Structure and Potential Mutagenicity of New Hydantoin Products from Guanosine and 8-Oxo-7,8-Dihydroguanine Oxidation by Transition Metals

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In vitro **work in this laboratory has identified new DNA lesions resulting from further oxidation of a common biomarker of oxidative damage, 8-oxo-7,8-dihydroguanine (OG). The major product of oxidation of OG in a nucleoside, nucleotide, or single-stranded oligodeoxynucleotide using metal ions that act as one-electron oxidants is the new nucleoside derivative spiroiminodihydantoin (Sp). In duplex DNA an equilibrating mixture of two isomeric products, guanidinohydantoin (Gh) and iminoallantoin (Ia), is produced. These products are also formed by the overall four-electron oxidation of guanosine by photochemical processes involving O_2. DNA template strands containing either Sp or Gh/Ia generally acted as a block to DNA synthesis with the Klenow exo$^-$ fragment of pol I. However, when nucleotide insertion did occur opposite the lesions, only 2′-deoxyadenosine 5-triphosphate and 2′-deoxyguanine 5-triphosphate were used for primer extension. The *Escherichia coli* DNA repair enzyme Fpg was able to remove the Sp and Gh/Ia lesions from duplex DNA substrates, although the efficiency was depended on the base opposite the lesion.** ***Key words:*** **DNA damage, DNA repair, guanine, oxygen radicals, polymerases, transition metals.** ***Environ Health Perspect*** **110(suppl 5):713–717 (2002).**
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One consequence of life in an oxygen atmosphere is continual attack upon the genome by reactive oxygen species. Oxidizing agents may be endogenously present, as in the case of metabolic intermediates involved in the electron transfer cascade in mitochondria, endogenously induced in cells experiencing oxidative stress, or exogenously introduced by environmental exposure to transition metals, oxidants, and free radicals. For example, the more carcinogenic transition metals nickel and chromium have been the focus of numerous studies because their redox activity and pathways of biodistribution conspire to induce DNA damage. Oxidative DNA damage results in strand breaks, DNA–protein cross-links, and base lesions, all of which require the action of DNA repair pathways to maintain the integrity of the genome. Ultimately, mistakes made by polymerases in the absence of DNA repair lead to mutagenesis. Because of its relevance to cancer, aging, and neurological disorders, oxidative damage to DNA continues to receive a very high level of attention.

The mutational spectra associated with oxidative damage indicate that the most common base substitution is guanine:cytosine → adenine:thymine (G:C → A:T) transitions followed by G:C → T:A and G:C → C:G transversions, although the specific details of mutational frequencies depend on the reactive species (*1*). For example, G:C → A:T transitions are the most frequent mutations arising from γ radiation and superoxide- and hydrogen peroxide–mediated DNA damage, but the predominance of G:C → C:G transversions from Fe(II) (*2*) and peroxyl radical–induced damage (*3*) and the relatively high occurrence of G:C → T:A transversions from all reactive oxygen species (*2*) suggest that multiple pathways of mutation exist. This multiplicity may be due either to the large number of lesions that result from DNA oxidation, over 50 base lesions alone now having been identified (*4*), or to confused polymerases that may insert more than one incorrect base opposite a given lesion. Errors in replication may be compounded by the efficiency by which DNA repair enzymes recognize and excise lesions in various sequence contexts.

How do these mutational spectra relate to specific DNA lesions? Mutations in a G:C pair may be due to oxidative damage to either G or C, followed by miscoding. Dihydroxylation of C leads to deamination, forming 5,6-dihydro-5,6-dihydroxyuridine that can give rise to insertion of A in the original position of G. This process may account for a large fraction of the most common G:C → A:T transition mutations (*1*). On the other hand, one molecular origin of G:C → T:A transversions is 8-oxo-7,8-dihydroguanine (OG), a molecule hailed as the biomarker of oxidative damage in the cell (*4*). Depending on the polymerase, either A or C may be inserted opposite OG during replication and transcription, and the subsequent repair of the OG:A mismatch relies on the "GO" repair enzymes that remove the incorrect A and subsequently the OG (*5,6*). However, many different guanine lesions may manifest as a G → T transversion mutation because *a*) guanine is the most easily oxidized base in DNA (*7*) and *b*) many polymerases follow the "A rule" for insertion opposite unrecognizable lesions (*8,9*). For example, recent work on an oxazolone lesion derived from one-electron oxidation of G indicates that it too may lead to G → T transversion mutations (*10*).

