Damage to Isolated DNA Mediated by Singlet Oxygen

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Dedicated to Professor *André M. Braun* on the occasion of his 60th birthday

In the present work, we study the reaction of singlet oxygen ($^1O_2$) with isolated DNA. Emphasis is placed on the identification and quantitative measurement of the DNA modifications that are produced by the reaction of $^1O_2$ with DNA. For this purpose, calf-thymus DNA was incubated with the endoperoxide of *N,N′*-di(2,3-dihydroxypropyl)-1,4-naphthalenedipropanamide, a chemical generator of $^1O_2$. Thereafter, DNA was digested, and the resulting oxidized nucleosides were measured by means of a recently optimized high-performance-liquid-chromatography tandem-mass-spectrometry assay. It was found that, among the different DNA lesions observed, 7,8-dihydro-8-oxo-2′-deoxyguanosine is the major $^1O_2$-mediated DNA-damage product. Interestingly, cyclobutane pyrimidine dimers, oxidized pyrimidine bases, 7,8-dihydro-8-oxo-2′-deoxyadenosine, and 2,6-diamino-5-formamido-4-hydroxypyrimidine are not formed, at least not in detectable amounts, following treatment of DNA with the $^1O_2$ generator. The reported results strongly suggest that the decomposition of the endoperoxide provides a pure source of $^1O_2$, and that reaction of $^1O_2$ with isolated DNA induces the specific formation of 7,8-dihydro-8-oxo-2′-deoxyguanosine.

Introduction. – Singlet oxygen, ($^1O_2$ Δ_g), the lowest excited state of molecular oxygen (O_2), seems to play an important role in several biological systems, and could generate oxidative damage in a variety of biological targets. For instance, the biological consequences of UVA radiation are mediated, at least partly, by the occurrence of type-II photosensitization reactions [1], involving the transient formation of $^1O_2$ [2]. Singlet oxygen is known to react with electron-rich molecules [3]. Among other cellular targets, DNA is of particular importance, due to its key role in cell survival and reproduction. Evidence has been accumulated for the genotoxic [4][5] and carcinogenic effects of reactive oxygen species [6] that strongly indicates that $^1O_2$ is able to oxidize cellular DNA [7–9]. Confirmation has been obtained recently, and 7,8-dihydro-8-oxo-2′-deoxyguanosine (8-oxodGuo)[1]) has been shown to be produced by

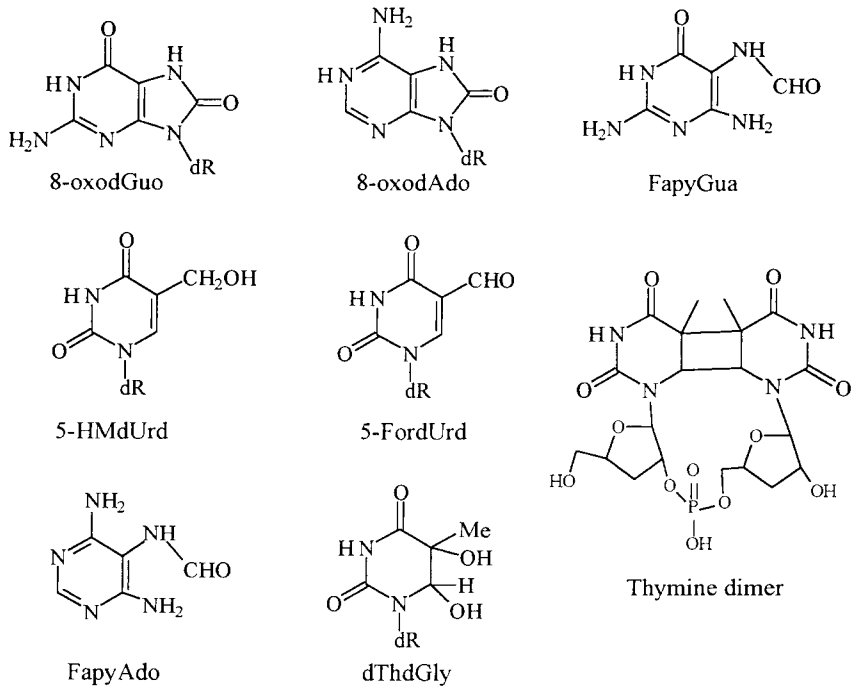
[1]) Abbreviations: $DHPNO_2$: endoperoxide of *N,N′*-di(2,3-dihydroxypropyl)-1,4-naphthalenedipropanamide; DHPN: *N,N′*-di(2,3-dihydroxypropyl)-1,4-naphthalenedipropanamide; dGuo: 2′-deoxyguanosine; 8-oxodGuo: 7,8-dihydro-8-oxo-2′-deoxyguanosine; 4-OH-8-oxodGuo: 4,8-dihydro-4-hydroxy-8-oxo-2′-deoxyguanosine; dThdGly: 5,6-dihydro-5,6-dihydroxythymidine; FapyGua: 2,6-diamino-5-formamido-4-hydroxypyrimidine; FapyAde: 4,6-diamino-5-formamidopyrimidine; 5-FordUrd: 5-formyl-2′-deoxyuridine; 5-HmdUrd: 5-(hydroxymethyl)-2′-deoxyuridine; 5-OHdUrd: 5-hydroxy-2′-deoxyuridine; 8-oxodAdo: 7,8-dihydro-8-oxo-2′-deoxyadenosine; HPLC-MS/MS: high-performance-liquid-chromatography tandem mass spectrometry; dR: 2-deoxyribose.

