Chemical biology of mutagenesis and DNA repair: cellular responses to DNA alkylation

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The reaction of DNA-damaging agents with the genome results in a plethora of lesions, commonly referred to as adducts. Adducts may cause DNA to mutate, they may represent the chemical precursors of lethal events and they can disrupt expression of genes. Determination of which adduct is responsible for each of these biological endpoints is difficult, but this task has been accomplished for some carcinogenic DNA-damaging agents. Here, we describe the respective contributions of specific DNA lesions to the biological effects of low molecular weight alkylating agents.

Introduction

DNA damage can be caused by radiation, by organic and inorganic chemical agents and by enzymes that have the roles of promoting natural methylation and deamination, such as members of the S-adenosylmethionine (SAM)-dependent methyltransferases, the activation-induced deaminase and the apolipoprotein B editing complex (1,2). Because DNA is abundantly equipped with nucleophilic sites, reaction with extracellularly generated and endogenously produced electrophiles results in an amazingly diverse array of covalent chemical-DNA adducts. These lesions compromise cellular welfare in three major ways (Figure 1). First, misreplication or misrepair of the lesions triggers mutations, which can be the initiating lesions of genetic diseases, including cancer. Second, the lesions can jeopardize the epigenetic program imprinted by natural enzymatic DNA modifications. Finally, the lesions can block RNA and DNA polymerases and can lead directly or indirectly to DNA strand breaks, which tend to be lethal in most cells. The biological importance of DNA damage is evidenced by the large commitment of the genome to protection of informational integrity; such genoprotective networks include electrophile scavengers, recombination complexes that permit DNA lesion tolerance, specialized polymerases that afford lesion bypass and a large battery of DNA repair proteins. Loss of one or more of these networks results in loss of informational integrity and, ultimately, the onset of disease (1).

Once it was appreciated that DNA lesions cause mutagenic and toxic events, researchers sought to understand the relationships between the structure of each lesion in DNA and the biological endpoints indicated above (3). For example, discovery of the mutagenic lesion of a carcinogenic DNA-damaging agent might lead to strategies to reduce the level of that lesion in DNA and hence reduce the likelihood of carcinogenesis. Studies on the DNA adducts of aflatoxin B1 led to intervention strategies at the population level that offer promise of reducing liver cancer burden (4). As a second example, knowledge of the relationship between the structures of DNA adducts of anticancer drugs and cytotoxicity endpoints can aid drug development efforts in clinical pharmacology. While it is obvious that establishing the relationships between DNA adducts and their biological endpoints is important, it proved very difficult to develop an experimental strategy to address the problem. Even a single simple DNA-damaging agent such as the aforementioned aflatoxin results in nearly a dozen DNA adducts, which frustrated early attempts to determine which adducts are the biologically important ones (5).

Dissection of the relative biological importance of individual DNA lesions proved to be a tractable problem with the advent of methodology whereby investigators could place one lesion at a time into synthetic DNA (Figure 2). In early in vitro studies, the oligonucleotides with adducts at known sites were acted upon with purified polymerases (Figure 2A) and repair proteins, which gave results that helped predict the biological relevance of a lesion and helped define the cellular repair systems that might protect against it. A second step involved the use of shuttle vectors that were globally modified by a DNA-damaging agent (Figure 2B). Chemical or enzymatic tools allowed the mapping of some (but not all) lesion sites along a stretch of DNA. The damage spectrum was then compared with the spectrum of mutations that arose when the modified vector was replicated within cells. Often multiple types of mutation were observed at a single site and it was impossible to ascertain if a single lesion gave rise to multiple mutations at, for example, a guanine site or whether there were several distinct guanine adducts each of which had its own signature and singular mutation. Nevertheless, this approach was and continues to be a cornerstone of mutation research.

The fusion of chemistry and biology, termed ‘chemical biology’, gave rise to a more advanced technology in which synthetic oligonucleotides containing well-characterized single DNA lesions were genetically engineered into the genomes of viruses or plasmids, which could be introduced into bacterial or mammalian cells (Figure 2C). Within the cell, the lesion would encounter the host repair and replication systems much in the same way that the lesion would be treated if it had formed endogenously. Lethal endpoints could be measured as a decrease in viral or plasmid progeny. Mutagenic outcomes could be determined by interrogating the vector genomes in the vicinity of the genomic site that originally contained the adduct. The relative importance of various DNA repair and polymerase systems to deal with or process the adduct could be determined by introduction of the vector into cell strains with known defects in repair or replication. In time, the quantitative and qualitative features of mutagenesis and toxicity of a wide array of DNA-damaging agents were profiled by this new technology.