Although OG is one of the major products formed by ionizing radiation, one-electron oxidation and singlet oxygen attack at G residues, more than a dozen other lesions have been identified or proposed as minor products of the above pathways or as major products with other oxidants (*11*). For example, the one-electron oxidation pathway apparently leads to OG when the intermediate guanine radical cation is hydrated but forms imidazolone (Iz), which is slowly hydrolyzed to oxazolone (Z; Figure 1) if $G^{\bullet+}$ deprotonates and reacts with O_2 (*12*). The "oxazolone" product Z, likely present as the ring-opened structure shown in Figure 1 rather than the heterocyclic structure originally proposed, is now recognized as a major G-derived lesion from *in vitro* DNA oxidation (*10,12*).

Beyond 8-Oxoguanine

The combination of good synthetic methods for incorporation of DNA lesions into oligomers coupled with the availability of sensitive mass spectral methods of analysis have led to a considerable broadening of the oxidized guanine field in the past 5 years. Part of this interest stems from the recent recognition that OG is highly reactive toward further oxidation; several *in vitro* studies now claim OG as a "hot spot" for oxidized damage (*13*). Indeed, the redox potentials shown in Figure 2 (*13,14*) suggest that the common oxidized bases OG, 5-OH–cytosine, 5-OH–uridine, and possibly 8-oxoadenine might all be considered reactive intermediates under conditions of oxidative stress. For example, the difference in redox potentials of G versus OG has been estimated as a 13 kcal/mol

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driving force in favor of OG oxidation in a one-electron pathway (*13*). This means that if electron transfer could readily occur (see below) between $G^{\bullet+}$ and OG,

$$G^{\bullet+} + OG \rightleftarrows G + OG^{\bullet+}$$
$$\Delta G = -13 \text{ kcal/mol}; \quad K_{eq} = 10^9 \qquad [1]$$

the equilibrium constant in favor of OG oxidation would be about 10^9. Of course, the reason that OG is commonly observed instead of instantly being further oxidized is because G residues are present in large excess, and the equilibrium shown in Equation 1 would have to compete with quenching of the G radical cation to form products. Clearly, however, OG is intrinsically the strongly preferred site of oxidation. Similarly, the high reactivity of OG compared with G in $^1O_2$ chemistry has been reported (*15*). Recent studies also show that OG is highly reactive toward peroxynitrite, a relatively abundant cellular oxidant (*16*). This high sensitivity of OG to further oxidation likely accounts for some of the problems of accurately quantifying levels of OG in cells, because the process of isolation and characterization of oxidized DNA can lead not just to more G oxidation (i.e., formation of OG) during manipulation but to oxidative destruction of OG as well.

Characterization of New Oxidized Lesions

Prompted by earlier investigations of metal-catalyzed guanine oxidation, studies in our laboratory have recently focused on the one-electron pathway of OG oxidation (*17–21*). In this mechanism, $OG^{\bullet+}$ is formed by loss of an electron from OG. One-electron oxidation may occur by type I photoprocesses (*22*), including ionizing radiation, high-valence transition metals [Fe(III–V), Ni(III), Cu(III), Cr(V), Ir(IV), etc.], and any pathway that involves attack by $RO^{\bullet}$ (alkoxyl radicals) followed by loss of RO^-. Our work has shown that iridium [Ir(IV)] is a convenient *in vitro* one-electron oxidant because its redox potential (~0.9 V) is sufficient to oxidize OG (as well as other oxidized bases) without reacting with undamaged bases (*17*). We have identified two major pathways of OG one-electron oxidation in which we propose 5-hydroxy-OG as an intermediate that can either isomerize to spiroiminodihydantoin (Sp) or hydrate and decarboxylate to guanidinohydantoin (Gh), as shown in Figure 3 (*21*). The formation of Gh is further complicated by its isomerization to iminoallantoin (Ia) (*23*). Overall, the two pathways Sp versus Gh/Ia are favored under different conditions: Sp is the exclusive product from nucleoside studies at pH 7, 22°C and is the preferred product in DNA oligomers at higher temperatures or higher pH (Table 1). Conversely, the Gh/Ia pathway is strongly preferred in duplex DNA at pH 7, 22°C. All of the lesions mentioned, with the exception of OG (*24*), are alkali labile under Maxam-Gilbert conditions (1 M piperidine, 90°C, 30 min).