the reaction of $^1O_2$ with the guanine moiety of cellular DNA [10]. In contrast to the hydroxyl radical (•OH), which reacts almost indifferently with all the nucleobases and the sugar moieties of DNA, $^1O_2$ oxidizes mainly the guanine base [11][12]. 8-OxodGuo has been shown to be a major $^1O_2$ oxidation product of isolated DNA [13][14].

However, such results need to be confirmed for two main reasons. First, it must be remembered that it is difficult to specifically produce $^1O_2$. Photosensitization reactions have been mainly used for this purpose [15][16]. However, even when predominant type-II photosensitizers are used, a competitive type-I reaction, involving an electron-transfer mechanism, may contribute to oxidation reactions. In this respect, 8-oxodGuo is known to be the major type-I oxidation product of the guanine moiety in DNA [17]. Second, various analytical methods are available for the measurement of 8-oxodGuo [18], but, until recently, only the GC-MS assay was used to monitor the formation of other DNA lesions such as pyrimidine and adenine oxidation products. However, it was recently shown that the latter assay suffers from a major drawback, *i.e.*, the occurrence of an artefactual oxidation of normal bases during the derivatization reaction that is required to produce volatile compounds [19–21] for GC separation.

In the present work, we have reassessed the reactivity of $^1O_2$ toward isolated DNA. For this purpose, we have used the water-soluble endoperoxide $DHPNO_2$ [22] that is known to release $^1O_2$ upon thermal decomposition [23]. Therefore, DNA was incubated with the thermolabile endoperoxide, and different DNA modifications were measured by means of an accurate and sensitive HPLC-MS/MS method [24][25]. Cyclobutane thymine dimers (detected as the dinucleoside monophosphates [26]) were monitored to determine whether the thermal decomposition of the endoperoxide could induce the formation of DNA excitation products. In addition, during attempts to determine whether electron transfer or •OH radical formation could occur, the formamidopyrimidine derivative of dGuo (FapyGua) and thymidine oxidation products (dThdGly, 5-FordUrd, 5-HmdUrd) as well as 8-oxodAdo were measured. It was found that 8-oxodGuo is the only targeted DNA damage that is detected following incubation of isolated DNA with $DHPNO_2$. Therefore, decomposition of the naphthalene endoperoxide provides a clean source of $^1O_2$, which, upon reaction with the guanine moiety of DNA, induces the formation of 8-oxodGuo.

Results and Discussion. – Different modified DNA bases, structures shown below, have been measured in DNA isolated after treatment with $DHPNO_2$. The levels of 8-oxodGuo and dThdGly measured in DNA upon incubation at 37° with increasing amounts of $DHPNO_2$ are reported in *Fig. 1*. An almost linear increase in 8-oxodGuo is noticed, whereas the level of dThdGly remains unchanged. This also applies to other DNA-damage products, including 8-oxodAdo, 5-HmdUrd, 5-FordUrd, FapyGua, and cyclobutane thymine dimers detected as their corresponding dinucleoside monophosphates (data not shown). The involvement of $^1O_2$ in the formation of 8-oxodGuo was confirmed upon treatment of DNA with either 50 μl of $DHPNO_2$ or heat-deactivated $DHPNO_2$ (*Fig. 2*). No increase in 8-oxodGuo could be detected when DNA is treated with heat-deactivated $DHPNO_2$. The time course of formation of 8-oxodGuo in isolated DNA incubated with 50 μl of $DHPNO_2$ is shown in *Fig. 3*.



