induced cleavage.³⁷ However, the structure of the modification(s) at T₁₂ and the final fate of dA• were undetermined. Structural and mechanistic studies on this novel tandem lesion, as well as the scope of its formation are described herein. The observations raise the possibility that traceless involvement (via product analysis) of nucleobase radicals in DNA damage induced by ionizing radiation may be a common process.

RESULTS AND DISCUSSION

Preliminary characterization and sequence dependence for tandem lesion formation from dA•

Tandem lesions resulting from an independently generated reactive intermediate at a specific site within ^{32}P -labeled DNA are readily detected by denaturing PAGE. Only the damaged nucleotide that is closest to the ^{32}P -label in a particular molecule is detected (as a cleavage site) by denaturing PAGE. Hence, tandem lesion formation can be inferred by comparing the cleavage pattern in 5'- and 3'- ^{32}P -labeled substrates (Figure 1A). (The strand in which dA• is generated is radiolabeled in all experiments.) The ratio of cleavage detected at two positions within one DNA strand is independent of which terminus is labeled if the damage results from independent processes. However, the cleavage ratio at dG₁₁ versus T₁₂ in **2** is dependent on whether the 5'- or 3'-terminus is labeled (Figure 1B). This is indicative of a tandem lesion (Figure 1A).

Oxidative strand damage within a dGGG triplet is consistent with this sequence's lowest ionization potential of any trinucleotide sequence.³⁸ However, Fpg-induced strand damage at the corresponding 3'-terminal 2'-deoxyguanosine (dG₁₂) in photolyzed 5'- ^{32}P -**3** (2.9 $\pm$ 0.3 %, Figure S4) was ~10-fold less than in 5'- ^{32}P -**2**. Digestion of the photolysates of **2** and **3** suggests that the disparity in strand cleavage is not caused by the difference in the conversion of **1**. The conversion of **1** is approximately 80% after 8 h photolysis in each duplex (Figure S6, and S7). The primary difference between duplexes **2** and **3** is the absence of a thymidine between dA• and the dGGG triplet in the latter. This suggests that despite the favorable thermodynamic driving force, dA• does not directly oxidize dG, such as by proton coupled electron transfer.^{39–42} This also indicates that the thymidine (T₁₂) in **2** is critical for efficient damage at dG₁₁, which is not adjacent to the original position at which dA• is produced.

The spin density of dA• is mostly localized at N6,⁴³ and conformational restrictions imposed by the right-handed helix were expected to have a significant effect on tandem lesion formation from dA• by controlling the juxtaposition of reactive species. Indeed, inverting the sequence from 5'-d(GGGT**1**) (**2**) to 5'-d(1T₁₄G₁₅GG) (**4**) results in no alkali-labile strand damage at T₁₄ or dG₁₅ upon photolysis in **4**, and only a small amount of piperidine cleavage is observed at T₁₂ (1.7 ± 0.1 %, Figure S8).⁶ This is attributed to the importance of the juxtaposition of reactive species.

Further illustration of the preference for a 5'-d(GT**1**) sequence is reflected by the absence of any alkali-labile damage at dA₁₁ in 5'-³²P-**5**. Damage is not detected at dA₁₁ in **5**, despite it being flanked on the 5'-side by a dGGG triplet. In addition, the alkali-induced cleavage at T₁₂ in 5'-³²P-**5** (5.4 ± 0.2 %, Figure S10) is less than that observed at the corresponding thymidine in 5'-³²P-**2** (Figure 1B). The lack of tandem lesion formation in a 5'-d(AT**1**) sequence is attributed to the less favorable oxidation potential of dA compared to dG.⁴¹ Furthermore, the reduced damage at the thymidine that is 5'-adjacent to the site at which dA• is generated in **5** compared to **2**, also suggests more distal sequence effects on the nucleotide's reactivity.

