Title
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Summary

Exocyclic DNA adducts are mutagenic lesions that can be formed by both exogenous and endogenous mutagens/carcinogens. These adducts are structurally analogs but can differ in certain features such as ring size, conjugation, planarity and substitution. Although the information on the biological role of the repair activities for these adducts is largely unknown, considerable progress has been made on their reaction mechanisms, substrate specificities and kinetic properties that are affected by adduct structures. At least four different mechanisms appear to have evolved for the removal of specific exocyclic adducts. These include base excision repair, nucleotide excision repair, mismatch repair, and AP endonuclease-mediated repair. This overview highlights the recent progress in such areas with emphasis on structure–activity relationships. It is also apparent that more information is needed for a better understanding of the biological and structural implications of exocyclic adducts and their repair. ***BioEssays*** **26:1–14, 2004.**

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Abbreviations: ε, etheno; E, ethano; Hm, hydroxymethyl; HO, hydroxyl; PdG, 1,N^2-propanoguanine; M_1G, pyrimido[1,2-α]purin-10(3*H*)-one; 3mA, 3-methyladenine; 5mC, 5-methylcytosine; O^6mG, O^6-methylguanine; 8-oxoG, 7,8-dihydro-8-oxoguanine; Hx, hypoxanthine; AP, apurinic/apyrimidinic; VC, vinyl chloride; CAA, chloroacetaldehyde; CNU, chloroethylnitrosourea; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; 4-HNE, 4-hydroxynonenal; *p*BQ, parabenzoquinone; Mug, mismatch-specific uracil-DNA glycosylase; TDG, thymine-DNA glycosylase; AlkA, *E. coli* 3-methyladenine DNA glycosylase II; ANPG, alkyl-*N*-purine DNA glycosylase; Ung, uracil-DNA glycosylase; APE1, major human AP endonuclease; BER, base excision repair; NER, nucleotide excision repair; MMR, mismatch repair.

Introduction

Exocyclic DNA adducts are a unique class of ring-extended modifications formed by a wild range of chemicals,[1] most of which have been classified as animal and/or human carcinogens.[2,3] Examples are vinyl halides, benzene, nitrosoureas, and α,β-unsaturated aldehydes. Since the initial identification of exocyclic adducts in 1960s,[4–6] numerous studies have been reported on the identification, chemistry and biology of various exocyclic adducts originating from both environmental and industrial sources.[1] Lately, attention has also focused on the formation of such adducts by endogenous metabolic processes (Fig. 1).[1,7,8] Many exocyclic adducts have now been identified in genomic DNA of cells exposed to carcinogens or in DNA of our "healthy" cells, largely owing to the development of ultrasensitive detection methods.[1] Therefore, an understanding of the biological impact of these adducts is of great importance in elucidating etiological mechanisms of both chemical and spontaneous tumorigenesis.

Exocyclic ring derivatives are formed by bifunctional electrophilic compounds that attack at one site of the base moiety followed by ring closure at the other site. The common sites for forming an exocyclic ring are *N*-1 and N^6 of dA, *N*-3 and N^4 of dC, *N*-1 and N^2 of dG, as well as N^2 and *N*-3 of dG.[1,9] Structurally, these adducts are analogous but can differ in ring structure such as size (e.g. 5- versus 6-membered), number (e.g. one ring versus two rings), saturation (e.g. etheno versus ethano), angularity (e.g. linear versus angular), and substituents' nature (e.g. –OH versus -CH_2OH) and location (e.g. 6-HO-PdG versus 8-HO-PdG). Certain exocyclic adducts are also present in stereoisomers.

The immediate consequences of these adducts on replication, if unrepaired, are anticipated to be polymerase blockage, base substituions or frameshift deletions since the exocyclic ring(s) disrupts Watson-Crick hydrogen bonding. Indeed, when examined in various systems, all the known exocyclic adducts are mutagenic, albeit with varying efficiencies.[1,10] There is also evidence that exocyclic adducts may be responsible for specific mutations in certain cancer genes such as *ras* and *p53*[11,12] and that some adducts can be formed preferentially at certain mutational hotspots in such a gene.[13] Although the role of each of the known exocyclic adducts in causing apoptosis, mutagenic and carcinogenic effects has not been clearly understood, their occurrence and

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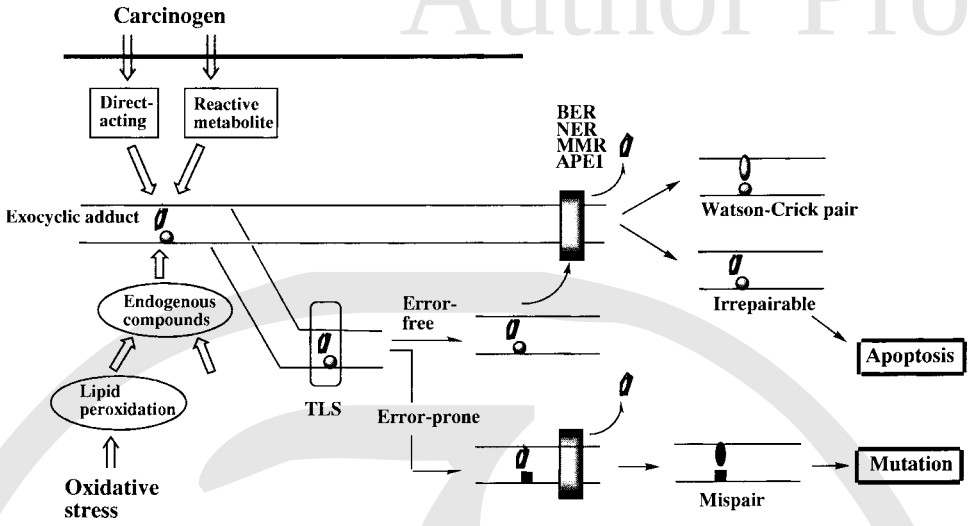

Figure 1. Schematic presentation of the formation, repair and mutagenic potential of exocyclic adducts in genomic DNA. Note that TLS (translesion DNA synthesis) occurs if the adduct is not repaired promptly. TLS itself can prevent a mutation by incorporation of a correct nucleotide opposite the adduct. Error-prone incorporation at the adduct site is the source for mutation, even though the adduct still can be removed by repair mechanisms. A base–base mismatch from such error-prone synthesis may further be corrected by MMR or mismatch-specific glycosylases from BER. Only those adducts that finally escape all the defense mechanisms may lead to biologically important end-point events such as mutation and apoptosis.

persistence on cellular DNA are believed to be critical for mechanisms of these processes.

DNA repair is one of the major defenses against the deleterious effects of these adducts (Fig. 1), which (1) ensures the removal of a heavy load of DNA adducts resulting from exposure to a carcinogen and (2) removes chronic or endogenously formed adducts at a faster rate faster than their formation. In the last decade, considerable progress has been made in understanding the specificities and mechanisms of such repair, facilitated by the major advances in cloning of novel enzymes, construction of site-directed DNA substrates, high resolution structures of repair proteins and adducted DNA, and targeted deletion of repair genes. Repair studies on exocyclic adducts have largely focused on the base excision repair (BER) pathway which is the main mechanism for removing alkylated, oxidized and deaminated bases.[14–17] Since these lesions are often miscoding and mostly produced endogenously, BER is considered to be the primary defense system for avoiding mutagenesis. Certain exocyclic adducts are also repaired by the nucleotide excision repair (NER) and mismatch repair (MMR) pathways. NER has long been known to remove helix-distorting lesions such as ultraviolet (UV) products, bulky adducts, and a variety of other types of damage.[14,15,18] MMR primarily corrects single base mispairs from miscoding and short loops from insertion/deletion and also acts on alkylated and platinated bases.[19,20] It should be noted that many of the exocyclic adducts, while identified, have not yet been studied for their repair.

In general, the identification, chemistry and mutagenicity of many exocyclic adducts have been studied extensively. Detailed description of these areas will not be covered in this overview, which will focus mainly on repair, with emphasis on how its specificity and efficiency are affected by exocyclic ring structures.

The five-membered etheno and ethano derivatives are substrates for DNA glycosylases

Etheno adducts and their repair

The etheno (ε) bases (Fig. 2) are the most extensively studied exocyclic adducts and are formed by a variety of exogenous chemicals such as vinyl chloride (VC) and ethyl carbamate, as well as by products generated from lipid peroxidation. These highly mutagenic lesions[10] are present not only in rodents treated with carcinogens but also in the unexposed mammals.[1] Increased levels of ε-adducts are observed in women consuming diets rich in polyunsaturated fatty acids (PUFAs) or in abnormalities with persistent oxidative stress such as Wilson's disease, hepatitis and familial adenomatous polyposis.[1,21] These adducts are therefore considered as oxidative stress markers.[1] Decreased repair of ε-adducts is also discovered in lung adrenocarcinoma.[22] There is evidence that ε-adducts may be responsible for *ras* and *p53* mutations in liver tumors of VC-exposed humans.[11,12]

In DNA treated with a major VC metabolite, chloroacetaldehyde (CAA), the efficiency of formation of the four known