All of the heterocycles in Figure 3 present numerous hydrogen-bonding opportunities for base (mis)pairing. The most mutagenic lesion will be the one whose high frequency of formation, low fidelity during replication, and high error rate from DNA repair activity combine to give the greatest mutation rate. In this sense, all of the lesions in Figure 3 are of interest in order to completely characterize the biochemistry of oxidized lesions, particularly in light of recent suggestions that multiple oxidation events can be clustered in localized regions of DNA (*25*). However, we

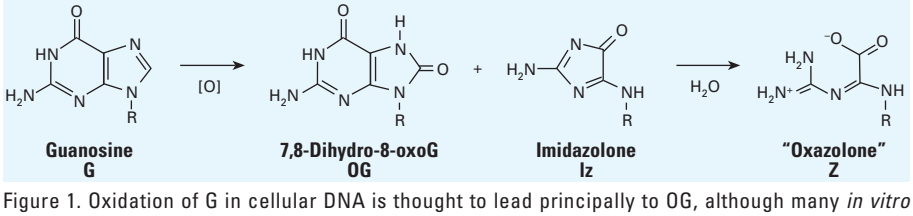

Figure 1. Oxidation of G in cellular DNA is thought to lead principally to OG, although many *in vitro* processes also lead to Iz and Z.

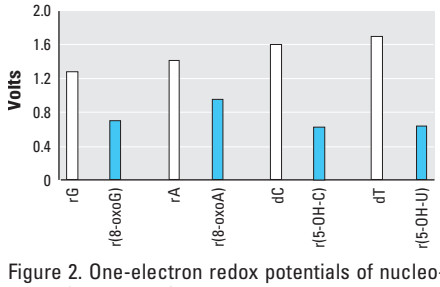

Figure 2. One-electron redox potentials of nucleosides (V vs. NHE) and common oxidized base lesions (*13,14*).

Table 1. Product distribution of OG oxidation by Ir(IV).

OG substrate	pH	Temperature (°C)	Product
Nucleoside	4	22	50% Gh 40% Ia
Nucleoside	7	22	90% Sp
ss-Oligomer	7	4	95% Gh/Ia
ss-Oligomer	7	22	55% Gh/Ia 45% Sp
ss-Oligomer	7	50	95% Sp
ds-Oligomer	7	22	95% Gh/Ia

Abbreviations: ds, double-stranded; ss, single-stranded.