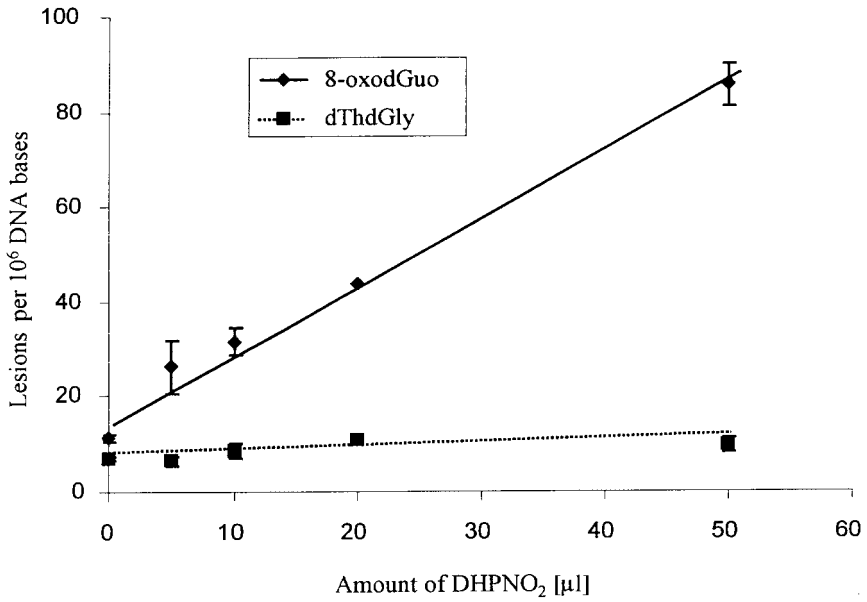


Fig. 1. *Formation of 8-oxodGuo and dThdGly in isolated calf-thymus DNA treated for 2 h at 37° with different amounts of* $DHPNO_2$ (results expressed as the number of modification per 10^6 DNA bases represent the average and standard deviation of three independent determinations)

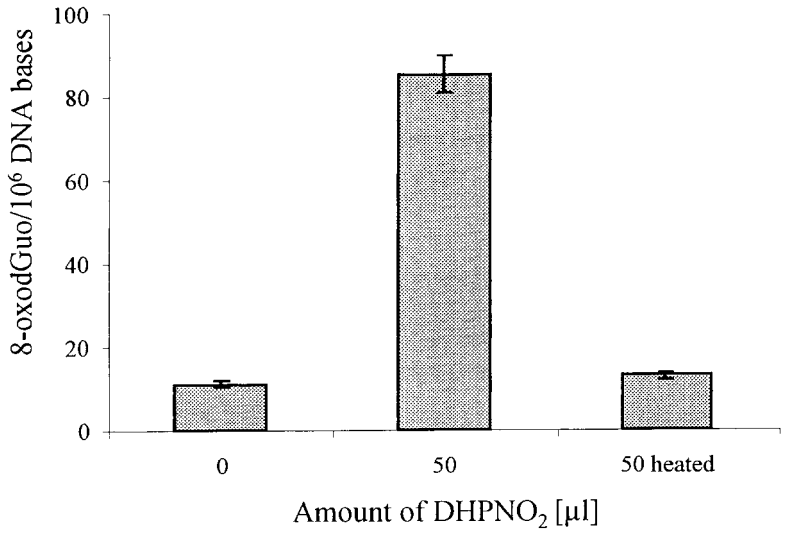


Fig. 2. *Formation of 8-oxodGuo in isolated DNA treated with 50 µl of $DHPNO_2$ with and without decomposition by heating at 70° for 20 min*