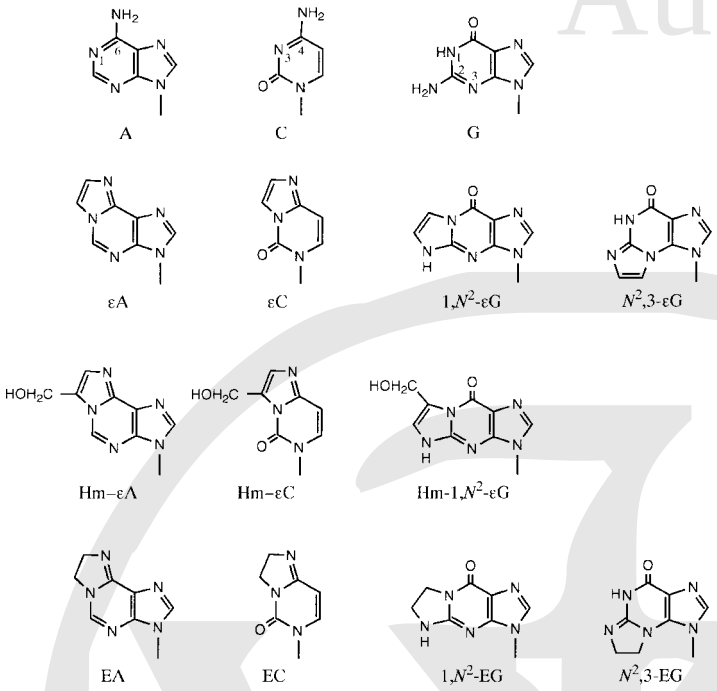

Figure 2. Structures of representative five-membered exocyclic DNA adducts. Repair of most of these adducts is mediated by two families of monofunctional glycosylases, the ANPG/AlkA and TDG/Mug proteins, which specifically recognize the purine and pyrimidine exocyclic derivatives, respectively, with limited cross-activities between the two families. The efficiency of the related activities of a glycosylase (e.g. the εC, 8-Hm-εC and EC activities of the Mug protein) is affected by adduct structure and complexity.

e-adducts was: 3,N^4-eC ≥ 1,N^6-eA > N^2,3-eG ≫ 1,N^2-eG.[23] The yield of these adducts is less in double-stranded than in single-stranded DNA. It should be pointed out that, in addition to the above ε-adducts, various substituted ε-derivatives have been identified. The effect on replication and repair of these adducts has not yet been reported except for 8-hydroxymethyl-εC (8-Hm-εC) (see next section).

Excision repair of a number of known five-membered exocyclic bases is mediated by specific DNA glycosylases, which recognize an adduct and hydrolyze the glycosidic bond between the adduct and the sugar moiety.[17] In most in vitro studies, repair experiments testing a glycosylase activity either measure the release of a radiolabeled or fluorescent exocyclic base from globally modified DNA or detect the cleavage of end-labeled DNA containing a site-specific adduct.

Excision of εA

The first report of repair of an ε-adduct was from Oesch et al.[24] in 1986, who described release of εA and N^2,3-εG by rat brain cell-free extracts from CAA-treated DNA. Later, Singer and co-workers identified an εA-DNA binding and glycosylase activity from human cell-free extracts.[25,26] Subsequent studies on cross-activities suggested that this activity resides in the human alkyl-*N*-purine DNA glycosylase (ANPG, also known as alkyladenine DNA glycosylase, AAG, and *N*-methylpurine DNA glycosylase, MPG).[27] This was confirmed shortly afterwards by Laval's group using a purified recombinant hANPG.[28] In addition, they showed that εA is removed by hANPG homologs in rat, yeast and *E. coli*. These activities are evolutionarily enhanced inasmuch as the mammalian glycosylases excise εA two to three orders of magnitude more efficiently than their yeast and bacterial functional homologs.[28] Both opposite base[28–30] and sequence context[30,31] can affect the εA activity of ANPGs but data from various studies differed in the magnitude of such effects. Recently, it was reported that the *E. coli* mismatch-specific uracil-DNA glycosylase (Mug) could remove εA but with extremely low efficiency.[32] εA can also be excised from DNA by mammalian mitochondrial extracts, possibly by one of the spliced forms of ANPG or an unknown enzyme.[33] Using in vitro assays with HeLa cell extracts, εA was shown to be repaired via both short- and long-patch BER.[34] Data from mice deficient in NER and MMR do not support the involvement of these two pathways in εA removal.[35]

Of all the known DNA glycosylases, ANPGs, which comprise a structurally diverse group of DNA lesions, probably have the broadest substrate range.[36] Independent of enzyme origins, these include 3mA, 3mG, 7mG, O^2mT, O^2mC, Hx, 8-oxoG, εA, 1,N^2-εG, N^2,3-εG, EA, and N^2,3-EG. The primary activity of these glycosylases, however, has generally been thought to remove *N*-3- and *N*-7-alkylated bases. Earlier Dosanjh et al.[37] reported that εA is excised 10- to 20-fold more efficiently than 3mA by hANPG, whereas data from several other studies[28,38,39] showed that hANPG prefers 3mA over

any other substrates, including εA. One reason for this contradiction could be the differences in the nature of the DNA substrates used. In general, it could be quite challenging to define a primary substrate or substrate range of a repair enzyme, since new DNA substrates may continue to be found and many factors, such as sequence context and opposite base, can affect repair efficiency greatly.

The structural studies have provided significant insights into how ANPG/AlkA may accomplish their specificity. The two-dimensional NMR structure of DNA containing an εA · T basepair[40] showed that both bases are in the normal *anti* orientation but in a nonplanar alignment, which disrupts any hydrogen bonding. The crystal structure of hANPG bound to DNA containing such a basepair[41] suggests that hANPG "flips" the modified nucleotide out of the helix and into the active site. Such "flip-out" is facilitated by the bending of the DNA (22°) by the enzyme and by the insertion of Tyr 162 into the minor groove of DNA to occupy the space left by the flipped nucleotide.[41,42] Once inside the active site, the εA adduct stacks between the aromatic side chains of residues and its position is stabilized by a key hydrogen bond between His 136 and N^6 of εA, which offers a unique acceptor lone pair essential for hydrolysis of the C1′–N glycosidic bond. Interestingly, the ring-opened derivatives of εA,[43,44] which are produced even at physiological conditions due to chemical rearrangement of the adduct, are no longer recognized by hANPG, but are substrates for *E. coli* formamidopyrimidine DNA glycosylase (Fpg) and thymine glycol-DNA glycosylases from *E. coli* (Nth) and *S. cerevisiae* (Ntg2).[44]

Part of the above biochemical results on substrate specificity was verified when $ANPG^{-/-}$ knockout mice were generated independently by the groups of Elder and Samson.[45,46] Using cell-free extracts and synthetic oligonucleotides/modified DNA, ANPG was shown to be the primary glycosylase excising εA, 1,N^2-εG, 3mA and Hx.[46–48] Such analysis provides an unambiguous means for the designation of the substrate specificity and for the exploration of backup activities for the missing enzyme. Biologically, however, it is surprising that these knockout mice did not show any overt phenotypic abnormalities[45,46] or significant increase in the spontaneous mutation rate, even increased mutations were observed in the *hprt* gene of the T lymphocytes of $ANPG^{-/-}$ mice treated with methyl methanesulfonate.[45] When the same mice were challenged with vinyl carbamate[49] or ethyl carbamate,[50] levels of εA were significantly higher and persisted longer in DNA from $ANPG^{-/-}$ mice than wild-type mice, indicating the cellular removal of εA by ANPG. It is puzzling, though, that the increased levels of adducts were not paralleled by the increased incidence of liver tumors in these mice.[49] Interestingly, even though no other glycosylase activity against εA was detected using the in vitro approach,[46,47] one study reported that there was residual repair of εA adducts in the $ANPG^{-/-}$ mice.[50] Whether this residual activity is from another DNA glycosylase or from other repair pathway(s) remains to be determined.

Excision of εC

The first evidence that εC is excised by a different glycosylase from ANPG came in 1996 when Hang et al noticed that εC and εA were excised by different column fractions.[51] Through an extensive purification, the εC-DNA glycosylase was identified as a 55 kDa polypeptide by SDS-PAGE,[52] which is the exact molecular mass of the previously purified human mismatch-specific T(U) · G-DNA glycosylase, termed thymine-DNA glycosylase (TDG).[53] Moreover, the T · G and U · G mismatch glycosylase activities co-eluted with the εC activity in the same fractions, and competition studies suggested that they all reside in the same protein.[52] It was then proposed that εC is a substrate for hTDG.[52] This was supported by the finding that the functional homolog of hTDG in *Methanobacterium thermoautotrophicum*, a thermostable mismatch glycosylase (Mig), also excises εC.[52] In a separate study, Saparbaev and Laval[54] purified an εC activity to homogeneity from *E. coli*, and identified it as the previously known Mug protein (also termed as double-stranded uracil-DNA glycosylase, dsUDG), a hTDG homolog. These authors also showed that the purified recombinant hTDG excises εC, indicating the recognition of this adduct by both hTDG and Mug proteins.

Of all the substrates for *E. coli* Mug, the εC activity is by far the most efficient and is considerably higher than the U · G activity,[32,54] for which the enzyme was named. hTDG also excises εC paired with G with greater efficiency than T from T · G, but less than U from U · G.[52,54] Both proteins can remove εC opposite each of the four bases but with varying efficiencies, with εC · G the preferred substrate.[52,54,57]

Recently, Kavli et al.[55] described excision of εC by the human single-strand-selective monofunctional uracil-DNA glycosylase (SMUG1), which is only found in higher eukaryotes. In another study, the human methyl-CpG binding domain protein (MBD4 or MED1) also shows a weak activity toward εC but only when the opposite base is G.[56] The biochemical details of these two activities have not been reported. It is interesting that TDG/Mug, together with SMUG1 and UNG, but not MBD4, belong to the same UDG superfamily. MBD4 and the thermophilic Mig, instead, are homologous to the helix–hairpin–helix (HhH) DNA glycosylases such as MutY, endonuclease III and AlkA.

