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When DNA Repair Goes Wrong: BER-Generated DNA-protein Crosslinks to Oxidative Lesions

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Abstract

Free radicals generate an array of DNA lesions affecting all parts of the molecule. The damage to deoxyribose receives less attention than base damage, even though the former accounts for ~20% of the total. Oxidative deoxyribose fragments (e.g., 3'-phosphoglycolate esters) are removed by the Ape1 AP endonuclease and other enzymes in mammalian cells to enable DNA repair synthesis. Oxidized abasic sites are initially incised by Ape1, thus recruiting these lesions into base excision repair (BER) pathways. Lesions such as 2-deoxypentose-4-ulose can be removed by conventional (single-nucleotide) BER, which proceeds through a covalent Schiff base intermediate with DNA polymerase β (Pol β) that is resolved by hydrolysis. In contrast, the lesion 2-deoxyribonolactone (dL) must be processed by multinucleotide ("long-patch") BER: attempted repair via the single-nucleotide pathway leads to a dead-end, covalent complex with Pol β crosslinked to the DNA by an amide bond. We recently detected these stable DNA-protein crosslinks (DPC) between Pol β and dL in intact cells. The features of the DPC formation *in vivo* are exactly in keeping with the mechanistic properties seen *in vitro*: Pol β -DPC are formed by oxidative agents in line with their ability to form the dL lesion; they are not formed by non-oxidative agents; DPC formation absolutely requires the active-site lysine-72 that attacks the 5'-deoxyribose; and DPC formation depends on Ape1 to incise the dL lesion first. The Pol β -DPC are rapidly processed *in vivo*, the signal disappearing with a half-life of 15–30 min in both mouse and human cells. This removal is blocked by inhibiting the proteasome, which leads to the accumulation of ubiquitin associated with the Pol β -DPC. While other proteins (e.g., topoisomerases) also form DPC under these conditions, 60–70% of the trapped ubiquitin depends on Pol β . The mechanism of ubiquitin targeting to Pol β -DPC, the subsequent processing of the expected 5'-peptidyl-dL, and the biological consequences of unrepaired DPC are important to assess. Many other lyase enzymes that attack dL can also be trapped in DPC, so the processing mechanisms may apply quite broadly.

Keywords

Base excision DNA repair; DNA polymerase β ; AP lyase; oxidative DNA damage; 2-deoxyribonolactone; DNA-protein crosslinks

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Introduction

The genome is under continual threat by DNA damage from a variety of endogenous and environmental sources. The most frequent endogenous DNA lesions are chemical alterations to the nitrogenous bases that include products of oxidation, alkylation and deamination reactions (1). Many types of base damage destabilize the *N*-glycosylic bond, leading to the formation of abasic (AP) sites, and these add to the estimated 10,000 AP sites per day in each human cell arising from the hydrolytic loss of undamaged purine bases (2–4). Many of the hydrolytic, oxidative or alkylation base lesions cause minimal structural effects on DNA, so they escape effective processing by nucleotide excision repair (*see article by Sugawara in this issue*). Instead, the mutagenic and genotoxic threat of these lesions is offset by mechanisms such as the base excision DNA repair (BER) system (5, 6) (Fig. 1).

The BER pathway can be initiated by any of the eleven DNA glycosylases in mammalian cells (7). Each glycosylase detects a specific range of base lesions and hydrolyzes the *N*-glycosylic bond between the base and DNA deoxyribose, forming an AP site (Fig. 1, **step 1**). These AP sites and those generated by spontaneous purine hydrolysis are then incised by an AP endonuclease, with the main activity in mammalian cells residing in the Ape1 protein (8, 9). Ape1 hydrolyzes the phosphodiester on the immediate 5' side of the AP site to generate a single-strand break bracketed by 3'-OH and 5'-deoxyribose-5-phosphate (5'-dRp) residues (Fig. 1, **step 1A, B**). In "classical" BER, only the missing nucleotide is replaced by DNA polymerase β (Pol β) (Fig. 1, **step 2A**), and the 5'-dRp residue is excised by a 5'-dRp lyase activity in a separate domain of Pol β (Fig. 1, **step 3A**) (10–12). The result is a nicked DNA that is competent for ligation, usually by DNA ligase III α (Fig. 1, **step 4A**).