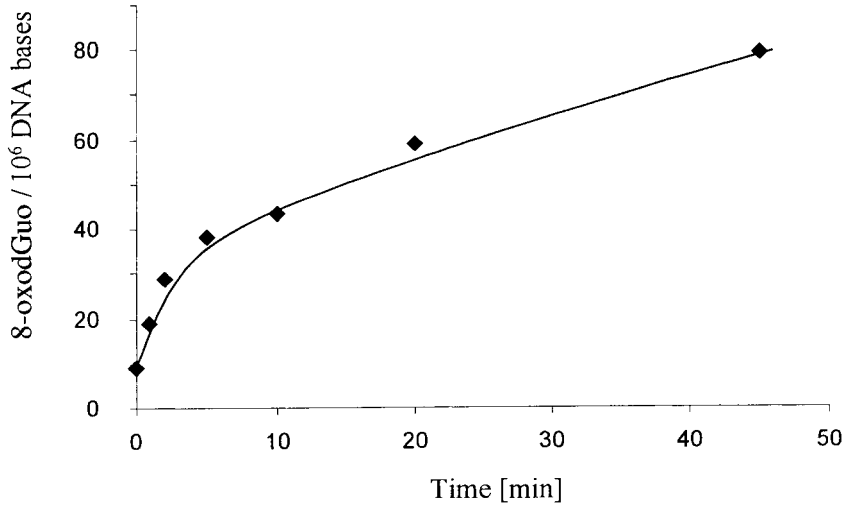


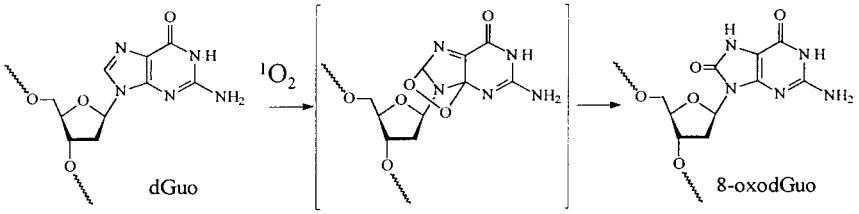
Fig. 3. *Time course for the formation of 8-oxodGuo in calf-thymus DNA incubated at 37° in presence of 50 µl of $DHPNO_2$*

The purpose of the current work was to study the mechanistic aspect of the reaction of $^1O_2$ oxidation of the guanine moiety of isolated DNA. For this purpose, thermal decomposition of $DHPNO_2$ has been used as a pure source of $^1O_2$. Emphasis has been placed on the delineation of the reactions induced by the thermal decomposition of the naphthalene endoperoxide derivative. For such a purpose, different DNA modifications (see above) were monitored. First, to evaluate the occurrence of a putative DNA-

excitation process during incubation of DNA with $DHPNO_2$ at 37°, the formation of cyclobutane thymine dimer was evaluated. Such a lesion is known to be produced when thymine is excited in DNA, usually by a low intensity UVB or UVC radiation. Since no cyclobutane thymine dimers are produced upon incubation of DNA with $DHPNO_2$, this strongly suggests that such a treatment does not induce DNA excitation. In addition, to check whether side-reactions, including one-electron oxidation and •OH formation, may occur during incubation with $DHPNO_2$, other DNA oxidation products were investigated. The absence of the formation of dThdGly (*Fig. 1*), HmdUrd, FordUrd, 8-oxodAdo, and FapyGua is consistent with the absence of •OH formation or one-electron-oxidation reactions. Altogether, the data suggest that thermal decomposition of $DHPNO_2$ leads to the formation of $^1O_2$ that, upon reaction with the guanine moiety of DNA, induces the formation of 8-oxodGuo.