Structurally, both NMR and crystallographic studies of εC · G-containing duplexes show that the incorporation of the adduct causes perturbation mainly at the adduct site.[58,59] One conventional hydrogen bond involving the O2 of εC and N1 of G is observed. A common characteristic of several base mispairs recognizable for Mug/TDG, i.e. εC · G (and aslo other εC mispairs), T · G and U · G, is the formation of a sheared basepair, which was proposed to be a potential structural feature that may facilitate enzymatic recognition.[58,59] From

the crystal structures of Mug and the complex of Mug with DNA containing a non-hydrolyzable dU analog (βFU),[60,61] it is evident that Mug has a significant structural homology to Ung, despite their low sequence homology (≈10%). However, the UNGs have very narrow specificity while the Mug/TDG family has a broad substrate range. In addition to U · G and T · G, Mug and/or TDG recognize a variety of substituted U or T mismatches as well as base-pairs containing exocyclic bases: εC, EC, 8-Hm-εC, or 1,N^2-εG. For Mug, such broad specificity seems to rely on a significant degree of flexibility of its active site, as a result of lacking the conserved catalytic residues as well as those residues important for specificity determination at the active site of the UNGs.[61,62] While no experimental structure of Mug or TDG complexed with DNA containing an εC is available, molecular modeling based on the structure of Mug–βFU-DNA complex demonstrated that εC can be readily accommodated in the space of this non-specific pyrimidine-binding pocket and makes necessary interactions with key residues.[61] As for the base pair specificity of Mug/TDG, it is proposed that the ease with which the basepair can be disrupted and the nature of the 'widowed' base after disruption plays an important role.[62]

Thus far, the biological role of TDG/Mug in the repair of ε-adducts is not yet clear. In *E. coli*, using a *mug* mutant, Lutsenko and Bhagwat[63] found that Mug appears to be the only εC activity and may not act on U · G or T · G. However, the lack of a strong mutator phenotype for *mug* suggests that endogenously ε-adducts are not significantly formed. In fact, previous studies have failed to identify εC from *E. coli*.[64] Thus, Mug may primarily be responsible for repair of exogenously formed ε-adducts. As with hTDG, its main biological role appears to repair the T · G mispair resulting from the deaminated 5mC in CpG sites, as suggested by Abu and Waters,[65] who found that both 5′-CpG · T and 5′-CpG · εC are much better substrates for hTDG than any other 5′ sequences flanking the same lesions and that the excision of T is actually faster than that of εC when in such a sequence.

Recently, a substituted εC derivative, 8-Hm-εC (Fig. 2), which can be formed by glycidaldehyde in vitro but not yet identified in vivo, has been synthesized and incorporated into defined oligonucleotides.[66] It was then found by Hang and co-workers that the Mug protein efficiently excises 8-Hm-εC from DNA.[66] This activity is only 2.5-fold lower than the εC activity, which could be attributed to the steric effect of the CH_2OH group on the Mug active site. Most recently, similar to Mug, hTDG was also shown to excise 8-Hm-εC at a slower rate than εC (unpublished data).

It has been demonstrated that TDG/Mug activities could be enhanced by a 5′ AP endonuclease,[17,67–69] which cleaves the AP site generated by the DNA glycosylase. For εC and 8-Hm-εC, their excision efficiency can be increased by several folds.[67–69] This feature is due to the fact that TDG and Mug, like many other glycosylases,[17] bind very tightly to their reaction product, an AP site, thereby reducing the enzyme turnover. An AP endonuclease could minimize such product inhibition by displacing the bound glycosylase, although the exact mechanism for this is still not clear.[17] hTDG activity can also be stimulated in vitro by other factors such as ubiquitin-like proteins SUMO-1 and SUMO-2/3.[70] In vivo, these interactions may be particularly useful for those so-called "poor substrates" as defined in vitro and/or for coordinating a specific repair activity/pathway with other repair pathways or cellular processes.[17]

Removal of the εG adducts

N^2,3-εG represents the predominant ε-adduct in the livers of VC-exposed animals.[1] 1,N^2-εG, however, has not yet identified in vivo, but can be formed in vitro by various compounds. Chemically, the angular N^2,3-εG is labile and the stability of its glycosyl bond is much lower than that of the isomeric 1,N^2-εG.[23] Due to such instability, it has been difficult to study the biochemical properties of N^2,3-εG in DNA.

Ludlum's group[71] first reported release of N^2,3-εG from CAA-treated DNA by the purified *E. coli* AlkA protein (3mA-DNA glycosylase II) and estimated that this novel activity is only 1/20th of the 3mA activity. A low-level release of this adduct was also found by Singer's laboratory in cell-free extracts of both HeLa cells and an *E. coli* strain expressing hANPG.[72] In agreement with these data, animal studies by Swenberg and co-workers[73] showed that the in vivo repair of N^2,3-εG is fairly slow. The nature of the human enzyme[Q1] excising this adduct has not been clarified, although hANPG is the most likely candidate.

It was shown that the expression of ANPG mRNA was induced in the hepatocytes of rat exposed to VC, while the non-parenchymal cells, the target for VC, had much lower expression of this enzyme.[74,75] N^2,3-εG is readily induced in these target cells by VC and there is a correlation between the levels of this adduct and the incidence of VC-induced angiosarcoma in rodent livers.[74] Understanding the underlying mechanisms for these cause–effect relationships may be crucial for elucidating the mechanism of VC-induced hepatocarcinogenesis.

It was earlier demonstrated that release of 1,N^2-εG from CAA-modified DNA by human cell-free extracts was similarly low.[72] When 1,N^2-εG was incorporated into a defined oligonucleotide, it was poorly excised by human cell-free extracts as well as by a purified hANPG.[36] However, a recent investigation[48] showed that this adduct, when present in a different sequence, is an efficient substrate for both hAPNG and Mug but not for AlkA and hTDG. The reason for this discrepancy is not clear. Interestingly, the 1,N^2-εG activity of hANPG requires its non-conserved N-terminal region; this region is dispensable for activities toward εA and other substrates,[48] which explains why the AlkA protein does not act on 1,N^2-εG. A detailed description of functions of various truncated versions of hANPG can be found in Ref. 35.

In *E. coli*, the NER pathway is also implicated in repair of 1,N^2-εG based on in vivo experiments in which the mutagenicity of this adduct increased when the adducted vectors were transfected into strains deficient in NER.[76] However, neither NER nor MMR in mammalian cells appears to play a significant role in repairing ε-adducts as shown from mice deficient in these two pathways.[35]

Ethano adducts and their repair

Ethano (E) adducts are saturated etheno ring derivatives. One important source for their formation is the antitumor agents chloroethylnitrosoureas (CNUs), such as BCNU.[77] These compounds directly react with DNA bases to form ethano adducts, monosubstituted bases and cross-links.[77] The stable ethano adducts identified include 1,N^6-EA, 3,N^4-EC and N^2,3-EG (Fig. 2). 1, N^2-EG is a model structure. The hydroxy EC and EG adducts (HO-EC and HO-EG) are the hydrated derivatives of εC and 1,N^2-εG, respectively, and are formed in DNA by reactive VC metabolites.[1] In contrary to the ε-adducts, the mutagenic potential and biological role of ethano adducts are much less understood.

Release of N^2,3-EG from CNU-treated DNA by the purified *E coli* AlkA protein was first reported in 1991.[78] The same enzyme also releases the closely related N^2,3-εG,[71] although the relative efficiency of these two activities has not been compared. Recently, EA in DNA was shown to be excised by both hANPG and AlkA, but with significantly lower efficiencies as compared with εA.[79,80] EC is also found to be excised by *E. coli* Mug at a rate 20-fold lower than excision of εC.[80] The HO-EC adduct can be released by human cell-free extracts.[72]

Ethano adducts differ from ε-adducts in certain features. Saturation of the ε-ring converts it from a planar to a puckered form. Also, the two extra hydrogens on the saturated ethano ring increase its van der Waals surface area. However, the conformational changes of the duplexes imposed by ε- or ethano adducts appear to be similar as shown by molecular modeling.[79,80] Molecular dynamics simulations of hANPG complexed to DNA containing an EA adduct[79] demonstrated that the stacking interactions between EA and the aromatic side chains of the key residues in the active site are reduced, as compared with those for the planar εA residue. This might contribute to the observed lower EA activity of hAPNG.

Thus far there is no clear evidence for the in vivo repair of CNU-induced ethano adducts by a specific glycosylase that could increase cellular resistance to CNUs. Data from various studies on the role of ANPGs in cellular protection of CNU-induced toxicity are controversial. It should be noted that ANPGs can excise not only the EA and EG adducts, but also several other CNU-induced lesions such as 7-alkylguanine bases and a dideoxyguanosinylethane.[81] The hTDG glycosylase, if it removes EC in a similar manner to Mug, could also be involved in such a function.

1,N^2-EG, similar to 1,N^2-εG, is found to be a substrate for bacterial NER, as shown in mutagenesis assays performed by Langouët et al.[76] In the same study, whether NER is involved in removal of HO-EG was not conclusive. These studies using repair mutant cell lines not only define the repair specificity toward the target adducts but also reveal the biological consequences as a result of lacking the specific repair. So far there is apparently no available information on repair of any of the ethano adducts by the mammalian NER.

Repair of six-membered propano-G derivatives and M_1G by the NER and MMR pathways

The six-membered propeno and substituted propano derivatives of dG are an important group of endogenously formed mutagenic lesions (Fig. 3). The 1,N^2-propanoG (PdG) derivatives are mainly formed by lipid peroxidation products, such as acrolein, crotonaldehyde and 4-hydroxynonenal.[7,8] PdG itself is not naturally occurring and rather serves as a model for the chemically unstable substituted PdGs and M_1G (pyrimido[1,2-α]purin-10(3*H*)-one). The latter is the major product produced by malondialdehyde (MDA), an endogenous mutagen/carcinogen from lipid peroxidation and prostaglandin biosynthesis.[7,82] M_1dA and M_1dC are also formed by MDA but as oxopropenyl derivatives without cyclization.[82] M_1G is among the most abundant exocyclic adducts identified in normal cells (levels ranging from 1 to 120/10^8 bases).[82] Therefore, cellular repair of these inescapable lesions is expected to be crucial for counteracting spontaneous mutagenesis.