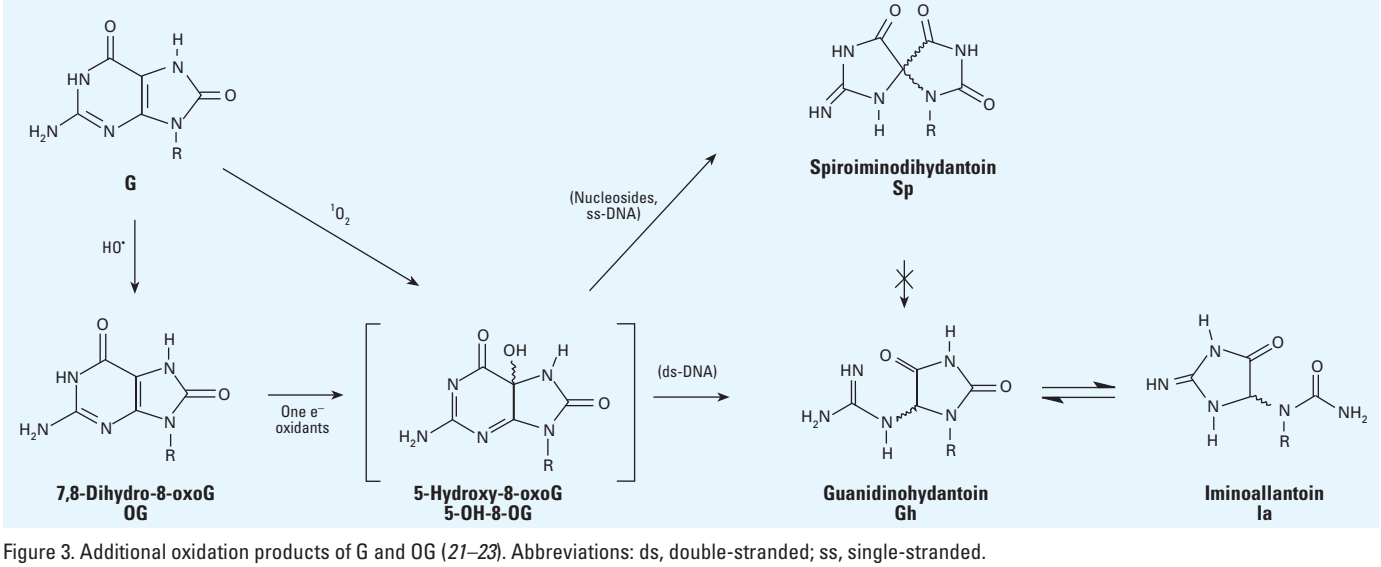

Figure 3. Additional oxidation products of G and OG (*21–23*). Abbreviations: ds, double-stranded; ss, single-stranded.

would argue that Gh and Ia in duplex DNA [and, to a lesser extent, Sp in the 2′-deoxynucleoside 5-triphosphate (dNTP) pool] are of special relevance. This is because of the ability of oxidative damage to accumulate at 8-oxoguanosine over hundreds of angstroms in duplex DNA via one-electron transfer (Equation 1). Although the exact mechanism of long-range electron transfer has been the subject of recent debate, it is now well established that ejection of an electron from the DNA duplex can result in rapid migration of the electron "hole" over tens, and possibly hundreds, of base pairs (*26*). Electron transfer is relevant to both steps of the two-step process for formation of Gh/Ia from G via OG: OG can be formed from G by one-electron transfer in which the radical cation site equilibrates to the most easily oxidized site in the duplex, typically a 5′-GG-3′ or 5′-GGG-3′ sequence (G = preferred oxidation site) (*27*). Then, a second oxidative event can occur anywhere within a few hundred angstroms of the OG site, with OG being the ultimate "electron hole sink" yielding the products Gh + Ia (Figure 4). However, only one-electron mechanisms will result in this equilibration to $OG^{\bullet+}$; other mechanisms such as $^1O_2$ oxidation of G cannot transmit damage over a long distance (*18*).

Thus, Gh + Ia might represent significant lesions in the genome, particularly under conditions of oxidative stress, although they have not yet been identified from cellular extracts. Furthermore, the relatively high concentration of the dNTP pool in the cell suggests that the 2′-deoxyguanine 5′-triphosphate → 2′-deoxy-8-oxo-7,8-dihydroguanine 5′-triphosphate (dOGTP) → 2′-deoxyspiroiminodihydantoin 5′-triphosphate (dSpTP) process may also occur to a substantial extent.

Preparation of Lesions in Oligomers

Before 1996, OG was thought to be an alkali-labile lesion in DNA as a result of depurination and β-elimination under standard Maxam-Gilbert conditions of hot piperidine treatment. However, a careful study by Cullis et al. (*24*) showed that OG was stable in hot piperidine if care was taken to prevent aerobic oxidation. In the presence of O_2, about 5–10% of an OG-containing oligodeoxynucleotide underwent strand scission with hot piperidine. Although the sensitivity of OG to further oxidation had been known (*28*), the species responsible for piperidine-induced scission had not been investigated until very recently (*29*). We found that OG could be selectively oxidized in quantitative yield in an oligomer by selecting a one-electron oxidant (Na_2IrCl_6, commercially available) whose redox potential lies between that of G and OG (*17*). This forms the basis of gel electrophoretic methods for detection of OG and deoxy-8-oxo-7,8-dihydroadenine because the oxidation products are both piperidine-labile lesions and polymerase stops in a primer extension assay under polymerase chain reaction conditions (*17*).

Ir(IV) compounds are convenient, stable, water-soluble complexes for *in vitro* oxidation of DNA. The anionic complexes used have no binding interaction with nucleic acids, and their inorganic mechanism of one-electron, outer-sphere reduction to the corresponding stable Ir(III) compounds has been well studied. Very little competing G oxidation occurs with $IrCl_6^{2-}$, even if easily oxidized GG and GGG sequences are present in the same strand (*18*).

