In vitro and in vivo effects of oxidative damage to deoxyguanosine

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Abstract
Biochemical, biophysical and biological studies of oligonucleotides containing lesions at defined sites provide a molecular basis for the effects of DNA lesions. dG (deoxyguanosine) is the most easily oxidized of the four native nucleotides. The chemical reactivity of dG correlates with compilations of mutations, which reveal that a significant fraction of transitions or transversions involve dG. OxodG (7,8-dihydro-8-hydroxy-2′-deoxyguanosine) is widely recognized as an important lesion derived from the oxidation of dG, and significant effort has been expended in studies of its effects on DNA structure and function. Recently, the properties of other lesions derived from dG and/or the oxidation of OxodG have been uncovered. Studies on these lesions reveal that they too are biologically significant.

Introduction
As the carrier of genetic information, maintaining the chemical integrity of DNA is vital to life. DNA is constantly exposed to endogenous and exogenous species that react with the biopolymer and alter its structure [1,2]. A variety of repair mechanisms and alternative polymerases have evolved to guard against DNA modification [3–5]. There are a number of redundancies in these enzyme systems, and in many instances they are conserved throughout species. Despite this sophisticated system of checks and balances, DNA damage has been implicated in aging and in a variety of diseases, such as cancer [6–8]. There is also a strong connection between faulty DNA repair and some genetically based diseases, such as xeroderma pigmentosum [9]. The formation of DNA lesions can result in mutagenesis and carcinogenesis through improper replication of lesion-containing DNA and/or ‘misrepair’ of the damaged biopolymer.

A great deal has been learned about the effects of specific lesions on the structure and function of DNA [10,11]. In vitro studies include biochemical analysis of the lesions’ effects on purified polymerases and repair enzymes, thermodynamic analysis of duplex melting, and atomic-level structural studies (NMR, X-ray). In vivo effects of specific lesions are often analysed utilizing shuttle vector technology in either single-stranded or double-stranded systems [12,13]. All of these in vitro and in vivo experiments are greatly facilitated by the ability to prepare pure oligonucleotides containing a DNA lesion at a defined site. This is often accomplished via chemical synthesis of oligonucleotides using the appropriate phosphoramidites [14–17].

Dozens of DNA lesions have been characterized, but this review focuses on a small number that are derived from oxidative damage to dG (deoxyguanosine). dG is the most readily oxidized of the four native deoxyribonucleotides [18]. Computational studies reveal that the oxidation potential of dG in DNA can be lowered even further, and is affected by local sequence [19]. The relatively facile oxidation of dG and modulation of its oxidation potential due to interactions with neighbouring nucleotides in DNA also plays a role in electron transfer. The ‘holes’ produced upon one-electron oxidation rapidly migrate to and localize at dG sites in DNA [20–23]. The proclivity of dG to oxidation correlates with data that reveal that G→T transitions and G→T transversions are the most commonly observed mutations in oxidatively damaged DNA ([11,24]; see also the p53 website http://p53.Curie.fr). OxodG (7,8-dihydro-8-oxo-2′-dG) is the most well studied lesion derived from dG. However, other dG modifications, including Fapy·dG (formamidopyrimidine), imidazolone, oxaluric acid, oxazolone, cyanuric acid, Gh (guanidinohydantoin), and Sp1 (spiroiminodihydantoin) and Sp2 (Figure 1) have been found to have significant effects on the properties of DNA.

Formation of dG lesions
OxodG results from the formal addition of a hydroxyl radical to the C-8 position of dG (Scheme 1), but it is also formed by other oxidative stress mechanisms (e.g. UV irradiation, singlet O2) [26–28]. The C-8 hydroxyl radical adducts formed initially partition between OxodG and the Fapy·dG lesion, with the former lesion produced in larger amounts in vitro under oxygen-rich conditions [29]. Fapy·dG is formed via a reductive pathway, and is the major product when chromatin is exposed to γ-rayolysis under anoxic conditions [28]. Fapy·dG was also formed in ~3 times greater amounts than OxodG when a human leukaemia cell line was exposed to...
γ-radiolysis [30]. Quantification of DNA lesions, particularly in cellular DNA, is a difficult exercise [1,2]. For instance, different research groups have detected a broad range of levels of OxodG in cells, even from identical tissue samples [31]. Determining the amounts of OxodG is made more difficult by its facile oxidation to ‘secondary oxidation products’, such as Gh, Sp1 and Sp2 [32,33]. In contrast, imidazolone is formed directly by oxidation of dG, as well as being a secondary oxidation product of OxodG [34]. Other dG lesions are formed when the nucleoside is exposed to oxidants such as peroxynitrite or singlet O₂ (¹O₂) [35,36].