Structurally, M_1G and PdG derivatives are similar to the five-membered ring adduct 1,N^2-εG (Fig. 3). The latter can be excised by DNA glycosylases,[36,48] in addition to NER.[76] In contrast, M_1G, PdG and HO-PdG are not recognized by glycosylase-mediated BER, as tested in vitro[36] or in vivo,[83–85] suggesting that BER may not be involved in repair of six-membered adducts. However, not all known DNA glycosylases have been tested for their activities toward these adducts and it is still unknown as to what repair mechanism(s) is involved in removal of other six-membered exocyclic adducts of dA and dC.

Instead, NER is found to be important in repair of several dG adducts. In 1997, Marnett and co-workers[83,86] reported that both PdG and M_1G are repaired in vivo by the *E. coli* UvrABC-mediated NER system, with similar efficiencies, based on the mutagenesis assays using M13 genomes containing a single adduct. The involvement of NER is indicated by both increased mutation frequency and increased adducted-template replication in the NER-deficient strains. Similarly, it was recently shown[84–87] that *E. coli* NER is also implicated in repair of the major acrolein-derived DNA adduct, γ-HO-PdG (Fig. 3), which has been detected in DNA from healthy human tissues.

Results from in vitro assays also indicated that PdG is a substrate for the purified UvrABC proteins although relatively

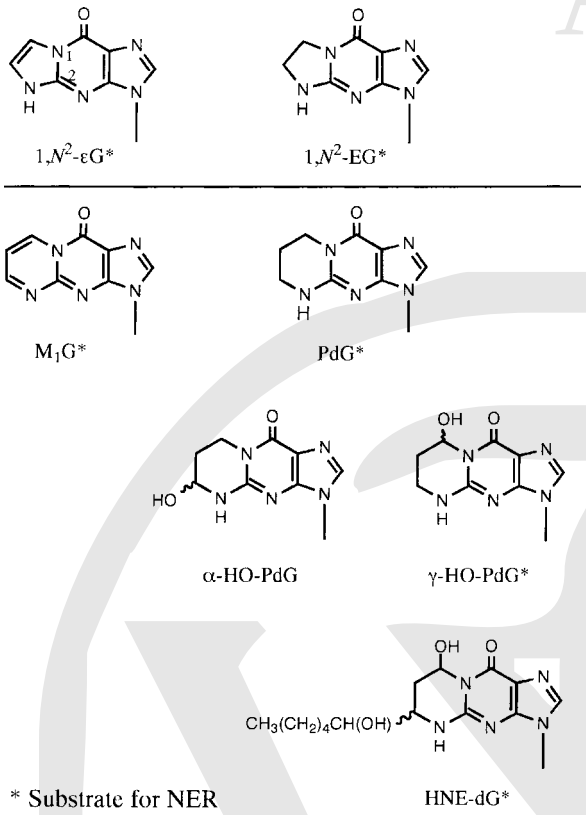

Figure 3. Structures of six-membered exocyclic M_1G and PdG derivatives. The top two structures are their five-membered analogs. Majority of these adducts are found to be substrates for NER. Two chemical features of these adducts may affect their repair significantly. One is that adducts such as acrolein- or 4-HNE-dG are present in multiple stereoisomers, which can lead to stereoselective repair.[88] The other is the spontaneous ring-opening process reported for M_1G and γ-HO-PdG. They convert to ring-opened forms when opposite C in duplex DNA, which is apparently due to the chemical rearrangement induced by the duplex formation.[89,90] This process is reversible upon thermal denaturation of the duplex. Such a mechanism has been shown to affect mutagenicity of these adducts and could similarly affect their repair.

poor.[83] However, a cell-free extract from Chinese hamster cells excised the same substrate as efficiently as with the cyclobutane thymine dimer (CPD), one of the primary substrates for NER.[83] A study by Tang, Chung and co-workers[13] also showed that 4-hydroxynonenal (4-HNE)-derived exocyclic dG adduct is excised by the UvrABC proteins efficiently and quantitatively. Using an in vitro repair synthesis assay, 4-HNE-dG in plasmid DNA can be readily repaired by HeLa but not XPA nuclear extracts, indicative of human NER involvement.[8,88] Since there is a preferential formation of this adduct at codon 249 of human *p53* gene,[13] which is a mutational hotspot in human cancers, it would be of interest to examine repair of 4-HNE-dG by human NER for sequence specificity. Recently, Roy and co-workers demonstrated that the four stereoisomers of 4-HNE-dG are repaired at differential initial rates, suggesting the importance of the stereo configuration of the 8-hydroxy group in enzymatic recognition and excision by NER proteins.[88]

In addition to NER, Marnett's laboratory has shown that PdG and M_1G in DNA are recognized by the bacterial MutS-dependent mismatch repair (MMR), which could lead to either the removal of these adducts by the pathway or the protection of such adducts from repair by NER.[91] In their study, mutations caused by both adducts were reduced when M13 genomes containing single PdG or M_1G were transfected into *mutS*-deficient *E. coli* strains, suggesting that MutS binds to the adduct, leading to the block of its repair by NER. This is supported by the finding that purified MutS protein binds to DNA containing these two adducts in vitro.[91] MutS also binds to a DNA duplex containing an εC or 8-Hm-εC (unpublished data). Whether MMR repairs other exocyclic adducts remains to be determined. Nevertheless, it is quite intriguing that the change from a five-membered to six-membered ring structure has major effect on the specificity of repair, i.e. a shift from BER to NER/MMR. It appears that a seemingly minor structural change(s) in DNA damage can cause a major difference in repair specificity.

It should be mentioned that there are a number of other six-membered ring adducts were also seen in the reactions of various compounds. Data on repair of these adducts, when available, should aid in getting a clear picture of the repair specificity as a function of the ring size.

Processing of bulkier exocyclic pBQ-adducts by damage-specific DNA endonucleases

A number of exocyclic adducts with two extra rings have been identified from the reaction of DNA bases with the metabolites of benzene, an ubiquitous human carcinogen/leukemogen.[1] Its metabolites, such as hydroquinone (HQ) and muconaldehyde, accumulate in the bone marrow, where HQ can undergo further oxidation to *p*-benzoquinone (*p*BQ). In vitro, both HQ and *p*BQ form hydroxy[92] (Fig. 4) or dihydroxy benzetheno adducts.[93] In DNA reacted with *p*BQ, the relative abundance was: *p*BQ-C ≫ *p*BQ-A ≫ *p*BQ-G.[94] The in vivo existence of these adducts has not yet been proved. Muconaldehyde also forms pyrrole ring-containing exocyclic adducts with purine nucleosides,[95] although their biochemical properties are unknown. Nevertheless, the formation of these adducts, if occurring in vivo, could contribute to benzene-related genotoxicity.

Syntheses of oligonucleotides containing a single *p*BQ-adduct of dA, dC and dG have greatly facilitated the repair studies.[92] Initially, such work by Singer, Hang and colleagues was directed toward testing whether the known glycosylase(s) excising etheno adducts would also act on these structurally related but bulkier adducts.[36,96,97] Although they were not

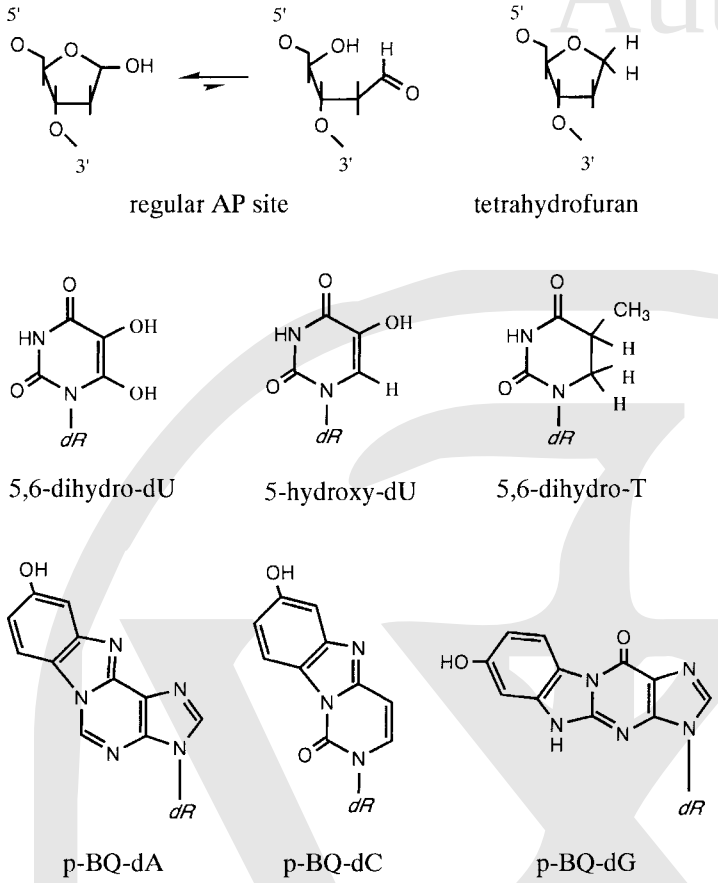

Figure 4. Partial list of DNA lesions that are substrates for the human APE1. The regular AP site is a mixture of the major ring-closed form and the minor ring-opened form. *p*BQ-A: 3′-hydroxy-1,N^6-benzetheno-A; *p*BQ-C: 3′-hydroxy-3,N^4-benzetheno-C; *p*BQ-G: 3′-hydroxy-1,N^2-benzetheno-G.