This review examines in detail the application of a variety of experimental systems, primarily the use of site-specifically modified vector genomes, to categorize the mutagenic and toxic properties of DNA alkylation agents. Such agents are common environmental carcinogens, some are formed endogenously and cause spontaneous DNA damage and some have found use as cancer chemotherapeutic agents. The paper specifically reviews current knowledge of the biological properties of each of the lesions formed by low molecular weight alkylating agents. The structures of the relevant lesions are shown in Figure 3. By compiling data on lesion mutagenicity, genotoxicity and reparability, we develop a biological ‘fingerprint’ for each lesion (Table 1). It is noteworthy that some lesions have mutagenicities at or approaching 100%, whereas others display comparably mutagenic properties; however, it must be kept in mind that a lesion with a mutagenicity of only 0.1% creates multiple mutations at a rate that is five orders of magnitude greater than the basal or spontaneous rate of mutagenesis. In the review, exocyclic monoadducts are covered first, followed by adducts in...
which endocyclic atoms are the points of attachment to the alkyl residue. The final sections of the review cover small cyclic adducts. To keep the manuscript of a manageable size, we have limited our attention to adducts of one or two carbon residues, avoiding larger adducts and some of the lipid-derived adducts that have been reviewed elsewhere (6).

**O^6^-methylguanine and O^4^-ethylguanine**

O^6^-methylguanine (O^6^MeG), which causes G → A transitions (7), is the primary mutagenic lesion under most conditions of alkylation damage to the genome (8). O^6^MeG is formed from both endogenous (9,10) and exogenous sources (11), and studies have correlated its persistence to organ-specific tumorigenicity in rats (12). O^4^-ethylguanine (O^4^EtG) is the major mutagenic lesion formed by ethylating agents (13) and also primarily causes G → A transitions (14).

*Escherichia coli* has two O^6^MeG-DNA methyltransferases that can repair the adduct—the constitutive Ogt protein and the inducible Ada protein, which directly reverse methylation damage by transferring the alkyl group to one of the internal cysteine residues on each repair protein. This transfer reversibly inactivates the repair proteins, making the non-enzymatic stoichiometric reaction ‘suicidal’ (15). Ada is part of the adaptive response, which was discovered when *E.coli* treated with a low dose of a methylating agent acquired resistance to the mutagenicity and toxicity of subsequent higher doses (16). The alkyl groups from O^6^AlkGua and O^6^AlkThy are transferred to Cys-321 at the C-terminus, whereas those from a third substrate, methylphosphotriester (MePT), is transferred to the N-terminus of Ada. It was initially believed that the methyl group from MePT was transferred to Cys-69 on the protein (17) but recent evidence identifies Cys-38 as the acceptor residue (18). Methylation at this site reduces the overall negative charge on Ada. Methylation of Cys-38 residues in Ada since it is not part of a network of hydrogen bonds.

The ability of a DNA lesion (lollipop structure) to block polymerases *in vitro* and cause mispairing during DNA synthesis can be evaluated in a system in which a template containing the lesion is primed with a complementary oligonucleotide that terminates to the 3′ side of the lesion. DNA synthesis may result in incorporation of non-complementary bases or in truncated products, which can be evaluated on sequencing gels. The same *in vitro* constructs, in double-stranded or single-stranded form, can also be used as substrates for DNA repair reactions using purified DNA repair proteins or cellular extracts. (B) To determine the mutagenic properties of the full population of adducts that form from treatment of DNA with a mutagen, a plasmid or viral vector is treated with the damaging agent. Replication of the vector in cells results in repair of some adducts but those that evade repair can possibly be converted into mutations. Sequencing the genomes of progeny can generate the mutational spectrum, which indicates the types and frequencies of specific mutations along the DNA sequence being studied. In parallel, one can map the locations of some of the DNA adducts by using enzymatic or chemical probes. The corresponding damage spectrum is often compared with the mutational spectrum in order to formulate hypotheses with regard to which DNA adduct might have caused specific mutations. (C) The most sophisticated system for analysis of mutagenesis involves chemical or enzymatic synthesis of an oligonucleotide that contains a candidate for mutagenesis (often the candidate is nominated based on the data from experiments shown in part B). The oligonucleotide is inserted into the genome of a virus or plasmid, which is later replicated within cells, either intra- or extra-chromosomally. Progeny are analyzed to determine the type, amount and genetic requirements for mutagenesis by the lesion. In parallel, the reduction in viable progeny is determined as an estimate of the extent to which each lesion inhibits replication of the genome.

Converting it to a transcriptional activator of the genes encoding the ‘adaptive response’ to alkylating agents, namely, ada, alkA, alkB and aidB. This is the most nucleophilic of all available cysteine residues in Ada since it is not part of a network of hydrogen bonds. Methylation at this site reduces the overall negative charge on Ada. Reduction in charge density is important for the role of Ada as a transcription factor as it enhances its interaction with negatively charged DNA by 1000-fold (19). The number of Ada molecules is estimated to rise from one to two molecules in an unadapted state to ~3000 molecules in a fully adapted cell (20,21). It was initially found that Ada preferentially repairs O^6^MeG as compared with O^4^-methylthymine (O^4^MeT) (22) but recent evidence suggests that it repairs both lesions with equal efficiency (23).