This possibility was examined further by comparing the strand damage detected in **2** with that in **6–8** (Figure 2). These substrates each contain a thymidine at T₁₂, the nucleotide bonded to the 5'-phosphate of dA•. However, the oxidation potential of the sequence flanking the 5'-phosphate of T₁₂ varies.³⁸ Strand damage at dG₁₁ and T₁₂ in 5'-³²P-**8** was considerably lower than in 5'-³²P-**2** (Figure 2A), but analysis of 3'-³²P-**8** applying the aforementioned criteria indicates that damage at dG₁₁ is attributable to tandem lesion formation (Figure S11 and S12). Comparing tandem lesion formation in a series of 3 substrates that differ by a single base at the position most remote from dA• in a 5-nucleotide span (5'-d(XGG₁₁T₁₂**1**)) reveals a correlation with ionization potential (Figure 2B). The effect of the flanking sequence ionization potential is evident at T₁₂ (Figure 2A) as well. Overall, these data show that the extended π-system affects the redox properties of DNA.

Structure determination of the major tandem lesion formed in 5'-d(GTA•) sequences

More detailed structural analysis was carried out using dodecamer **9** containing the 5'-d(GGGT**1**) sequence, and when possible the findings were corroborated using 5'-³²P-**2**.

Insight into the nature of the damaged nucleotide(s) at T₁₂ was obtained by the observation that NaBH₄ treatment of 5'-³²P-**2** photolyzate prior to piperidine cleavage practically eliminated strand scission at this position (0.4 ± 0.1 %, Figure S15). Reaction of photolyzed 5'-³²P-**9** (T_m = 35.8 °C) with aldehyde reactive probe (ARP) produced a product (8.1 ± 0.5 %, Figure S16) that migrated more slowly in a denaturing polyacrylamide gel. These observations are consistent with formation of a piperidine labile carbonyl-containing compound at the thymidine bonded to the 5'-phosphate of dA•. 5-Formyl-2'-deoxyuridine (fdU) was a good candidate for this product.⁴⁴

More definitive identification of the tandem lesion was obtained by LC-MS/MS analysis of photolyzed **9** (Figure 3). LC-MS/MS is a powerful method for identifying nucleic acid modifications. It is most frequently used to identify isolated lesions following enzyme digestion of the DNA. However, DNA lesion location is lost upon enzyme digestion. Tandem lesions have been identified in this manner as well by taking advantage of their resistance to the enzyme digestion conditions and/or covalent bonding between nucleotides.^{3,4,21,23,26,32,45} Analysis of intact oligonucleotides using collision-induced dissociation (CID) enables identifying where a lesion is located, but is less common.^{46–48} We took advantage of the HFIP-TEA ion-pairing system, in conjunction with reverse-phase UPLC to provide product separation and reduced ion suppression to analyze the crude photolyzate of **9**.⁴⁹ The major product observed is that resulting from reduction of dA• (**10**), and is consistent with denaturing PAGE analysis of photolyzed **2**. However, we also observed a product with $m/z = 3688.8921$ that is consistent with the expected mass for the tandem lesion containing 5'-d(oxodGuo-fdU) and dA at the position where dA• (**12**) is generated (**11**, calculated $m/z = 3688.6137$) (Figure 3). Treatment of the photolysate with NaBH₄ yielded a

new product that was two mass units higher than **11** ($m/z = 3690.9253$, Figure S21). This is consistent with the presence of fdU. Finally, collision-induced dissociation of the ion corresponding to $z = 3$ of **11** (Figure 3, Table S1) was also consistent with the assigned structure.

The LC-MS/MS and more inferential gel electrophoresis data all support **12** as the major tandem lesion upon generation of dA• flanked by 5'-d(GGGT). To our knowledge, this is the first example in which a nucleotide radical intermediate participates in tandem lesion formation but is ultimately repaired during the process. If one were to rely only upon product analysis of randomly damaged DNA, such as when the biopolymer is exposed to γ -radiolysis, the involvement of dA• would be traceless.

Mechanistic investigation of the formation of tandem lesion **12**

The formation of a tandem lesion composed of 8-oxodGuo and fdU suggested that dA• abstracts the hydrogen atom from the C5-methyl group of the 5'-adjacent thymidine (Scheme 2, step 1), and the resulting 5-(2'-deoxyuridiny)methyl radical (T•) ultimately transfers damage to the 5'-adjacent dG. Based upon the approximate BDEs of the N6-H bond (~97 kcal/mol) in dA and the allylic C5-H bond (BDE ~90 kcal/mol) of thymidine, this process should be exothermic.^{50,51} In addition, a molecular model suggests that the methyl group of the 5'-adjacent thymidine is approximately 2 Å closer than the corresponding hydrogen in the 3'-adjacent thymidine to the radical at N6 of dA• (Figure 4).