Another sub-pathway called long-patch BER (LP-BER) also exists, in which DNA repair synthesis replaces 2–10 nucleotides, Fen1 ("flap") endonuclease excises the displaced strand (Fig. 1, **3B**), and DNA ligase I seals the nick (13–15) (Fig. 1, **step 4B**). LP-BER repair synthesis can be initiated by Pol β , which may (inefficiently) insert a second nucleotide. When the 5'-dRp strand is displaced by two or more nucleotides, the 5'-dRp lyase of Pol β no longer functions, requiring Fen1 to remove the single-stranded flap. There is evidence that DNA polymerase δ and DNA polymerase ϵ can participate in LP-BER in a PCNA-dependent fashion, but the real contributions of the different DNA polymerases have not been established *in vivo* (16–19). When LP-BER was discovered (20–22), its role was unclear, with speculation about unknown modified 5' termini possibly requiring LP-BER. A clear case came for the major oxidative lesion 2-deoxyribonolactone (dL) (Fig. 1, **step 2B**), which cannot be repaired by classical single-nucleotide BER (14, 23).

1. The Formation of BER-Mediated DNA-protein Crosslinks

The formation of oxidative Pol β -DPC *in vitro* and *in vivo*

Free radicals, such as the by-products of aerobic metabolism, damage all the components of DNA including the deoxyribose (24). The latter products include strand breaks with deoxyribose fragments attached to the termini, and AP sites with oxidized sugars. An important example is dL (24), which almost 50 years ago was the first chemically defined

lesion of H₂O₂ damage (25). Other oxidative agents, such as X-rays, also form dL as one of many products (26–28), while certain agents can generate the lesion more selectively. These include some genotoxic compounds that attack C1' of deoxyribose specifically, such as neocarzinostatin (NCS) (28–30), tirapazamine (TPZ) (31–33) and the organometallic 'chemical nuclease' 1,10-copper-*o*-phenanthroline [(Cu(OP)₂] (34–37). However, the chemical instability of dL and other features prevented a systematic study of its processing for decades. Finally, three labs report generally similar methods for generating dL site-specifically in synthetic oligodeoxynucleotides (38–40) which allowed a more detailed understanding of its effects *in vitro* (41–45). (23, 46, 47).

In vitro reactions with dL-oligonucleotide substrates showed that Ape1 cleaved the lesions about as well as it does a regular AP site (37). Subsequent BER steps were stymied, however, and molecular analysis showed that Polβ had been captured in a stable, covalent complex with the DNA (23). This study revealed a form of enzyme mechanism-based cross-linking, dependent on the lyase active-site residue lysine-72 (K72) (Fig. 1, **step 2C**, and Fig. 3A). In mouse embryonic fibroblast (MEF) extracts incubated with dL-DNA, Polβ-DPC were the major species detectable, dependent on the *POLB* gene (14, 48, 49). Around the same time, it was reported that *E. coli* endonuclease III can be trapped at (uncleaved) dL sites via that enzyme's lyase activity (46). We later showed that mitochondrial DNA polymerase γ (Polγ), which harbors a 5'-dRp lyase similar to that of Polβ, is also trapped by 5'-dL (15). In contrast to enzymatic AP lyases, the cross-linking of lysine-rich proteins such as histones to dL-DNA occurs much less efficiently, at a rate ~100-fold lower than that of Polβ (47). Thus, dL in DNA constitutes a specific threat to the various lyases that act on DNA abasic sites, especially significant because dL would be generated *in vivo* at a rate similar to that for 8-oxoguanine (28).

The finding that dL traps DNA repair lyases in DPC suggested that cells ought to have mechanisms to avoid this hazard. Consistent with this hypothesis, we showed that dL residues incubated with cell-free extracts are processed exclusively through an LP-BER mechanism that is dependent on the activity of Fen1 (14) (Fig. 1, **step 2B**), while the Polβ-DPC product itself is (not surprisingly) resistant to removal by LP-BER (14) (Fig. 1, **step 2C**). Consequently, a first line of defense against the genotoxicity of dL in DNA is its channeling into LP-BER, which highlights the importance of understanding the mechanism that governs this channeling. Conversely, these observations raise the question of whether imbalances in LP-BER protein expression, or conditions of oxidative stress generating large numbers of dL residues, could cause the formation of Polβ-DPC *in vivo*.