found to be excised by the glycosylases,[36,96] it nevertheless led to the finding of a novel human repair activity efficiently cleaving DNA containing *p*BQ-C.[96] This activity was further purified to apparent homogeneity and surprisingly found to be identical to the major human AP endonuclease (APE1, also termed as HAP1, APEX, and Ref-1).[97] Thus, *p*BQ-C is a "new" substrate for an "old" enzyme. hAPE1 also acts on *p*BQ-A and *p*BQ-G but with much lower efficiency.[98] Regardless, the AP site still remains the primary substrate for the enzyme.[99] Interestingly, the two 5′ AP endonucleases in *E. coli,* exonuclease III and endonuclease IV, are much more efficient toward the three *p*BQ-adducts than hAPE1. These two enzymes appear to be the only 5′ cleavage activities in *E. coli* that act on these adducts, as shown by studies using mutants lacking *xth* and/or *nfo*.[98] It would be interesting to see whether the recently identified hAPE2 acts on these *p*BQ-adducts. hAPE2 differs from hAPE1 at the N and C terminus but retains many of the essential active site residues.[100]

The difference in size and structure between an AP site and the *p*BQ-adduct is large,[99] yet both are efficiently repaired by the same proteins. The AP endonucleases now recognize a structural diversity of substrates as shown partially in Fig. 4.[36,101,102] It also appears that the structural requirements for *p*BQ-adduct recognition are highly specific, since APE1 does not act on other five- or six-membered exocyclic adducts.[36] Reaction mechanisms by which hAPE1 recognizes and cleaves an AP site have been proposed based on crystal structures of APE1.[103–105] A recent work[106] using MD simulations, based on the high resolution X-ray coordinates for hAPE1 complexed to DNA containing an AP analog tetrahydrofuran,[104] showed that pBQ-dC can be accomodated at the active site with certain structural rearrangements. The APE1–*p*BQ-C complex forms a similar hydrogen bond network at the active site as in the crystallographically determined APE1/AP-DNA.[106] In addition, site-directed mutagenesis showed similar requirements of those key active-site residues for both AP and *p*BQ-C endonuclease activities.[99,107]

hAPE1 is a multifunctional enzyme with functions in repair, transcription and other cellular processes.[100] In BER, APE1 cleaves the AP site resulting from a glycosylase action and removes 3′ replication-blocking moieties. In processing a

*p*BQ-adduct, APE1 specifically recognizes the adduct and cleaves the phosphodiester bond 5′ to it, leaving the adduct as a "dangling base" on the 5′ terminus.[97] Therefore, APE1 could serve as an initial recognition and incision step of a pathway that repairs *p*BQ-adducts. A similar mechanism, namely, alternative excision repair, was previously proposed for a damage-specific endonuclease-initiated repair of thymine dimer and other lesions in *S. pombe*.[108,109] Recently, both bacterial and human AP endonucleases were found to act on several oxidized bases (Fig. 4) using a similar mode of action.[101,102] It therefore appears that this type of repair mechanism is implicated in removal of various classes of DNA damage.

Conclusions and perspectives

Many chemicals can form exocyclic adducts in DNA by virtue of their bifunctionalities. These adducts are analogous in structure, yet at least four different mechanisms: BER, NER, MMR and AP endonuclease-mediated repair, may be involved in their repair (Table 1). It is noteworthy that, so far, many identified adducts have not been tested for their repairability and also not every repair enzyme/pathway has been tested for their activity toward all the presently known exocyclic adducts. A nonrepairable adduct for a specific repair enzyme/pathway can be useful for structure–function analyses. It is also possible that some adducts might be refractory to any repair.

Repair of many five-membered ring adducts is mediated by two families of monofunctional DNA glycosylases, the ANPG/AlkA and TDG/Mug proteins, which specifically recognize the purine and pyrimidine exocyclic derivatives, respectively, with limited cross-activities between the two families (Table 1). Thus far, data do not indicate the involvement of BER in repair of six-membered ring adducts and those with two extra rings. Instead, six-membered dG adducts are found to be repaired by the NER and MMR pathways, which is not surprising since NER is highly versatile and, as for MMR, it is likely that these exocyclic adducts in duplexes may have structures similar to Watson-Crick base–base mispairs. Therefore, it is worthwhile to test whether these two pathways also act on other classes of exocyclic adducts. For the two extra ring *p*BQ-adducts, the AP endonucleases may serve as damage-specific endonucleases, initiating a process similar to the so-called alternative excision repair pathway.[108,109] This mechanism seems to be limited as AP endonucleases have not been found to act on five- and six-membered adducts. Overlapping repair also exists for some exocyclic adducts, since, chemically, most of the exocyclic adducts are nonbulky, which makes them potential targets for various pathways. For example, both NER and MMR are implicated in repair of PdG and M_1G,[83,86,91] whereas both BER and NER can act on 1,N^2-εG.[36,48,72,76] Overlap between glycosylases is also common as examplified by the in vitro identification of the εC-activity in three human DNA glycosylase, TDG, SMUG1 and MBD4.[52,54–56] Although the biological function of these activities remains to be seen, such redundancy of repair is expected to be useful for backing up in vivo.

Although we seem to have had considerable information on the in vitro repair of many exocyclic adducts, there is only limited evidence for their in vivo repair. To identify the cellular existence of these adducts and to confirm their cellular repair are two primary goals in establishing an initial biological relevance. Obviously it will be far more difficult to interpret in vivo findings. In real life, when a chemical carcinogen attacks cellular DNA, base modification will be likely in more than one type and on various sequences of the genome. Repair can be influenced by multiple factors such as the specificity or multiplicity of repair enzymes/pathways, sequence context and strandedness, saturation or cytotoxic inhibition of repair proteins, protein–protein or pathway–pathway interplays, modulation of repair by gene expression or cell cycle control. This is only a partial list.

The relevance of any data on repair of specific exocyclic adducts, obtained in vivo or in vitro, needs to be considered with regard to the overall mechanism of mutagenesis and carcinogenesis by the parental compound. In some cases, the major lesion(s) formed by a carcinogen is not exocyclic adducts but other type(s) of damage. It is well known that, in addition to exocyclic adducts, bifunctional alkylating agents can also cause DNA damage such as interstrand cross-links and react with cellular proteins.[1] Since most of the repair data are from cell-free systems, in order to have a clear mechanistic understanding of carcinogenicity of a compound, much work remains to be done in vivo to connect the dots into the full picture of the formation, repair and mutagenic effects of all the DNA lesions formed[Q2].

A unique array of structural variations in exocyclic adducts (Fig. 5) provides a sound platform for studying the structural basis of substrate specificity. Structural studies on a number of such adducts, using NMR, crystallography and thermodynamics, have provided detailed information on both adduct structure and adduct-imposed duplex conformational changes.[e.g. 40,59,89,90] Overall, exocyclic adducts examined cause structural perturbations around the adduct in DNA duplex. Attempts have been made on identifying specific structural features, which could be from both adduct structure and localized conformation, that serve as initial "signal" for enzyme recognition. As with BER, the crystal and co-structures of several glycosylases excising exocyclic adducts (e.g., ANPG, AlkA, Mug, Mig) have been solved, which enables scientists to make a detailed analysis, looking for general requirements of substrate recognition as well as specific interactions for an individual substrate. It is generally assumed that damage recognition may involve initial groove contacts by a glycosylase and subsequent adduct "flipping" into a specific binding pocket to check for proper fit. For exocyclic adducts, their shape, size, aromaticity, hydrogen-bonding capacity, as

Table 1. Substrate specificity of DNA repair enzymes/pathways acting on exocyclic adducts

Repair enzymes/pathways	Origin	Exocyclic substrates			Other substrates
		5-membered	6-membered	2 extra rings	
BER					
AlkA	*E. coli*	εA, N^2,3-εG, EA, N^2,3-EG			*N*-3 and *N*-7 alkylpurines, O^2-alkylpyrimidines, FoU, HmU, Hx, Xan, fragmented T
ANPG (AAG, MPG)	Human	εA, 1,N^2-εG, EA			*N*-3 and *N*-7 alkylpurines, Hx, 8-oxoG
Mug (dsUDG)	*E. coli*	εC, 8-Hm-εC, EC, 1,N^2-εG, εA			U•G, 5-substituted U•G pairs (e.g., FU•G, OHU•G, ClU•G), FoU•A, U•Hx, U•A, U•2AP, T•G
TDG	Human	εC, 8-Hm-εC			U•G, HmU•G, OHU•G, T•G, FU•G, T•O^6mG, T•6-thioG, T•AMAP, Tg•G
SMUG1	Human	εC			U•G, HmU•G, OHU•G, U•A, HmU•A, FoU•A, ssU, ssHmU, ssFoU, ssOHU
MBD4 (MED1)	Human	εC•G			T•G, U•G, FU•G, FoU•G, Tg•G
NER	*E. coli*	1,N^2-εG, 1,N^2-EG	PdG, M_1G, γ-HO-PdG, 4-HNE-dG		UV products: CPD, (6-4) photoproduct intrastrand cross-links (e.g. 1,2-d(GpG) cisplatin cross-link)Bulky adducts (e.g. AAF-G) Nonbulky lesions: AP site, Tg, 8-oxoG, O^6mG
	Human		PdG, 4-HNE-dg		
MMR	*E. coli*		PdG, M_1G		Base-base mismatches, insertion/delesion loops, alkylated and platinated bases
APE1 (HAP1, APEX, Ref1)	Human			pBQ-dC, pBQ-dA, pBQ-dG	AP sites, 3′-phosphate, 3′-deoxyribose-5′-phosphate, 3′-phosphoglycolaldehyde, DHdU, DHT, OHdU, αA, αT

ss, single-stranded; Xan, xanthine; FoU, 5-formyluracil; HmU, 5-hydroxymethyluracil; OHU, 5-hydroxyuracil; FU, 5-fluorouracil; ClU, 5-chlorouracil; 2AP, 2-aminopurine; AMAP, 2-amino(6-methylamino)purine; CPD, cyclobutane pyrimidine dimmer; AAF, acetylaminofluorene; Tg, thymine glycol; DHdU, 5,6-dihydro-2′-dU; DHT, 5,6-dihydrothymidine; αA, alpha-2′-deoxyadenine; αT, alpha-thymidine.