Therefore, our results confirm that $^1O_2$ specifically induces the formation of 8-oxodGuo in isolated DNA. The generation of 8-oxodGuo is correlated to the amount of endoperoxide used (*Fig. 1*). In addition, the decomposed endoperoxide (20 min at 70°) does not induce the formation of 8-oxodGuo. This strongly suggests that $^1O_2$ is involved in the formation of 8-oxodGuo. The time course for formation of 8-oxodGuo is not linear with the time of incubation of DNA with $DHPNO_2$. This could be explained by the exponential decomposition of $DHPNO_2$ with a half-life of about 20 min [22]. Thus, the amount of $^1O_2$ is initially high and decreases with time, in agreement with the initially rapid formation of 8-oxodGuo followed by a decrease in the rate of formation over longer incubation times. Altogether, our results could be rationalized in term of specific formation of 8-oxodGuo in isolated DNA upon incubation with a chemical generator of $^1O_2$. The formation of 8-oxodGuo could be explained by the transient formation of an endoperoxide derivative (*Scheme*) according to a [4 + 2] *Diels-Alder* reaction. Such an unstable endoperoxide has been detected at low temperature in a reaction with a 8-methylguanosine derivative as a model compound [27]. At the nucleoside level, decomposition of the endoperoxide leads mainly to the formation of the two 4*R* and 4*S* diastereomers of 4-hydroxy-8-oxo-4,8-dihydro-2′-deoxyguanosine (4-OH-8-oxodGuo) [16], which may further rearrange into spiroiminodihydantoin nucleosides [28]. In addition, 8-oxodGuo, whose formation requires a reduction of the initially formed endoperoxide, is generated as a minor product. However, the two diastereomers of 4-OH-8-oxodGuo (or the spiroiminodihydantoin nucleosides) have never been detected in isolated DNA treated with $^1O_2$ [14][16][29]. Therefore, this indicates that, in isolated DNA, the decomposition of the initially formed guanine endoperoxide predominantly gives rise to formation of 8-oxodGuo. However, we could

Scheme. *Mechanism Proposed for the Reaction of $^1O_2$ with the Guanine Moiety of Isolated DNA That Specifically Produces 8-OxodGuo*



not exclude that other yet-unidentified guanine-oxidation product(s) are formed upon $^1O_2$-mediated oxidation of the guanine moiety of isolated DNA.

In a previous work, additional (and sometime unidentified) products were detected [13] following treatment of DNA with $^1O_2$ generated by photosensitization. The origin of the discrepancy could be due, at least partly, to how difficult it is to obtain a pure source of $^1O_2$ (*vide supra*). Photosensitization reactions with type-II photosensitizers have been mainly used for this purpose, and a competitive type-I reaction might partly induce side reactions [30][31]. Therefore, the use of a chemical generator of $^1O_2$ represents an interesting alternative to overcome this problem. Thermal decomposition of naphthalene endoperoxide derivatives is known to release only O_2 in the triplet state and $^1O_2$ [32–34]. It was shown in the present work that side reactions ($^{\bullet}OH$ formation, one-electron oxidation, or energy transfer) are not involved, at least not in detectable yields. Interestingly, FapyGua was not generated when DNA was incubated in the presence of $DHPNO_2$. This lesion is known to be one of the two major products arising from the 8-hydroxy-7,8-dihydro-2′-deoxyguanosyl radical that may be generated through hydration of the 2′-deoxyguanosine radical cation [35][36]. Our data indicate that $^1O_2$ is unable to induce the formation of FapyGua, ruling out the possibility of one-electron oxidation of the guanine moiety.

The mutagenicity of $^1O_2$ has been also extensively studied, either by photosensitization or with chemical generation of singlet oxygen, and mutation spectra indicate that this reactive oxygen species induces mainly G-C to T-A transversion [4][5]. Such a mutation spectrum is in good agreement with the known mutagenicity of 8-oxodGuo [37]. However, in addition to that, one G deletion has been observed [4], but the nature of the lesion responsible for such a mutation is yet unknown. However, according to recent data dealing with the tendency of 8-oxodGuo to react with $^1O_2$, it may be postulated that secondary oxidation products of 8-oxodGuo could be responsible for such a mutation. In that respect, it is important to note that oxaluric acid, the main oxidation product of 8-oxodGuo in small oligonucleotides [38], most probably formed by a type-II mechanism, also produces G to T transversion [39]. Therefore, another yet-unidentified $^1O_2$-oxidation product of the guanine (or 7,8-dihydro-8-oxoguanine) moiety is formed in DNA. In addition, other $^1O_2$-induced mutations were detected in relatively high amounts, mainly at GC sequences [40]. The production of such mutations strongly suggests the formation of DNA modifications other than 8-oxodGuo that may include tandem DNA lesions and/or secondary 8-oxodGuo oxidation products.