Hence, hydrogen atom abstraction from the methyl group of thymidine would explain the directional preference for tandem lesion formation, as well as the requirement that dA• is repaired in **12**.

This possibility was explored by examining strand damage in duplexes in which the 5'-adjacent thymidine in **2** was substituted either by 5,6-dihydrothymidine (dHT, **13**) or 2'-deoxyuridine (dU, **14**). The C-H bond strength of C5-methyl group in dHT is higher than that in dT, and hydrogen atom abstraction from its methyl group by dA• should be disfavored. Since dHT is cleaved by piperidine or Fpg, photolyzates of 5'-³²P-**13** and 5'-³²P-**2** were treated with hOGG1, followed by NaOH to assay for damage at dG₁₁ (Figure S23).⁵² Strand scission at dG₁₁ was significantly reduced from 26.1 ± 0.2 % in **2** to 2.0 ± 0.6 % in the duplex containing dHT (**13**). The low level of strand damage that is still detected at dG₁₁ in 5'-³²P-**13** could be attributed to hydrogen atom abstraction by dA• from the C5-methyl group in dHT, but this is uncertain. Replacement of the 5'-adjacent thymidine by dU has an even more definitive effect on tandem lesion. Strand damage at dG₁₁ in 5'-³²P-**14** photolyzates is completely eliminated (Figure S23). Overall, these observations are consistent with the major pathway for tandem lesion formation from dA• involving initial C5-methyl hydrogen atom abstraction from the 5'-adjacent thymidine (Scheme 2, step 1).

FdU formation and the O₂ dependence of tandem lesion formation suggest that the corresponding peroxy radical of T• (Tp•) oxidizes the 5'-adjacent dG (Scheme 2, steps 2, 3). The viability of this process was investigated by independently generating T• from **17** within DNA (**15**, **16**).^{53–55} These duplexes were designed to replicate the environment in which T• was produced from dA• (above). Indeed, alkali-labile lesions were generated at dG₁₁ in photolyzed 5'-³²P-**15** and 5'-³²P-**16** under aerobic conditions (Figure S24–26). Yields of alkali-labile lesion products were lower than when dA• was generated from **1**

(cleavages at G₁₁ are 6.7 ± 0.7 % in **15** and 1.3 ± 0.2 % in **16**). This is consistent with the generation of carbocation from **17** in addition to T•.⁵⁵ The alkali-labile cleavage yield in the substrate containing the 5'-d(GGG) sequence (**15**) was considerably higher than in **16**. This is consistent with the above experiments (Figure 2) in which the flanking sequence up to 4 nucleotides away affects dA• reactivity. Cleavage by Fpg, as well as the reduction in alkali-induced cleavage at dG₁₁, when the photolyzate was treated with piperidine/BME was consistent with 8-oxodGuo formation at this site, as was observed when tandem lesion formation was initiated by dA•. In addition, the elimination of alkali-labile lesions at T₁₂ following NaBH₄ treatment is consistent with fdU formation at this position. Overall, independent generation of T• at T₁₂ (**15**) within the otherwise identical sequence in which dA• is produced (**2**), supports that the T• generated by hydrogen atom abstraction is trapped by oxygen (Scheme 2, step 2), and that the resulting peroxy radical (Tp•) oxidizes the 5'-adjacent dG (Scheme 2, step 3), ultimately resulting in a tandem lesion (**12**) consisting of 5'-oxodGuo-fdU.

Although peroxy radicals have been suggested to oxidize dG via an outer sphere process,⁵⁶ studies on related nucleobase peroxy radicals indicate that addition to the purine ring is more likely.²⁷ Outer sphere and inner sphere mechanisms are distinguishable by product analysis. The former involves formation of G^{•+} that gives rise to hole migration.⁵⁷⁻⁵⁹ Hole migration would result in preferential damage at the 5'- and middle-dG's of a dGGG sequence. The absence of strand damage at dG₉ and dG₁₀ in **2** argue against this process.⁶⁰ Furthermore, transformation of G^{•+} into 8-oxodGuo requires incorporation of oxygen from H₂O.⁶¹ However, ¹⁸O-incorporation was not detected by LC-MS/MS following photolysis of **9** in H₂¹⁸O (Figure S27). These observations are also consistent with damage on dG₁₁ arising by an inner sphere mechanism.