The actual products of one-electron oxidation of OG in both nucleosides and oligomers have been rather complicated to sort out because the product mixture varies as a function of temperature, pH, and base stacking. The best clues came from two related purine pathways: *a*) uric acid oxidation, whose uncatalyzed mechanism has been studied by Poje and co-workers (*30*) [the related enzyme-catalyzed oxidation to form allantoin was studied in depth by Tipton's laboratory (*31*)]; and *b*) the aerobic oxidation of carcinogenic aryl amine adducts at C8 of G studied by Johnson and co-workers (*32*). These electron-rich purines share with OG the propensity to form products derived from opening of the six-membered ring. In all cases, the 5-hydroxypurine is proposed as an intermediate.

The ultimate product distribution is highly dependent on the reaction conditions used (Table 1). In a nucleoside at pH 7 at 22°C, 5-OH–OG isomerizes via acyl migration to the spirocyclic product Sp in essentially quantitative yield. This product was identified by tandem mass spectrometry using electrospray ionization ^1H and ^{13}C nuclear magnetic resonance and by independent synthesis (*21*). A crucial part of the study was analysis of the mass spectrometric fragmentation of the spirocycle formed in $H_2{}^{16}O$ versus $H_2{}^{18}O$, which provides a "fingerprint" of the base lesion and allows distinction of the "oxo" and "imino" halves of the spirocycle because of ^{18}O labeling at C5. dSpTP is expected to be the predominant product of oxidation of dOGTP in the nucleotide pool under physiological conditions.

In contrast, oxidation of OG in the duplex region of a hairpin-forming oligomer by Ir(IV) at pH 7, 22°C led to formation of Gh/Ia as the major products (*23*). It is perhaps not surprising that the pathway leading to a bulky spirocycle is disfavored in duplex DNA. In single-stranded oligos the reaction was highly temperature dependent, leading to Gh/Ia at low temperature (4°C) and Sp at high temperature (50°C). Because these two base-labile lesions can be formed in nearly quantitative yields in a single-stranded oligomer by simply controlling the reaction temperature, we now have a convenient means of synthesis of essentially pure lesions for biochemical studies. Representative mass spectra of lesion-containing oligomers are shown in Figure 5.

Misinsertion Opposite Lesions by Polymerases

To understand the potential for miscoding of the new lesions Sp and Gh/Ia, single-nucleotide primer extension studies were carried out using Klenow exo⁻ and oligodeoxynucleotides containing a lesion "X" in the template strand opposite the next point of insertion of a nucleotide on a 3′ end of the primer strand. A qualitative assessment showed insertion of 2′-deoxyadenosine 5′-monophosphate (dAMP) > 2′-deoxyguanine 5′-monophosphate (dGMP) > > 2′-deoxycytidine 5′-monophosphate (dCMP), 2′-deoxythymidine 5′-monophosphate (dTMP) opposite an oxidized form of OG (*20*). Interestingly, both Gh/Ia and Sp show nearly the same pattern of insertion of nucleotides opposite the lesion, always with an approximately 2:1 preference for dAMP over dGMP. Use of calf thymus pol α or human pol β resulted in no extension of the primer, likely due to the proofreading activity of these enzymes.

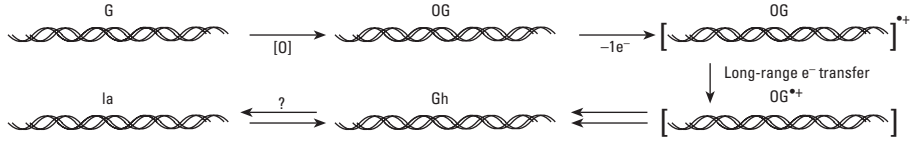

Figure 4. OG as an electron hole sink for oxidative damage in duplex DNA occurring by a one-electron mechanism.

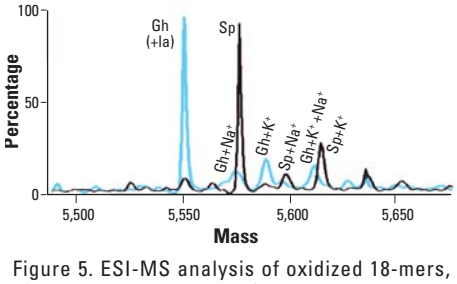

Figure 5. ESI-MS analysis of oxidized 18-mers, d(TCATGGGTC**O**TCGGTATA), where **O** = OG, after oxidation with Ir(IV) at 4°C (blue) and at 50°C (black).