In addition to oxidized DNA bases, DNA strand breaks have been also detected in DNA treated with $^1O_2$ [41]. However, the time course for formation of these strand breaks indicates that their generation required two molecules of $^1O_2$. Therefore, they probably arise from further oxidation of 8-oxodGuo initially formed in the DNA. Such a hypothesis is supported by the observation that 8-oxodGuo is selectively formed by the reaction of $^1O_2$ with either isolated DNA (present work) and cellular DNA [10]. Specific analytical tools aimed at measuring other DNA lesions are required to better understand the reactivity of $^1O_2$ toward DNA, and to determine whether secondary oxidation of 8-oxodGuo occurs in isolated and cellular DNA. In that respect, the HPLC-MS/MS approach is well-suited to the measurement of modified DNA bases, including for example the oxaluric acid derivative. In addition, the comet assay

associated with specific DNA-repair enzymes [42] (*e.g.* the Fpg protein that recognizes 8-oxodGuo) would allow determination of formation of strand breaks relative to oxidized bases (mainly 8-oxodGuo). Such information is required to better assess the role of $^1O_2$ in biological processes. In that respect, we have shown that the thermal decomposition of a water-soluble endoperoxide represents a suitable method to produce a clean source of $^1O_2$.

Experimental Part

Chemicals. Nuclease P1 (*Penicillium citrium*) and calf-thymus DNA were obtained from *Sigma* (St. Louis, MO). Alkaline phosphatase, calf-spleen phosphodiesterase (SPDE), and snake-venom phosphodiesterase (VPDE) were purchased from *Roche Molecular Biochemicals* (Mannheim, Germany), and edta was from *Interchim* (Montluçon, France). Water was deionized with a *Millipore/Milli-Q* system (*Millipore*, Molsheim, France). The endoperoxide $DHPNO_2$ was synthesized as previously described [22] by methylene-blue-mediated photosensitization of DHPN in the presence of unlabeled O_2. The modified DNA derivatives, including 8-oxodGuo, 8-oxodAdo, 5-HmdUrd, 5-FordUrd, and FapyGua as well as the corresponding labeled internal standards, were prepared as previously described [25].

DNA Oxidation. In the first experiment, DNA (0.5 mg/ml, 100 µl final volume) was treated in deionized distilled H_2O with different amounts of 55 mM $DHPNO_2$. The resulting solns. were incubated at 37° for 2 h prior to DNA precipitation (*vide infra*). In the second experiment, DNA (0.5 mg/ml, 1 ml) was incubated at 37° in the presence of 250 µl of $DHPNO_2$. Then, 100 µl (50 µg of DNA) aliquots were periodically removed from the incubated solution. To each aliquot, 5 µl of 0.1M NaN_3 (a well-known $^1O_2$ scavenger) was added to stop the reaction, and samples were stored at 4°. Thereafter, DNA was precipitated from each aliquot by addition of 250 µl cold EtOH, and resuspended in 100 µl of H_2O prior to DNA digestion.

Sample Analysis. DNA Digestions were performed as previously described [25][42]. On-line HPLC-MS/MS measurements were carried out with a *7100 Hitachi-Merck* pumping system (*Merck*, Darmstadt, Germany). Loop injections were performed with a *SIL 9A* (*Shimadzu*, Tokyo, Japan) autosampler equipped with a 20 µl loop. Separations were performed on an Uptisphere ODB (5 µm, 150 × 2 mm i.d.) octadecylsilyl silica-gel column (*Interchim*, Montluçon, France) for the simultaneous measurement of dThdGly, 5-HmdUrd, 5-FordUrd, 8-oxodGuo, and 8-oxodAdo as previously described [25]. The same chromatographic system was used to monitor cyclobutane thymine dimers as their corresponding dinucleoside monophosphates [26]. For FapyGua, separations were achieved with a *Hypersil* NH_2 (5 µm, 150 × 2 mm i.d.) silica-gel column (*Interchim*, Montluçon, France) [25][43]. In both cases, the output of the chromatographic system was fed into a *L-4000 Merck-Hitachi* UV detector set at 280 nm to monitor the elution of normal nucleosides that enables quantification of the amount of DNA. Thereafter, elution buffer was introduced without split into a turbo-ion-spray source (*MDS SCIEX*, Concord, ON) of a *API 3000* triple-quadrupole mass spectrometer [25][26]. DNA Lesions were specifically detected with the multiple-reaction-monitoring mode and quantified with either internal or external standards for oxidized-DNA lesions [25] and cyclobutane thymine dimers, resp. [26].

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