Inspection of molecular models suggested that Tp• was well positioned to add to C8 of the 5'-adjacent dG (**18**) (Figure 5). The second one electron oxidation can be achieved by addition of O₂, followed by superoxide elimination (Scheme 2, step 4).^{62,63} We propose that 1,2-hydride migration (Scheme 2, step 5) in the carbocation (**19**), followed by deprotonation from **20** (Scheme 2, step 6) produces the tandem lesion (**12**).

The efficiency with which Tp• reacts with dG₁₁ was explored by measuring the effect of BME on the yield of alkali-labile lesions at the purine position in photolyzed 5'-³²P-**2** (Figure 6). Assuming that the maximum rate constant for trapping T• by BME is $1 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$, even the highest concentration of BME (0.5 mM) employed would not compete with O₂ (0.2 mM), which reacts with alkyl radicals at $\sim 2 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$, for the alkyl radical.⁶⁴ In addition, BME reacts inefficiently with dA•.³⁶ Hence, we are confident that the reduction in cleavage at dG₁₁ as a function of BME concentration is a reflection of the competition between the reaction of Tp• with the purine (k_{Add}) and the thiol (k_{Red}). The amount of Tp• reduction product was calculated to be the difference in alkali-induced cleavage at dG₁₁ in the absence of BME and the presence of a given thiol concentration. The slope of the line ($4.8 \times 10^3 \text{ M}^{-1}$, Figure 6) is the ratio of rate constants ($k_{\text{Red}} / k_{\text{Add}}$, Eqn. 1), and if we assume that $k_{\text{Red}} = 2 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$, then $k_{\text{Add}} = 4.2 \times 10^{-2} \text{ s}^{-1}$.⁶⁵ The estimated rate constant for addition of Tp• to the 5'-adjacent dG₁₁ is slightly slower than that estimated for other nucleobase peroxy radicals using competitive kinetics.²⁷ The trapping of Tp• by BME

is further corroborated by LC-MS (Figure S30, and 31). When **9** is photolyzed in the presence of 1 mM BME, the tandem lesion is replaced by a product with $m/z=3674.8979$, which is consistent with the reduction $\text{Tp}^\bullet$ yielding 5-hydroxymethyl-2'-deoxyuridine (**21**), calculated $m/z = 3674.6344$).

CONCLUSIONS

Tandem lesions are an important type of DNA damage. 2'-Deoxyadenosin-*N*6-yl radical ($\text{dA}^\bullet$) is the first neutral purine nitrogen radical shown to unequivocally produce tandem lesions. The involvement of $\text{dA}^\bullet$ in this process is only detectable because it is independently generated. To our knowledge, this is the first time that a radical has been shown to play a traceless role in tandem lesion formation.

The use of LC-MS/MS on intact oligonucleotides was also vitally important for elucidating the structure of the tandem lesion. Employing the more common approach of digesting the biopolymer would have provided significantly less information on tandem lesion (**12**) formation. In addition, utilizing relatively long hybridized oligonucleotides (25 bp) enabled us to examine this chemistry in duplex substrates, as well as to investigate the effects of extended sequences (e.g. 5'-d(NGG)) on the oxidation chemistry. Tandem lesions from $\text{dA}^\bullet$ are formed relatively efficiently when flanked on the 5'-side by 5'-d(NGGT) sequences, which based upon statistics will appear $>10^6$ times in the human genome. Thus, this type of tandem lesion could be potentially relevant to human health.

The role of nitrogen-centered purine radicals in DNA damage has been overshadowed by other nucleoside reactive intermediates, carbon centered radicals and radical cations. Unlike radical cations, nitrogen-centered radicals do not react with water. Nitrogen radicals also do not react rapidly with O_2 , unlike carbon radicals. Consequently, other reaction pathways, including hydrogen atom abstraction by $\text{dA}^\bullet$, may be general for nitrogen-centered radicals in DNA. Considering the frequent formation of neutral purine radicals (Scheme 1), their heretofore minor role in DNA damage is surprising.²⁹ The experiments described here raise the possibility that such radicals may play important, general roles in the formation of tandem lesions that are produced by γ -radiolysis.