The misinsertion of dAMP and dGMP opposite oxidized guanine lesions is not uncommon. Studies with a wide variety of oxidation conditions ranging from singlet oxygen to various metal ion catalysts with added oxidants indicate that dAMP > dGMP is almost always observed (Table 2). The exception is OG, for which no dGMP insertion is seen, although dAMP insertion is efficient in addition to the correct dCMP insertion. It is interesting that essentially no dCMP insertion occurs opposite the hydantion lesions generated from G and OG oxidation. Thus, in the absence of DNA repair, these G oxidation products are anticipated to be highly mutagenic. Indeed, a recent study indicates that oxazolone, oxaluric acid, and cyanuric acid lesions generated from peroxynitrite oxidation of OG lead to high frequencies of G to T transversions in *Escherichia coli* (*33*).

Additional *in vitro* studies in our laboratory with Klenow exo$^-$ showed that a small amount of full-length extension always occurred for primers annealed to lesion-containing templates. The amount of full extension increased if "running start" experiments were performed in which the polymerase could bind to the primer–template duplex and insert two nucleotides opposite normal bases before encountering the lesion. Current studies also indicate that both the efficiency of nucleotide insertion and the amount of full extension are somewhat sequence dependent. Overall, the hydantoin products of G/OG oxidation inhibit DNA synthesis, but when it does occur, only misinsertion of A > G is observed.

Base Excision Repair of Oxidized Lesions

Although bulky adducts to the DNA bases are typically removed from the genome by the nucleotide excision repair pathway, small adducts, oxidized bases, and ring-opened bases are excised by DNA glycosylases in the base excision repair (BER) pathway (*6*). Some of the best-characterized enzymes are those in *E. coli,* including Fpg (or MutM) and endo III (or Nth), which catalyze deglycosylation of damaged purines and pyrimidines, respectively (*6*). These enzymes appear to work in concert with MutY and endo VIII (also known as Nei), among other cellular repair enzymes, to correct oxidative damage (*5*). For example, OG when paired with C is a good substrate for Fpg, although OG:A pairs are not; instead, OG:A is a better substrate for MutY, which excises the incorrect A opposite OG. After a second round of synthesis, a resulting OG:C pair could then be properly repaired by Fpg.

Our initial experiments surveyed Fpg, MutY, and endo III as possible enzymes to process Sp and Gh/Ia lesions in duplex DNA. MutY is a simple glycosylase enzyme that has been the subject of intense research in recent years, and a crystal structure of a truncated form of the protein with adenine bound was published in 1998 (*34*). This portion of the protein shows high homology to endo III, although endo III has a wide spectrum of substrates, whereas MutY does not appear to. As for endo III, a wide range of substrates have now been characterized for Fpg; most of them share an oxo functionality at a position resembling C8 of a purine.

Preliminary studies in the David lab (*35*) have indicated that Gh:A and Sp:A were not substrates for MutY, nor were Gh and Sp-containing oligomers efficiently cleaved by endo III. In contrast, activity was found with Fpg. Using an 18-bp duplex (10 nM),

5′-d(TCATGGGTC**X**TCGGTATA)-3′
X = OG, Sp or Gh/Ia,

3′d(AGTACCCAG**Y**AGCCATAT)-5′
Y = A, C, G, or T,

in which the X-containing strand was 5′-end labeled, Fpg was found to effect strand cleavage for all X:Y pairs.

The observed rate constants obtained for a series of substrates are shown in Table 3 (*35*). The Sp and Gh/Ia lesions in duplex oligos opposite C, T, and G are all substrates for Fpg, although not to the extent that OG is. An intriguing observation is the 10-fold higher reactivity of Sp:A and Gh:A compared with OG:A, although all of the rate constants are substantially lower than those for the other base pairs studied. This surprising result suggests that polymerase incorporation of A opposite oxidized OG or oxidation of OG in an OG:A pair could be a potentially mutagenic event because Fpg would more readily excise the oxidized OG before MutY could repair the incorrect A. Interestingly, the Sp and Gh/Ia lesions were also found to be substrates for the BER enzyme endo VIII (*36*). In current work, we are attempting to learn more about the structure of duplex oligonucleotides containing the Sp and Gh/Ia lesions in order to fully understand how different structures lead both to misinsertion of nucleotides in the opposite strand and also to repair or misrepair.