To address the above issue, we adapted an immunoslot-blot approach that detects topoisomerase DPC in cells treated with agents such as camptothecin and etoposide (50, 51). This procedure worked very well for detecting and quantifying oxidative Polβ-DPC in human and mouse cells challenged with oxidants, especially those that generate dL preferentially (52). Such dL-inducing oxidants include TPZ, which damages DNA selectively under hypoxia, and the compound Cu(OP)₂, both generating significant levels of Polβ-DPC in a dose-dependent manner (52) (Fig. 2, **step 1**). Most importantly, the Polβ-DPC formation *in vivo* exhibited the mechanistic characteristics established *in vitro*: the DPC were not generated by non-oxidative agents such as methyl methane sulfonate; they

required the activity of the Ape1 AP endonuclease (Fig. 1, **step 1B**); and they depended completely on the Pol β lyase active-site residue, lysine-72 (52).

The repair and biological impact of oxidative Pol β -DPC

An attempt to identify a possible repair mechanism for Pol β -dL-DPC used HeLa whole-cell extracts competent for nucleotide excision repair (NER), which did not reveal any processing via NER (48). The authors suggested that the lack of NER repair was due to the presence of a pre-existing strand break at the DPC site (48). We approached this issue using the *in vivo* DPC assay, which revealed a role for the proteasome in the removing this lesion. Detectable oxidative Pol β -DPC were rapidly cleared from the genome of human and mouse cells, with half-times of 15–20 min (Fig. 2, **step 3A**). Treatment of cells containing Pol β -DPC with the proteasome inhibitor MG132 completely prevented this rapid removal, demonstrating proteolysis as a critical early step (52) (Fig. 2, **step 3B**). Involvement of the proteasome suggested that Pol β in oxidative DPC is targeted by ubiquitylation (Fig. 2, **step 2**), and indeed, a strong ubiquitin signal is found in the DPC of cells co-treated with Cu(OP) $_2$ and MG132 (52) (Fig. 2, **step 3B**). In fact, Pol β -DPC seem to account for most (60–70%) of the ubiquitin trapped in oxidative DPC (52), which is very striking in that Pol β is a low-abundance protein in most cell types (53). Identifying the specific lysine residues ubiquitylated in oxidative Pol β -DPC is an important task ahead. Additionally, investigating the enzymes responsible for this targeting (i.e., E3 ligases, Fig. 2, **step 2**) and the downstream repair processes that repair the adducted site (specific nucleases, direct reversal, or general repair pathways; Fig. 2, **step 4A**) will also be critical for understanding how cells cope with this class of DPC (Fig. 2, **step 5A**).

The accumulation of unrepaired oxidative Pol β -DPC is cytotoxic (Fig. 2, **steps 4B and 5B**). We compared the survival of cells expressing wild-type Pol β to those expressing the K72A (lyase-deficient) form after a combined treatment with Cu(OP) $_2$ and MG132, which demonstrated that cells with the lyase-deficient protein had substantially *higher* survival than those with WT-Pol β (52). Since other proteins (e.g., topoisomerases) are also covalently trapped on DNA in cells treated with Cu(OP) $_2$ and MG132, this result underscores the specific biological impact of the Pol β -DPC. The cytotoxic effects may reflect not only the effect of the accumulated DNA lesion (Pol β -DPC), but also the depletion of a critical repair enzyme. Future studies will seek to uncover the mechanism of cytotoxicity-induced by this process in order to understand how this class of DPC contributes to cell death (Fig. 2, **step 5B**).

2. Additional BER Related DNA-protein Cross-links

DPC formation with dL and its β -elimination product by BER-associated lyases

Other enzymes that possess either 5'-dRp lyase or AP lyase activity have also been shown to form DPC *in vitro* with both dL and with its β -elimination product, butenolide (54). DNA glycosylases arrive at the site of base damage and initiate BER by removing base lesions; the bifunctional subclass of glycosylases may cleave the resulting AP site through their intrinsic lyase activities (55, 56). *In vitro* studies with dL-containing oligonucleotide substrates revealed initially that *E. coli* endonuclease III is trapped in DPC with the lesion without

effecting cleavage of the DNA strand (46). Reactions of dL with *E. coli* Fpg or endonuclease VIII, yeast OGG1 or NTG2 (an endonuclease III homolog), and human OGG1 were all much less efficient than seen for *E. coli* endonuclease III (54). However, the AP lyases that can effect δ -elimination of AP sites (*E. coli* Fpg, NEIL1 and endonuclease VIII) are all robustly trapped by butenolide (54). Other *in vitro* studies indicate that (human) Nth1 and OGG1 form DPC when incubated with dL-DNA, while Fpg-DPC with dL were not detected (56). Together, these studies suggest that dL and its degradation product butenolide are capable of trapping enzymes with AP lyase activity *in vitro*, although their contribution *in vivo* has not been established.