EXPERIMENTAL SECTION

Materials and methods

All solvents were distilled before use. Dichloromethane, DIPEA, DMF and pyridine were distilled from CaH_2 . THF is distilled from sodium. T4 polynucleotide kinase (T4 PNK), human 8-oxoguanine DNA N-glycosylase 1 (hOGG1) and terminal transferase were obtained from New England Biolabs. DNA Degradase Plus was obtained from Zymo Research. γ - ^{32}P -ATP and α - ^{32}P -cordycepin 5'-triphosphate were purchased from Perkin Elmer. C18-Sep-Pak cartridges were obtained from Waters. 5,6-Dihydrothymidine phosphoramidite was synthesized as described in the literature.⁶⁶ PBS buffer (0.1 M NaCl, 10 mM sodium phosphate, pH 7.2) and water were treated with Chelex[®] 100 resin (Bio-Rad). Oligonucleotides were synthesized on an Applied Biosystems Incorporated 394 oligonucleotide synthesizer. Oligonucleotide synthesis reagents were purchased from Glen

Research (Sterling, VA). Commercially available fast deprotecting phosphoramidites were used for DNA synthesis of oligonucleotides containing **1**. ESI-MS was carried out on a Thermoquest LCQDeca. UPLC-MS analyses were carried out on Waters Acquity/Xevo-G2 UPLC-MS system equipped with a ACQUITY UPLC HSS T3 Column (100 Å, 1.8 µm, 2.1 mm × 100 mm) or Oligonucleotide BEH C18 Column (130Å, 1.7 µm, 2.1 mm × 100 mm). Oligonucleotide masses were obtained via deconvolution using MassLynx 4.1 software. CID was processed using Microsoft Excel. MALDI-TOF analyses were carried out on a Bruker AutoFlex III Maldi-TOF. Quantification of radiolabeled oligonucleotides was carried out using a Molecular Dynamics Phosphorimager 860 equipped with ImageQuant Version TL software.

Oligonucleotide synthesis

A 5 min coupling time was used for the previously reported modified phosphoramidite.⁶ Deprotection of synthesized oligonucleotides containing **1** was performed with concentrated aqueous ammonia for 16 h at room temperature, followed by concentration under reduced pressure. The oligonucleotides were purified using 20% denaturing PAGE. Oligonucleotides containing **17** were synthesized, deprotected, and purified according to previously reported procedures.⁶⁷ Oligonucleotides were eluted from polyacrylamide gel and desalted using C18-Sep-Pak cartridges.

Photolysis of oligonucleotides

The strand (1 µM) containing the radical precursor was labeled at the 5' end with γ -³²P-ATP using T4 PNK in T4 PNK buffer (70 mM Tris-HCl, pH 7.6, 10 mM MgCl₂, 5 mM DTT, 45 min, 37 °C). The labeled strand was hybridized to the complementary strand (1.5 eq.) in PBS by heating at 90°C for 1 min and slowly cooling to room temperature. The hybridized duplexes were diluted to 0.1 µM in PBS before photolysis. All photolyses were carried out at 25 °C in Pyrex using a Rayonet photoreactor equipped with 16 lamps having a maximum output at 350 nm. All anaerobic photolyses were carried out in sealed Pyrex tubes, which were degassed by freeze-pump-thaw degassing (3 cycles). Samples were sealed while under vacuum at 77 K. Photolyses of DNA containing **1** and **17** were carried out for 8 h and 20 min respectively.

Post-photolysis treatments

Aliquots from photolyzed solutions or unphotolyzed controls were treated with piperidine (1 M, 30 min, 90 °C), Fpg (1.25 µM, 1 µL, 10 mM Bis Tris-Propane HCl (pH 7), 10 mM MgCl₂, 1 mM DTT, 100 µg/mL BSA, 1 h, 37 °C), or Ir⁴⁺ (0.1 mM of Na₂IrCl₆•6H₂O) for 1 h at 25 °C, quenched (2 mM Hepes, 10 mM EDTA, pH 7), and treated with piperidine (1 M, 30 min, 90 °C). Alternatively, photolyzates were treated with in the presence of 0.25 M BME, NaBH₄ (100 mM, 1 h, 4 °C) followed by piperidine (1 M, 30 min, 90 °C), or hOGG1 (8 units, 50 mM NaCl, 10 mM Tris-HCl (pH 7.9), 10 mM MgCl₂, 1 mM DTT, 100 µg/mL BSA) for 1 h at 37 °C, followed by NaOH (1 M, 30 min, 37 °C). NaOH treated samples were neutralized with HCl (1 equiv.). All samples treated with enzymes were precipitated (0.3 M NaOAc, pH 5.2, 0.1 g/mL calf thymus DNA) with ethanol. Piperidine treated samples were evaporated to dryness under vacuum, and washed with 2 × 10 µL water, which