Summary and Implications

Increasing evidence shows a direct link connecting DNA damage to mutations and cancer (*37*). DNA repair plays a central role in correcting damage to prevent mutagenesis and carcinogenesis. In the specific area of oxidized base lesions, a causal relationship between cancer and the lack of DNA repair was proposed by the observation that the human gene *hogg1*, which encodes for an oxidized guanine glycosylase, is located in a chromosomal region often deleted in lung cancers (*38*). More recently, it was suggested that defective transcription-coupled repair of oxidative DNA damage was linked with the breast and ovarian susceptibility gene *BRCA1* (*39*) as well as playing a role in Cockayne syndrome (*40*). These examples illustrate the critical role of repair of oxidized DNA damage for prevention of cancer.

Chromosomes in a single cell are estimated to suffer 1,000–10,000 oxidative events per day, and mitochondrial DNA damage has been argued to be at an even higher level (*1*). Much of the focus of DNA damage has centered on OG as the key lesion. Given its role as a central figure in mutagenesis, the chemistry and biology of OG's further oxidation products are also highly relevant, because some of the thousands of OG lesions per cell will likely go on to be further oxidized. The phenomenon of multiple oxidative damage at proximal sites has been discussed since 1981 to explain the clustering of endonuclease S1–sensitive sites in

Table 2. Nucleotide misinsertion by Klenow exo$^-$ fragment opposite oxidized G lesions generated by various metal complexes and oxidation systems.

Substrate	Oxidant added	Nucleotide inserted opposite lesion	Reference
OG	None	A ~ C	(*8*)
G	$^1O_2$	A	(*10*)
OG	$^1O_2$	A > G	(*20,44*)
OG	Na_2IrCl_6	A > G	(*20*)
OG	Ni(II) complexes + $KHSO_5$	A > G	(*20*)
OG	Ni(II) complexes + Na_2SO_3 + O_2	A > G	(*20*)
OG	$CoCl_2$ + $KHSO_5$	A > G	(*20*)
OG	Cr(V) = O complex	A	(*42*)

Table 3. Rate constants at 37°C for single-turnover experiments with Fpg and various central base pairs in an 18-mer duplex substrate (*35*).

Substrate	k_{obs} (min^{-1})
OG:C	> 7
Gh:C	1.4 ± 0.4
Sp:C	1.3 ± 0.2
OG:T	> 6
Gh:T	1.7 ± 0.6
Sp:T	1.6 ± 0.3
OG:G	> 6
Gh:G	1.9 ± 0.2
Sp:G	1.4 ± 0.2
OG:A	0.045 ± 0.007
Gh:A	0.34 ± 0.06
Sp:A	0.34 ± 0.06

DNA exposed to ionizing radiation (*41*). The possibility of electron transfer to concentrate damage at OG further underscores the relevance of Sp and Gh/Ia in understanding the full spectrum of oxidative lesions.

The new oxidative lesions Sp and Gh/Ia as oxidation products of OG and, under some conditions, of G itself have yet to be identified in cellular DNA extracts. Nevertheless, the ubiquity of their formation in the laboratory by multiple mechanisms ranging from singlet oxygen to peroxynitrite to transition metal complexes suggests that they must certainly be present in the genome. Recent *in vitro* studies have also demonstrated formation of Sp and Gh/Ia from chromium(V) (*42*) and HOCl (*43*) oxidation of DNA. These lesions might account for some of the non-G to T mutational spectra observed from oxidative damage to DNA, as well as contributing to a fraction of the G to T mutations because experiments so far indicate that A > G are inserted opposite Sp and Gh/Ia (*44*). In addition, these lesions are providing insight into substrate recognition by the BER family, and specifically about Fpg, because it is extremely interesting that this enzyme can recognize such a wide range of substrates from OG to hydantoins to cyanuric acid. Ultimately, we hope to gain a better molecular view of the processes leading from metal-mediated oxidative damage to cancer.

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