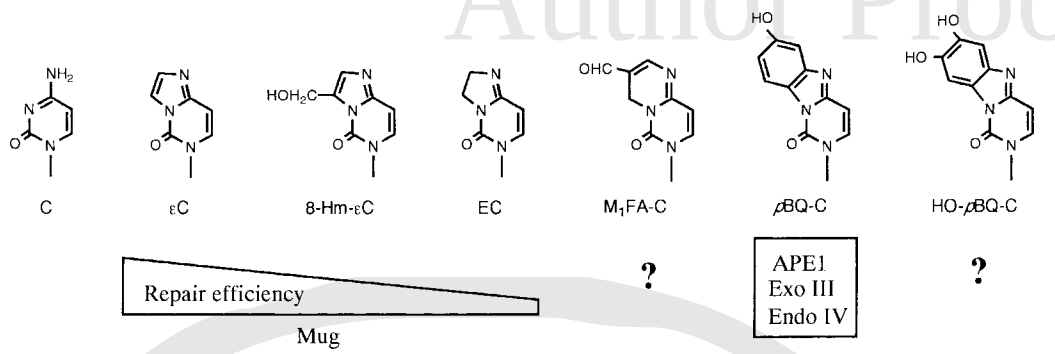

Figure 5. Change of specificity and efficiency of repair as a function of adduct structure. The question markers indicate that repair is unknown.

well as conformational changes imposed by their presence, may all be relevant to such specific recognition.

At present, it is evident that more structural studies, perhaps with the aid of new physical methods, as well as exploring new substrates, are both important for a better understanding of the mechanisms responsible for enzyme specificities and activities. In the near future, combined effort from chemistry, biochemistry, structure and allied fields could lead to the prediction of repair specificity and the design of novel substrates and inhibitors for various purposes.

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References

1. Singer B, Bartsch H, editor. 1999. Exocyclic DNA Adducts in Mutagenesis and Carconogenesis. Lyon: IARC Publication No. 150.
2. IARC. 1987. IARC Monographs on the Evaluation of the Carcinogenic Risks to Humans. Suppl. 7, Lyon, France: IARC.
3. National Toxicology Program, 10th Report on Carcinogens. U.S. Department of Health and Human Services, Public Health Service.
4. Shapiro R, Hachmann J. 1966. The reaction of guanine derivatives with 1,2-dicarbonyl compounds. Biochemistry 5:2799–2807.
5. Goldschmidt BM, Blazej TP, Van Duuren BL. 1968. The reaction of guanosine and deoxyguanosine with glycidaldehyde. Tetrahedron Lett 13:1583–1586.
6. Shapiro R. 1969. Reactions with purines and pyrimidines. Ann NY Acad Sci 163:624–630.
7. Marnett LJ. 2000. Oxyradicals and DNA damage. Carcinogenesis 21: 361–370.
8. Chung FL, Pan J, Choudhury S, Roy R, Hu W, et al. 2003. Formation of trans-4-hydroxy-2-nonenal- and other enal-derived cyclic DNA adducts from omega-3 and omega-6 polyunsaturated fatty acids and their roles in DNA repair and human p53 gene mutation. Mutat Res 531:25–36.
9. Leonard NJ. 1992. Etheno-bridged nucleotides in structural diagnosis and carcinogenesis. Biochem Mol Biol Chemtracts 3:273–297.
10. Barbin A. 2000. Etheno-adduct-forming chemicals: from mutagenicity testing to tumor mutation spectra. Mutat Res 462:55–69.
11. Marion MJ, Froment O, Trepo C. 1991. Activation of Ki-ras gene by point mutation in human liver angiosarcoma associated with vinyl chloride exposure. Mol Carcinog 4:450–454.
12. Hollstein M, Marion MJ, Lehman T, Welsh J, Harris CC, et al. 1994. p53 mutations at A:T base pairs in angiosarcomas of vinyl chloride-exposed factory workers. Carcinogenesis 15:1–3.
13. Hu W, Feng Z, Eveleigh J, Iyer G, Pan J, et al. 2002. The major lipid peroxidation product, trans-4-hydroxy-2-nonenal, preferentially forms DNA adducts at codon 249 of human p53 gene, a unique mutational hotspot in hepatocellular carcinoma. Carcinogenesis 23:1781–1789.
14. Friedberg EC, Walker GC, Siede W. 1995. DNA repair and mutagenesis. Washington, D.C: ASM Press. p 698.
15. Lindahl T, Wood RD. 1999. Quality control by DNA repair. Science 286: 1897–1905.
16. Nilsen H, Krokan HE. 2001. Base excision repair in a network of defense and tolerance. Carcinogenesis 22:987–998.
17. Hang B, Singer B. 2003. Protein-protein interactions involving DNA glycosylases. Chem Res Toxicol 16:1181–1195.
18. de Laat WL, Jaspers NGJ, Hoeijmakers JHJ. 1999. Molecular mechanism of nucleotide excision repair. Genes Dev 13:768–785.
19. Modrich P, Lahue R. 1996. Mismatch repair in replication fidelity, genetic recombination, and cancer biology. Annu Rev Biochem 65: 101–133.
20. Schofield MJ, Hsieh P. 2003. DNA mismatch repair: molecular mechanisms and biological function. Annu Rev Microbiol 57:579–608.
21. Bartsch H, Nair J. 2002. Potential role of lipid peroxidation derived DNA damage in human colon carcinogenesis: studies on exocyclic base adducts as stable oxidative stress markers. Cancer Detect Prev 26:308–312.
22. Speina E, Zielinska M, Barbin A, Gackowski D, Kowalewski J, et al. 2003. Decreased repair activities of 1,N^6-ethenoadenine and 3,N^4-ethenocytosine in lung adenocarcinoma patients. Cancer Res 63: 4351–4357.
23. Kusmierek JT, Singer B. 1992. 1,N^2-ethenodeoxyguanosine: properties and formation in chloroacetaldehyde-treated polynucleotides and DNA. Chem Res Toxicol 5:634–638.
24. Oesch F, Adler S, Rettelbach R, Doerjer G. 1986. Repair of etheno DNA adducts by *N*-glycosylases. Vol. 70. Lyon: IARC Sci Publ, pp 373–379.
25. Rydberg B, Dosanjh MK, Singer B. 1991. Human cells contain protein specifically binding to a single 1,N^6-ethenoadenine in a DNA fragment. Proc Natl Acad Sci USA 88:6839–6842.
26. Rydberg B, Qiu ZH, Dosanjh MK, Singer B. 1992. Partial purification of a human DNA glycosylase acting on the cyclic carcinogen adduct 1,N^6-ethenodeoxyadenosine. Cancer Res 52:1377–1379.
27. Singer B, Antoccia A, Basu AK, Dosanjh MK, Fraenkel-Conrat H, et al. 1992. Both purified human 1,N^6-ethenoadenine-binding protein and purified human 3-methyladenine-DNA glycosylase act on 1,N^6-ethenoadenine and 3-methyladenine. Proc Natl Acad Sci USA 89:9386–9390.
28. Saparbaev M, Kleibl K, Laval J. 1995. *Escherichia coli, Saccharomyces cerevisiae*, rat and human 3-methyladenine DNA glycosylases repair