**Effects of dG-derived lesions on purified polymerases**

Synthesis is often the greatest stumbling block when studying the effects of DNA lesions [14–17]. Studies on OxodG were greatly facilitated when Johnson and co-workers reported a reliable synthetic method for oligonucleotides containing the lesion more than 10 years ago [37]. Steady-state kinetic experiments using several polymerases revealed significant decreases in enzyme fidelity when the template contained OxodG (Table 1) [38–40]. All of the enzymes experienced a significant increase in misinsertion frequency for dA opposite OxodG. Furthermore, in all instances in which it was measured, the extension of primers containing OxodG:dA pairs was more efficient than with OxodG:dC pairs. In fact, the extension frequency was comparable with that of primers containing dG:dC pairs. The preference for misincorporating dA was rationalized by rotation about the glycosidic bond in OxodG, such that lesion exists in the syn conformation. syn-OxodG presents a hydrogen bonding pattern comparable with that of dT (Figure 2), which results in the observed G → T transversions.

Fewer studies have been carried out on other dG lesions, but these also reduce the fidelity of polymerases. Fapy·dG most closely mirrors the observations made using OxodG [41]. This lesion results in an increase in misincorporation of dA by Klenow exo⁻, albeit to a smaller extent (insertion frequency ~0.05). Klenow exo⁻ also extends a primer containing dA opposite Fapy·dG almost as efficiently as it does when dC is opposite the lesion. The in vitro synthesis
experiments using Klenow exo$^{-}$ indicated that Fapy·dG is $\sim$8 $\times$ 10$^{3}$-fold more likely to induce the enzyme to misinsert dA opposite itself than is dG. Miscoding for dA can be rationalized when the lesion occupies the syn conformation (Figure 3). Two rotamers of the formamide group enable syn-Fapy·dG to present thymidine-like hydrogen-bonding patterns. The secondary oxidation products Gh and Sps induce Klenow exo$^{-}$ to misinsert dG and dA to the exclusion of dC [42]. Imidazolone induces Klenow exo$^{-}$ to exclusively misincorporate dG [43]. Furthermore, imidazolone was selectively incorporated opposite dG during extension of a primer–template complex by Klenow exo$^{-}$ when deoxyimidazolone triphosphate was present.

**Correlations between the effects of lesions on polymerase fidelity and the thermodynamics of duplex melting**

Thorough studies have been carried out using duplexes containing OxodG opposite various nucleotides. The thermodynamics of duplex melting were determined using calorimetry and UV melting temperatures [44]. The $\Delta$G for melting of a duplex containing an OxodG:dC base pair was lower than that for an otherwise identical duplex containing dG opposite dC, indicating that duplexes containing the lesion are destabilized compared with those containing native nucleotides. However, a duplex containing OxodG:dA base pairs was more stable by approx. 10.4 kJ/mol (2.5 kcal/mol) than one containing a dG:dA base pair. These experiments support the proposal that OxodG forms hydrogen bonds to dA when it is in the syn conformation. Finally, this base-pairing scheme has been verified by X-ray crystallography [45].

van’t Hoff plots of dodecamers containing Fapy·dG also showed significant stabilization of the lesion when it is opposite dA, which is the nucleotide most often misincorporated opposite the lesion by Klenow exo$^{-}$ [41]. In contrast with OxodG duplexes, the $\Delta$G for melting of DNA containing Fapy·dG:dA base pairs was within the experimental error of the value for an otherwise identical duplex containing Fapy·dG:dC. Similar trends are observed for duplexes containing Gh and Sp [42]. Overall, DNA containing the lesions is destabilized thermally relative to that containing native nucleotides opposite the appropriate partner. However, duplexes containing the lesions opposite dA and/or dG are typically more stable than those with lesions opposite dC.

**Effects of dG lesions on base-excision repair enzymes**

The family of base-excision repair enzymes that repair oxidatively damaged dG nucleotides in *Escherichia coli* are referred to as the ‘GO’ (guanine oxidase) enzymes. Homologues of a number of these enzymes exist in eukaryotes, but additional enzymes that excise modified guanosines have been discovered in these organisms [5]. The GO family of enzyme consists of Fapy·dG DNA glycosylase (Fpg, MutM), the deoxyadenosine mismatch repair protein (MutY), and MutT. More recently a fourth enzyme, Endo VIII (endonuclease VIII), has been included in the family [46]. Fpg is a bifunctional enzyme that excises modified purines. The enzyme cleaves the glycosidic bond of the lesion and carries out a $\beta,\delta$-elimination of the resulting abasic site, leaving behind gapped DNA containing 5'- and 3'- phosphates. Repair is then completed by a polymerase and a ligase. It is important that Fpg (and any glycosylase) discriminates between different base pairs. For instance, Fpg selectively excises OxodG almost 20 times more efficiently when the lesion is opposite dC than when it is part of a promutagenic base pair with dA [47]. Such discrimination by base-excision repair enzymes is required for preventing misrepair, because excision of a lesion from a promutagenic base pair (e.g. OxodG:dA) leaves behind the incorrect nucleotide as the only source of sequence information for the repair polymerase responsible for filling in the gap. Instead, OxodG:dA base pairs are repaired preferentially by MutY, which hydrolyses the glycosidic bond of dA [48]. It has been proposed that MutT protects the nucleotide triphosphate pool by hydrolysing the triphosphate of OxodG. Although MutT selectively hydrolysates OxodGTP to its monophosphate in the presence of dGTP ($\sim$200 times more efficiently) *in vitro*, the biological relevance of this finding was recently questioned [49,50].