Oxanine-DPC formation with BER enzymes *in vitro*

Certain base lesions can also instigate DPC formation with various BER enzymes and other proteins *in vitro*. Oxanine (Oxa) is a significant product of guanine nitrosation by nitric oxide, nitrous acid or *N*-nitrosoindoles and can form diverse types of DPC (57, 58). Reaction of Oxa-containing DNA duplexes (Oxa-DNA) with proteins containing nucleophilic lysine or arginine residues produces DPC for both types of linkage. This reactivity appears to be due to the aromatic lactone formed by Oxa, which would form a stable amide bond between lysine and the carbonyl derived from the guanine C1 (57, 58) (Fig. 3B). Oxa-DPC form not only with lyase-active enzymes, but also with histones and high mobility group (HMG) proteins (57). Similar to what has been observed for dL, trapping of Fpg, hOGG1 and endonuclease VIII was also observed for Oxa-DNA *in vitro*, but it is worth noting that the lyase-inactive glycosylase *E. coli* AlkA was also trapped, and at a rate only a few fold lower than seen for the lyases (54). Although Oxa has the potential to be a significant suicide substrate for DNA repair enzymes, the possible physiological impact of Oxa-DPC remains to be determined.

DPC formation with 5-hydroxy-5-methylhydantoin in DNA

The thymine oxidation product 5-hydroxy-5-methylhydantoin acts as a suicide substrate, forming DPC with *E. coli* Fpg and endonuclease VIII, and with human NEIL1 (59). The crystal structure of a stabilized form of an Fpg-hydantoin DPC revealed cross-linking between the Fpg N-terminal proline (that enzyme's lyase nucleophile) and the C5 position of the hydantoin (Fig. 3C). As for Oxa, the biological as opposed to *in vitro* significance of these DPC needs to be addressed.

Topoisomerase-DPC formation in DNA with BER lesions *in vitro* and *in vivo*

DNA topoisomerases are capable of being trapped as the covalent DNA-protein intermediates that are part of their normal catalytic cycle, so-called topoisomerase cleavage complexes [(reviewed in (60))]. Under physiological conditions, topoisomerases break and rapidly rejoin DNA strands to relieve topological strain. Eukaryotic topoisomerase I (Top1) accomplishes this by nicking one DNA strand with the formation of a tyrosyl ester to the 3'-phosphate, followed by rejoining the DNA termini and releasing the enzyme. Topoisomerase 2 (Top2) carries out analogous reactions involving both strands, forming tyrosyl esters (one per subunit of the homodimer) with the 5'-phosphoryl termini, which are reclosed to release the enzyme. Normally, the Top1- and Top2-DPC intermediates are quickly resolved, but they

can become trapped in various ways and are associated with substantial cytotoxicity (Fig. 3D, E).

Reflecting this cytotoxicity, the well-known topoisomerase poisons camptothecin (against Top1) and etoposide (against Top2) are also widely used anticancer agents. However, DNA damage in general can also disrupt the catalytic cycle of topoisomerases, evidently through their effects on DNA structure, rather than the chemistry of the topoisomerases *per se*. Lesions such as 8-oxoguanine, AP sites, single-strand breaks and uracil residues, when placed suitably close to a topoisomerase cleavage site, lead to DPC formation *in vitro* (61–63). These effects are also reflected *in vivo* when cells are exposed to agents that generate other DNA lesions. For example, mammalian cells lacking the *O*⁶-alkylguanine-DNA alkyltransferase, the enzyme responsible for removing alkyl groups from the 6-position of guanines, are sensitive to treatment with the alkylating agent, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), and accumulate more Top1-DPC than wild-type cells (64). Furthermore, yeast cells overexpressing human Top1 display hypersensitivity to MNNG exposure, whereas knocking out Top1 confers resistance to the effects MNNG treatment (64). BER agents such as temozolomide, a clinically relevant methylating drug that forms *O*⁶-methylguanine, 7-methylguanine, and 3-methyladenine DNA adducts also induces robust Top2-DPC formation in cells (65).