1,N^6-ethenoadenine when present in DNA. Nucl Acids Res 23:3750–3755.
29. Abner CW, Lau AY, Ellenberger T, Bloom LB. 2001. Base excision and DNA binding activities of human alkyladenine DNA glycosylase are sensitive to the base paired with a lesion. J Biol Chem 276:13379–13387.
30. Wyatt MD, Samson LD. 2000. Influence of DNA structure on hypoxanthine and 1,N^6-ethenoadenine removal by murine 3-methyladenine DNA glycosylase. Carcinogenesis 21:901–908.
31. Hang B, Sági J, Singer B. 1998. Correlation between sequence-dependent glycosylase repair and the thermal stability of oligonucleotide duplexes containing 1,N^6-ethenoadenine. J Biol Chem 273:33406–33413.
32. O'Neill RJ, Vorob'eva OV, Shahbakhti H, Zmuda E, Bhagwat AS, et al. 2003. Mismatch uracil glycosylase from *Escherichia coli*: a general mismatch or a specific DNA glycosylase? J Biol Chem 278:20526–20532.
33. Dianov GL, Souza-Pinto N, Nyaga SG, Thybo T, Stevnsner T, Bohr VA. 2001. Base excision repair in nuclear and mitochondrial DNA. Prog Nucleic Acid Res Mol Biol 68:285–297.
34. Fortini P, Parlanti E, Sidorkina OM, Laval J, Dogliotti E. 1999. The type of DNA glycosylase determines the base excision repair pathway in mammalian cells. J Biol Chem 274:15230–15236.
35. Gros L, Ishchenko AA, Saparbaev M. 2003. Enzymology of repair of etheno-adducts. Mutat Res 531:219–229.
36. Singer B, Hang B. 1997. What structural features determine repair enzyme specificity and mechanism in chemically modified DNA? Chem Res Toxicol 10:713–732.
37. Dosanjh MK, Roy R, Mitra S, Singer B. 1994. 1,N^6-ethenoadenine is preferred over 3-methyl-adenine as substrate by a cloned human N-methylpurine-DNA glycosylase (3-methyladenine-DNA glycosylase). Biochemistry 33:1624–1628.
38. O'Connor TR. 1993. Purification and characterization of human 3-methyladenine-DNA glycosylase. Nucleic Acids Res 21:5561–5569.
39. Asaeda A, Ide H, Asagoshi A, Matsuyama S, Tano K, et al. 2000. Substrate specificity of human methylpurine DNA N-glycosylase. Biochemistry 39:1959–1965.
40. Kouchakdjian M, Eisenberg M, Yarema K, Basu A, Essigmann J, Patel DJ. 1991. NMR studies of the exocyclic 1,N^6-ethenodeoxyadenosine adduct (εdA) opposite thymidine in a DNA duplex. Nonplanar alignment of εdA(anti) and dT(anti) at the lesion site. Biochemistry 30:1820–1828.
41. Lau AY, Wyatt MD, Glassner BJ, Samson LD, Ellenberger T. 2000. Molecular basis for discriminating between normal and damaged bases by the human alkyladenine glycosylase, AAG. Proc Natl Acad Sci USA 97:13573–13578.
42. Lau AY, Schärer OD, Samson L, Verdine GL, Ellenberger T. 1998. Crystal structure of a human alkylbase-DNA repair enzyme complexed to DNA: mechanisms for nucleotide flipping and base excision. Cell 95:249–258.
43. Basu AK, Wood ML, Niedernhofer LJ, Ramos LA, Essigmann JM. 1993. Mutagenic and genotoxic effects of three vinyl chloride-induced DNA lesions: 1,N^6-ethenoadenine, 3,N^4-ethenocytosine, and 4-amino-5-(imidazol-2-yl)imidazole. Biochemistry 32:12793–12801.
44. Speina E, Kierzek AM, Tudek B. 2003. Chemical rearrangement and repair pathways of 1,N^6-ethenoadenine. Mutat Res 531:205–217.
45. Elder RH, Jansen JG, Weeks RJ, Willington MA, Deans B, et al. 1998. Alkylpurine-DNA-*N*-glycosylase knockout mice show increased susceptibility to induction of mutations by methyl methanesulfonate. Mol Cell Biol 18:5828–5837.
46. Engelward BP, Weeda G, Wyatt MD, Broekhof JL, del Wit J, et al. 1997. Base excision repair deficient mice lacking the AAG alkyladenine DNA glycosylase. Proc Natl Acad Sci USA 94:13087–13092.
47. Hang B, Singer B, Margison GP, Elder RH. 1997. Targeted deletion of alkylpurine-DNA-*N*-glycosylase in mice eliminates repair of 1,N^6-ethenoadenine and hypoxanthine but not of 3,N^4-ethenocytosine or 8-oxoguanine. Proc Natl Acad Sci USA 94:12869–12874.
48. Saparbaev M, Langouet S, Privezentzev CV, Guengerich FP, Cai H, et al. 2002. 1,N^2-ethenoguanine, a mutagenic DNA adduct, is a primary substrate of *Escherichia coli* mismatch-specific uracil-DNA glycosylase and human alkylpurine-DNA-N-glycosylase. J Biol Chem 277:26987–26993.
49. Barbin A, Wang R, O'Connor PJ, Elder RH. 2003. Increased formation and persistence of 1,N^6-ethenoadenine in DNA is not associated with higher susceptibility to carcinogenesis in alkylpurine-DNA-N-glycosylase knockout mice treated with vinyl carbamate. Cancer Res 63:7699–7703.
50. Ham AJ, Engelward BP, Koc H, Sangaiah R, Meira LB, et al. 2004. New immunoaffinity-LC-MS/MS methodology reveals that Aag null mice are deficient in their ability to clear 1,N^6-etheno-deoxyadenosine DNA lesions from lung and liver in vivo. DNA Repair (Amst) 3:257–265.
51. Hang B, Chenna A, Rao S, Singer B. 1996. 1,N^6-ethenoadenine and 3,N^4-ethenocytosine are excised by separate human DNA glycosylases. Carcinogenesis 17:155–157.
52. Hang B, Medina M, Fraenkel-Conrat H, Singer B. 1998. A 55-kDa protein isolated from human cells shows DNA glycosylase activity toward 3,N^4-ethenocytosine and the G/T mismatch. Proc Natl Acad Sci USA 95:13561–13566.
53. Neddermann P, Jiricny J. 1993. The purification of a mismatch-specific thymine-DNA glycosylase from HeLa cells. J Biol Chem 268:21218–21224.
54. Saparbaev M, Laval J. 1998. 3,N^4-ethenocytosine, a highly mutagenic adduct, is a primary substrate for *Escherichia coli* double-stranded uracil-DNA glycosylase and human mismatch-specific thymine-DNA glycosylase. Proc Natl Acad Sci USA 95:8508–8513.
55. Kavli B, Sundheim O, Akbari M, Otterlei M, Nilsen H, et al. 2002. hUNG2 is the major repair enzyme for removal of uracil from U:A matches, U:G mismatches, and U in single-stranded DNA, with hSMUG1 as a broad specificity backup. J Biol Chem 277:39926–39936.
56. Petronzelli F, Riccio A, Markham GD, Seeholzer SH, Genuardi M, et al. 2000. Investigation of the substrate spectrum of the human mismatch-specific DNA *N*-glycosylase MED1 (MBD4): fundamental role of the catalytic domain. J Cell Physiol 185:473–480.
57. Saparbaev M, Laval J. 1999. Enzymology of the repair of etheno adducts in mammalian cells and in *Escherichia coli*. Vol. 150. Lyon: IARC Sci Publ, pp 249–261.
58. Cullinan D, Johnson F, de los Santos C. 2000. Solution structure of an 11-mer duplex containing the 3, N^4-ethenocytosine adduct opposite 2′-deoxycytidine: implications for the recognition of exocyclic lesions by DNA glycosylases. J Mol Biol 296:851–861.
59. Freisinger E, Fernandes A, Grollman AP, Kisker C. 2003. Crystallographic characterization of an exocyclic DNA adduct: 3,N^4-etheno-2′-deoxycytidine in the dodecamer 5′-CGCGAATTεCGCG-3′. J Mol Biol 329:685–697.
60. Barrett TE, Savva R, Panayotou G, Barlow T, Brown T, et al. 1998. Crystal structure of a G:T/U mismatch-specific DNA glycosylase: mismatch recognition by complementary-strand interactions. Cell 92:117–129.
61. Barrett TE, Scharer OD, Savva R, Brown T, Jiricny J, et al. 1999. Crystal structure of a thwarted mismatch glycosylase DNA repair complex. EMBO J 18:6599–6609.
62. Pearl LH. 2000. Structure and function in the uracil-DNA glycosylase superfamily. Mutat Res 460:165–181.
63. Lutsenko E, Bhagwat AS. 1999. The role of the *Escherichia coli* Mug protein in the removal of uracil and 3,N^4-ethenocytosine from DNA. J Biol Chem 274:31034–31038.
64. Bartsch H, Nair J. 2000. Ultrasensitive and specific detection methods for exocylic DNA adducts: markers for lipid peroxidation and oxidative stress. Toxicology 153:105–114.
65. Abu M, Waters TR. 2003. The main role of human thymine-DNA glycosylase is removal of thymine produced by deamination of 5-methylcytosine and not removal of ethenocytosine. J Biol Chem 278:8739–8744.
66. Chenna A, Perry A, Singer B. 2000. Synthesis of 8-(hydroxymethyl)-3,N^4-etheno-2′-deoxycytidine, a potential carcinogenic glycidaldehyde adduct, and its site-specific incorporation into DNA oligonucleotides. Chem Res Toxicol 13:208–213.
67. Hang B, Downing G, Guliaev AB, Singer B. 2002. Novel activity of *Escherichia coli* mismatch uracil-DNA glycosylase (Mug) excising 8-