Although it is not known if Fapy·dGTP is a substrate for MutT, the lesion’s interactions with Fpg and MutY are remarkably similar with those of OxodG [51]. Fpg excises Fapy·dG from DNA with comparable efficiency to OxodG removal, and it discriminates against Fapy·dG:dA compared with Fapy·dG:dC base pairs by approx. 17-fold.

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**Figure 3 | Structure of the syn-Fapy·dG:dA base pair**

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Furthermore, Fapy·dG:dA base pairs are repaired by MutY. The adenine is removed faster than when it is opposite dG, but 3–4 times slower than when it is opposite OxodG.

Fpg exhibits less selectivity with respect to the nucleotide opposite the secondary oxidation products Sp and Gh than is observed when OxodG or Fapy·dG is excised [52]. Single-turnover experiments indicate that Fpg excises Sp or Gh approx. 4-fold more efficiently when they are paired with dC compared with dA. One cannot discount the possibility that the greater selectivity of Fpg for DNA containing OxodG and Fapy·dG opposite different nucleotides compared with these secondary oxidation products is due to differences in the experimental methods employed.

The newest member of the GO family is Endo VIII. [46] Unlike Fpg, Endo VIII excises OxodG when it is opposite dG or dA more rapidly than when the lesion is paired with dC [53,54]. However, the selectivity is much less and excision is slower overall than that exhibited by Fpg. It has been proposed that Endo VIII excises OxodG that is misincorporated via its nucleotide triphosphate during replication [46]. The activity of Endo VIII against other dG-derived lesions has not yet been reported.

**Effects of dG lesions in vivo**

The cytotoxicity and mutagenicity of DNA lesions are typically determined using shuttle vectors [12,13]. Shuttle vector experiments are routine provided that oligonucleotides containing the lesion of interest are obtainable. Given this requirement, it is not surprising that OxodG has been studied by several research groups in different cell lines. OxodG shows low levels of toxicity to *E. coli* and gives rise to modest levels of G→T transversions [10,55]. The exact levels of mutation are dependent on the cell line, and in some systems lower levels of G→C transversions and G→A transitions have been detected.

Recently, results of shuttle vector experiments with the OxodG oxidation products oxaluric acid, cyanuric acid, oxazolone, Gh, Sp1, and Sp2 in *E. coli* have been reported [35,56]. The experiments were carried out using the REAP (restriction endonuclease and post-labelling) assay developed by Essigmann and co-workers [12], which enables one to measure mutation frequencies using TLC analysis of radiolabelled nucleotides. Gh resulted in an extremely high rate of G→C transversions (98 ± 0.2%). In a side-by-side analysis, OxodG gave rise to ~3% G→T transversions. The Sps are also highly mutagenic. Each isomer (Sp1 and Sp2) gave rise to extremely high (>95%) frequencies of mutations, which were divided between G→C (major) and G→T transversions. However, Sp1 and Sp2 are considerably more lethal to the bacteria, giving rise to survival rates that were only marginally higher than that with an abasic site. The peroxynitrite-induced (secondary) oxidation products oxaluric acid, oxazolone and cyanuric acid also gave rise to much higher frequencies of G→T transversions than did their precursor OxodG [35]. In a side-by-side REAP experiment, oxaluric acid, cyanuric acid and oxazolone gave rise to 99%, 97% and 86% G→T transversions respectively, compared with 6.8% with OxodG. The secondary oxidation products were all more lethal than OxodG, but significantly less so than a model abasic site. *In vivo* data have not yet been published regarding the lethality and/or mutagenicity of Fapy·dG or imidazolone, but it is worth noting that, thus far, effects *in vivo* have correlated with *in vitro* effects on purified polymerses.

**Conclusions**

The proclivity of dG to oxidation gives rise to several modified nucleotides in DNA in addition to OxodG. OxodG is the most well studied DNA lesion, and results in modest levels of mutation in various cell lines. Studies on recently discovered lesions (e.g. Gh) and/or those whose study was not possible previously due to synthetic limitations (e.g. Fapy·dG) indicate that these other lesions may also exert significant effects on cells. The combination of organic chemistry, biochemistry and molecular biology creates a powerful triumvirate for understanding the molecular basis of the biological effects of DNA damage.

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**References**

25 Reference deleted
31 ESCODD (European Standards Committee on Oxidative DNA Damage) (2002) Carcinogenesis 23, 2129–2133

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