Consistent with these broad effects, both yeast and mouse cells challenged with the oxidant H₂O₂ display markedly increased levels of Top1-DPC (66). In accord with these data, we found that Cu(OP)₂ also induces Top1 and Top2-DPC, which can accumulate to a similar extent to that observed in camptothecin- or etoposide-treated cells (52). In this case, there could be additional effects of the *o*-phenanthroline ligands of Cu(OP)₂, as previously suggested by molecular simulation studies (67). Whatever the case, the contribution of these abundant proteins to the ubiquitin trapped in oxidative DPC is still less than that found for the less abundant Polβ protein.

3. Closing Remarks and Future Prospects

Although the details of oxidative Polβ-DPC formation and clearance *in vivo* are coming into focus, much information is lacking regarding the trapping of other DNA repair enzymes involved in the BER process. DPC formation with BER repair factors such as the bifunctional DNA glycosylases has been extensively studied *in vitro*, but much work remains to be done to understand the formation and fate of glycosylase-DPC involving dL, Oxa and 5-hydroxy-5-methylhydantoin in the genome. The *in vivo* formation of oxidative DPC by other DNA repair enzymes that harbor 5'-dRp or AP lyase activity such as DNA polymerase λ (68), DNA polymerase γ (69), DNA polymerases of the Y-family (κ, ι, and η) (70) Ku antigen (71, 72) and PARP1 (73, 74) have also been demonstrated *in vivo* from work in our lab (unpublished data), but the mechanism(s) by which they are cleared from cells has yet to be established. Since DNA repair enzymes with AP lyase activity are required for cell survival and are often aberrantly expressed in neoplastic tissue (75), oxidative trapping of these enzymes may prove to be an effective novel anticancer strategy in the future. Thus, identifying key protein targets involved in oxidative DPC formation, and

the mechanisms responsible for their potential removal *in vivo*, should open up new and significant avenues of research in this regard.

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Abbreviations

Cu(OP)₂	1,10 copper- α -phenanthroline
dL	2-deoxyribonolactone
5'-dRp	5'-deoxyribose-5-phosphate
AP	apurinic/apyrimidinic (abasic)
Polβ	DNA polymerase β
DPC	DNA-protein crosslink
MEF	mouse embryonic fibroblast
Oxa	oxanine
TPZ	tirapazamine
Top1	topoisomerase 1
Top2	topoisomerase 2

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Figure 1. BER processing of oxidative DNA damage and the formation of DPC

Following base damage and glycosylase processing, or the oxidation of DNA deoxyribose, the resulting abasic sites are cleaved by Ape1 (1A, B). The incised AP sites are then channeled through single-nucleotide (“short-patch”) or multinucleotide (“long-patch”) BER. In the former, following incision of the AP site, Polβ inserts a single-nucleotide to replace the damaged nucleotide (2A) and removes the 5'-dRp residue generated by Ape1 (3A). Synthesis of 2 nucleotides in multinucleotide BER generates a flap that prevents 5'-dRp excision and requires activities such as Fen1 (3B). Multinucleotide BER processes dL lesions effectively (2B), but in some circumstances attempted 5'-dL excision results in the formation of Polβ-DPC (2C). Short-patch BER is completed by DNA Ligase IIIα (4A) and in the case of long-patch BER, repair is completed by DNA Ligase I (4B) restoring the DNA backbone to its native condition.

Figure 2. The Fate and Impact of Oxidative Polβ-DPC *in vivo*

Exposure of cells to Cu(OP)₂ results in robust Polβ-DPC formation (1). Polβ-DPC are subsequently targeted for ubiquitylation (2) and rapid proteolysis, which is expected to generate 5'-peptidyl-dL-DNA adducts (3A) that would be removed from the genome by DNA repair mechanisms being investigated (4A). Together, such repair processes would allow cells to escape the toxic effects of oxidative Polβ-DPC accumulation (5A). However, inhibition of the proteasome prevents removal of Polβ-DPC (3B), leading to their toxic accumulation (4B) with cell killing and perhaps other consequences (5B). Biological and mechanistic questions requiring further definition are highlighted in *brown*.

Figure 3. Representative Structures of Some Mechanism-based DPC

Schematic depiction of the bonding chemistry of DPC due to DNA lesions or enzyme-inhibiting drugs (see main text). The structures are: Polβ-dL-DPC (A), hOGG1-Oxa-DPC (B), Fpg-cHyd-DPC (C), Top2-DPC (D), and Top1-DPC (E). The linking amino acid residues for Polβ, hOGG1 and Fpg are indicated using single-letter code (K = lysine; P = proline).