(hydroxymethyl)-3,N^4-ethenocytosine, a potential product resulting from glycidaldehyde reaction. Biochemistry 41:2158–2165.
68. Sung JS, Mosbaugh DW. 2000. *Escherichia coli* double-strand uracil-DNA glycosylase: involvement in uracil-mediated DNA base excision repair and stimulation of activity by endonuclease IV. Biochemistry 39:10224–10235.
69. Privezentzev CV, Saparbaev M, Laval J. 2001. The HAP1 protein stimulates the turnover of human mismatch-specific thymine-DNA-glycosylase to process 3,N^4-ethenocytosine residues. Mutat Res 480–481:277–284.
70. Hardeland U, Steinacher R, Jiricny J, Schar P. 2002. Modification of the human thymine-DNA glycosylase by ubiquitin-like proteins facilitates enzymatic turnover. EMBO J 21:1456–1464.
71. Matijasevic Z, Sekiguchi M, Ludlum DB. 1992. Release of N^2,3-ethenoguanine from chloroacetaldehyde-treated DNA by *Escherichia coli* 3-methyladenine DNA glycosylase II. Proc Natl Acad. Sci USA 89: 9331–9334.
72. Dosanjh MK, Chenna A, Kim E, Fraenkel-Conrat H, Samson L, et al. 1994. All four known cyclic adducts formed in DNA by the vinyl chloride metabolite chloroacetaldehyde are released by a human DNA glycosylase. Proc Natl Acad Sci USA 91:1024–1028.
73. Morinello EJ, Ham AJ, Ranasinghe A, Nakamura J, Upton PB, et al. 2002. Molecular dosimetry and repair of N^2,3-ethenoguanine in rats exposed to vinyl chloride. Cancer Res 62:5189–5195.
74. Swenberg JA, Bogdanffy MS, Ham A, Holt S, Kim A, et al. 1999. Formation and repair of DNA adducts in vinyl chloride- and vinyl fluoride-induced carcinogenesis. Vol. 150. Lyon: IARC Sci Publ, pp 29–43.
75. Holt S, Roy G, Mitra S, Upton PB, Bogdanffy MS, Swenberg JA. 2000. Deficiency of *N*-methylpurine-DNA-glycosylase expression in nonparenchymal cells, the target cell for vinyl chloride and vinyl fluoride. Mutat Res 460:105–115.
76. Langouet S, Mican AN, Muller M, Fink SP, Marnett LJ, et al. 1998. Misincorporation of nucleotides opposite five-membered exocyclic ring guanine derivatives by *Escherichia coli* polymerases in vitro and in vivo: 1,N^2-ethenoguanine, 5,6,7,9-tetrahydro-9-oxoimidazo[1, 2-α]purine, and 5,6,7,9-tetrahydro-7-hydroxy-9-oxoimidazo[1, 2-α]purine. Biochemistry 37:5184–5193.
77. Ludlum DB. 1990. DNA alkylation by the haloethylnitrosoureas: nature of modifications produced and their enzymatic repair or removal. Mutat Res 233:116–127.
78. Habraken Y, Carter CA, Sekiguchi M, Ludlum DB. 1991. Release of N^2,3-ethanoguanine from haloethyl-nitrosourea-treated DNA by *Escherichia coli* 3-methyladenine DNA glycosylase II. Carcinogenesis 12:1971–1973.
79. Guliaev AB, Hang B, Singer B. 2002. Structural insights by molecular dynamics simulations into differential repair efficiency for ethano-A versus etheno-A adducts by the human alkylpurine-DNA-N-glycosylase (APNG). Nucleic Acids Res 30:3778–3787.
80. Guliaev AB, Singer B, Hang B. Chloroethylnitrosourea-derived ethano cytosine and adenine adducts are substrates for *Escherichia coli* glycosylases excising analogous etheno adducts. DNA Repair, In press.
81. Habraken Y, Carter CA, Kirk MC, Ludlum DB. 1991. Release of 7-alkylguanines from *N*-(2-chloroethyl)-*N*′-cyclohexyl-*N*-nitrosourea-modified DNA by 3-methyladenine DNA glycosylase II. Cancer Res 51:499–503.
82. Marnett LJ. 1999. Chemistry and biology of DNA damage by malondialdehyde. Vol. 150. Lyon: IARC Sci Publ, pp 17–27.
83. Johnson KA, Fink SP, Marnett LJ. 1997. Repair of propanodeoxyguanosine by nucleotide excision repair in vivo and in vitro. J Biol Chem 272:11434–11438.
84. VanderVeen LA, Hashim MF, Nechev LV, Harris TM, Harris CM, et al. 2001. Evaluation of the mutagenic potential of the principal DNA adduct of acrolein. J Biol Chem 276:9066–9070.
85. Marnett LJ. 2002. Oxy radicals, lipid peroxidation and DNA damage. Toxicology 181–182:219–222.
86. Fink SP, Reddy GR, Marnett LJ. 1997. Mutagenicity in *Escherichia coli* of the major DNA adduct derived from the endogenous mutagen malondialdehyde. Proc Natl Acad Sci USA 94:8652–8657.
87. Yang I-Y, Hossain M, Miller H, Khullar S, Johnson F, et al. 2001. Responses to the major acrolein-derived deoxyguanosine adduct in *Escherichia coli*. J Biol Chem 276:9071–9076.
88. Choudhury S, Pan J, Amin S, Chung FL, Roy R. 2004. Repair kinetics of trans-4-hydroxynonenal-induced cyclic 1,N^2-propanodeoxyguanine DNA adducts by human cell nuclear extracts. Biochemistry 43:7514–7521.
89. Mao H, Reddy GR, Marnett LJ, Stone MP. 1999. Solution structure of an oligodeoxynucleotide containing the malondialdehyde deoxyguanosine adduct N^2-(3-oxo-1-propenyl)-dG (ring-opened M^1G) positioned in a (CpG)3 frameshift hotspot of the Salmonella typhimurium hisD3052 gene. Biochemistry 38:13491–13501.
90. de los Santos C, Zaliznyak T, Johnson F. 2001. NMR characterization of a DNA duplex containing the major acrolein-derived deoxyguanosine adduct gamma -OH-1,-N^2-propano-2′-deoxyguanosine. J Biol Chem 276:9077–9082.
91. Johnson KA, Mierzwa ML, Fink SP, Marnett LJ. 1999. MutS recognition of exocyclic DNA adducts that are endogenous products of lipid oxidation. J Biol Chem 274:27112–27118.
92. Chenna A, Maruenda H, Singer B. 1999. Synthesis of para-benzoquinone and 1,3-bis(2-chloroethyl)nitrosourea adducts and their incorporation into oligonucleotides. Vol. 150. Lyon: IARC Sci Publ, pp 89–101.
93. Gaskell M, Jukes R, Jones DJL, Martin EA, Farmer PB. 2002. Identification and characterization of (3″,4″-dihydroxy)-1,N^2-benzetheno-2′-deoxyguanosine 3′-monophosphate, a novel DNA adduct formed by benzene metabolites. Chem Res Toxicol 15:1088–1095.
94. Pongracz K, Bodell WJ. 1991. Detection of 3′-hydroxy-1,N^6-benzetheno-2′-deoxyadenosine 3′-phosphate by ^{32}P postlabeling of DNA reacted with *p*-benzoquinone. Chem Res Toxicol 4:199–202.
95. Bleasdale C, Kennedy G, MacGregor JO, Nieschalk J, Pearce K, et al. 1996. Chemistry of muconaldehydes of possible relevance to the toxicology of benzene. Environ Health Perspect 104(Suppl 6):1201–1209[Q3].
96. Chenna A, Hang B, Rydberg B, Kim E, Pongracz K, et al. 1995. The benzene metabolite p-benzoquinone forms adducts with DNA bases that are excised by a repair activity from human cells that differs from an ethenoadenine glycosylase. Proc Natl Acad Sci USA 92:5890–5894.
97. Hang B, Chenna A, Fraenkel-Conrat H, Singer B. 1996. An unusual mechanism for the major human apurinic/apyrimidinic (AP) endonuclease involving 5′ cleavage of DNA containing a benzene-derived exocyclic adduct in the absence of an AP site. Proc Natl Acad Sci USA 93:13737–13741.
98. Hang B, Chenna A, Sagi J, Singer B, 1998. Differential cleavage of oligonucleotides containing the benzene-derived adduct, 1,N^6-benzetheno-dA, by the major human AP endonuclease HAP1 and *Escherichia coli* exonuclease III and endonuclease IV. Carcinogenesis 19:1339–1343.
99. Hang B, Rothwell DG, Sagi J, Hickson ID, Singer B. 1997. Evidence for a common active site for cleavage of an AP site and the benzene-derived exocyclic adduct, 3,N^4-benzetheno-dC, in the major human AP endonuclease. Biochemistry 36:15411–15418.
100. Wilson DM 3rd, Barsky D. 2001. The major human abasic endonuclease: formation, consequences and repair of abasic lesions in DNA. Mutat Res 485:283–307.
101. Ischenko AA, Saparbaev MK. 2002. Alternative nucleotide incision repair pathway for oxidative DNA damage. Nature 415:183–187.
102. Gros L, Ishchenko AA, Ide H, Elder RH, Saparbaev MK. 2004. The major human AP endonuclease (Ape1) is involved in the nucleotide incision repair pathway. Nucleic Acids Res 32:73–81.
103. Gorman MA, Morera S, Rothwell DG, de La FE, Mol CD, et al. 1997. The crystal structure of the human DNA repair endonuclease HAP1 suggests the recognition of extra-helical deoxyribose at DNA abasic sites. EMBO J 16:6548–6558.
104. Mol CD, Izumi T, Mitra S, Tainer JA. 2000. DNA-bound structures and mutants reveal abasic DNA binding by APE1 and DNA repair coordination. Nature 403:451–456.
105. Beernink PT, Segelke BW, Hadi MZ, Erzberger JP, Wilson DM III, et al. 2001. Two divalent metal ions in the active site of a new crystal form of human apurinic/apyrimidinic endonuclease, Ape1: implications for the catalytic mechanism. J Mol Biol 307:1023–1034.

106. Guliaev AB, Hang B, Singer B. 2004. Structural insight into the recognition and repair of a benzene-derived DNA adduct, pBQ-C, by the major human AP endonuclease. Nucleic Acid Res 32:2844–2852.
107. Rothwell DG, Hang B, Gorman MA, Freemont PS, Singer B, Hickson ID. 2000. Substitution of Asp-210 in HAP1 (APE/Ref-1) eliminates endonuclease activity but stabilises substrate binding. Nucl Acids Res 28:2207–2213.
108. Bowman KK, Sidik K, Smith CA, Taylor JS, Doetsch PW, et al. 1994. A new ATP-independent DNA endonuclease from *Schizosaccharomyces pombe* that recognizes cyclobutane pyrimidine dimers and 6-4 photoproducts. Nucleic Acids Res 22:3026–3032.
109. Alleva JL, Zuo S, Hurwitz J, Doetsch PW. 2000. In vitro reconstitution of the *Schizosaccharomyces pombe* alternative excision repair pathway. Biochemistry 39:2659–2666.

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