was also removed under vacuum. Samples were analyzed by dissolving in formamide loading buffer prior to analyzing by 20% denaturing PAGE. BME, piperidine, NaBH₄, and Ir⁴⁺ solutions were prepared fresh on the day of the experiment.

Enzymatic digestion of oligonucleotides to 2'-deoxynucleosides for UPLC analysis

Duplexes **2**, **3**, and the corresponding photolysates (4 pmol) in 1.6 μ L H₂O, 10 $\times$ DNA degradase plus[®] buffer (5 μ L) and dU (2.5 μ L, 80 μ M) as internal standard were incubated with DNA degradase plus[®] (2 μ L, 5 U/ μ L) for 4 h at 37 °C. The reaction mixture was filtered through a nanosep[®] 3K filter by centrifuging the mixture for 5–10 min at 16000 $\times$ g. The filter was washed once with 25 μ L H₂O and the combined filtrate (50 μ L) was analyzed using an Agilent 1290 infinity UPLC equipped with the ACQUITY UPLC HSS T3 Column (A, 10 mM ammonium formate; B, acetonitrile; 5% B from t = 0 to t = 2 min; 5–80% B linearly over 7.5 min; 80% B from t = 9.5 to t = 12.5 min; 80–5% B linearly over 1 min; 5% B from t = 13 to t = 16 min; flow rate, 0.3 mL/min.)

UPLC-MS/MS analysis of oligonucleotides

Photolysates (8 μ L) containing 5 μ M of duplex dodecamer were analyzed by UPLC-MS using the Oligonucleotide BEH C18 Column (A, 100 mM HFIP and 8.6 mM TEA; B, Methanol; 2% B from t = 0 to t = 5 min; 2–9% B linearly over 3 min; 9% B from t = 8 to t = 20 min; 9–30% B linearly over 5 min; 30% B from t = 25 to t = 30 min; 30–2% B linearly over 5 min; 2% B from t = 35 to t = 40 min; flow rate, 0.2 mL/min.). The column temperature was 60 °C. The collision energy was set to ramp from 10 to 45 V.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Tandem lesion formation in photolyzed **2**. (A.) Schematic demonstration of tandem lesion detection in 5'- and 3'-³²P-labeled DNA following piperidine (Pip.) treatment. X and Y are alkaline labile. (B.) Piperidine-induced cleavage in photolyzed **2**. The values presented are the ave. ± std. dev. of 3 replicates.

Figure 2.

Local sequence effect on strand damage from dA•. (A.) Strand scission at dG₁₁ (Fpg) and T₁₂ (piperidine). (B.) Dependence of damage at dG₁₁ (Fpg) from dA• as a function of the trinucleotide sequence ionization potential.³⁸ The values presented are the ave. $\pm$ std. dev. of 3 replicates.

Figure 3.

CID mass spectrum of the ion ($m/z = 1229.2$, $z = 3$) of tandem lesion **11**. X = 8-oxodGuo, Y = fdU (see **12** in text).

Figure 4. Molecular modeling demonstrating hydrogen atom abstraction by dA• in the trinucleotide sequence 5'-T-dA•-T/3'-dA-T-dA.

Figure 5.
Molecular modeling demonstrating $\text{Tp}^\bullet$ addition to a 5'-dG.

5'-d(CGG GXA TAC GTC) 21

Figure 6.

Thiol effect on tandem lesion (**12**) formation in photolyzed 5'-³²P-**2**. Plot of reduced peroxyl radical (Tp•) versus tandem lesion formation as a function of [BME]. The values presented are the ave. ± std. dev. of 3 replicates.

Scheme 1. Formation of $dA^\bullet$ by γ -radiolysis and photolysis of **1**.

Scheme 2.
Tandem lesion (**12**) formation from dA•.