The second DNA methyltransferase, Ogt, was discovered by deletion of the *ada* operon (24,25). Unlike Ada, Ogt is constitutively expressed in *E.coli*, shows a preference for repair of O^6^MeT and larger alkyl adducts and does not repair MePT (25). It is estimated that there are ~30 molecules of Ogt in wild-type *E.coli* (21). The mammalian homolog of Ogt and Ada is O^6^-methylguanine-DNA methyltransferase (MGMT) (also referred to as AGT). This enzyme works in...
a similar suicidal fashion but is not inducible, and it shows a 35-fold higher preference for repairing $O^6$MeG over $O^4$MeT (23). Human MGMT can be silenced by epigenetic modifications (26). This silencing plays a dual role in carcinogenesis as tumors not expressing MGMT acquire a mutator phenotype but also become more susceptible to killing by alkylating agents (27).

Ogt is speculated to provide protection at low levels of sporadic exposure to alkylating agents, whereas the adaptive response becomes more important against higher chronic exposures or acute exposures that trigger the transcriptional switch of the adaptive response operon. In addition to the methyltransferases, the UvrABC nucleotide excision repair (NER) pathway can also repair $O^6$MeG. Excision of $O^6$MeG on duplex substrates has been shown to occur in vitro (28) and in vivo (29). When $O^6$MeG is present in a single-stranded context in vivo, NER does not affect mutation frequency of the lesion; the mutation frequencies in E.coli uvrB$^+$/ada$^+$/ogt$^+$/cells are very similar to those found in uvrB$^-$/ada$^-$/ogt$^-$/cells (30). Interestingly, Chambers et al. (31) found a 40-fold decrease in the G $\rightarrow$ A transition caused by an $O^6$MeG lesion introduced on a single-stranded ΦX174 genome in an NER-deficient (uvrA) cell strain versus wild-type. The authors suggest a shielding mechanism by which UvrA binds to the lesion and protects it from repair by Ada or Ogt, leading to elevated mutation frequencies. There is some evidence of the NER pathway playing a role in repair of $O^6$MeG in Drosophila melanogaster (32) and of $O^6$EtG in D.melanogaster (33) and mammalian cells (34).

The mismatch repair (MMR) pathway has also been implicated in the cellular response to $O^6$MeG (35). $O^6$MeG can be processed by post-replicative MMR in E.coli in a double-stranded context, but in a single-stranded context (a gapped plasmid), the mutation frequencies in wild-type and mutS$^-$ cells are the same (36). Using an M13 single-stranded system containing an $O^6$MeG lesion, Rye et al. (37) have shown that dam$^-$ and mutH$^-$ strains display the same mutation frequency as wild-type, but mutS$^-$ and mutL$^-$ strains show a decrease. This result suggests that MMR proteins may aid in the repair of $O^6$MeG in a cooperative fashion. Whereas early work suggested that $O^6$EtG is not repaired by alkyltransferases or MMR in E.coli (38), more recent studies suggest that it is repaired by the same machinery that repairs $O^6$MeG in mammalian cells (39). Nevertheless, in rat mammary cells, $O^6$EtG is repaired 20 times faster than $O^6$MeG by an unknown, MGMT-independent mechanism (40). In line with expectations based upon this finding, a G $\rightarrow$ A mutation is not seen as a frequent event at codon 12 of the H-ras gene in tumors initiated by N-ethyl-N-nitrosourea compared with tumors initiated by N-methyl-N-nitrosourea (MNU).

The toxicity of $O^6$MeG has been established by several studies, and it appears that abortive MMR or inhibition of replication systems may play roles in converting the adduct into lethal intermediates. Evidence that $O^6$MeG is potently toxic in mammalian cells comes from a number of studies, including those in MGMT knockout mice, which display hypersensitivity to the lethal effects of alkylating agents that generate $O^6$MeG (41). There are two proposed mechanisms by which this lesion contributes to the toxicity generated by alkylating agents. The first suggests that the lesion reduces the efficiency of replication by polymerases. This phenomenon has been studied using in vitro systems. The rates of replication by T4 and T5 phage DNA polymerases and E.coli polymerase I decrease linearly with increasing proportion of $O^6$MeG in the synthetic oligonucleotide used as a template (42). Also, human polymerase $\beta$, subcloned in an E.coli plasmid, is blocked by $O^6$MeG present on a single-stranded DNA template (43). The second mechanism leading to toxicity is that of futile cycling of the MMR system at an $O^6$MeG:T pair (44,45). The model proposes recognition of this base pair by the MMR enzymes, which results in the removal of the newly incorporated thymine from the nascent strand opposite the lesion. On re-replication, $O^6$MeG
preferentially pairs once again with an incoming thymine (7), reinitiating the repair and replication cycle. This persistent iteration of excision and synthesis is thought to result in a stabilized nick or small gap in one strand of DNA, which may activate damage signaling pathways (46). The recursive cycling mechanism is thought to be of practical significance in that it may explain the lethal effects of the anticancer drug, temozolomide (47). In *E.coli*, O\textsubscript{6}MeG is more toxic than O\textsubscript{6}EtG (38) but the mechanism underlying this differential toxicity is unknown.

### Table I. Mutagenicity, genotoxicity and repairability of DNA alkylation lesions

<table>
<thead>
<tr>
<th>Lesion</th>
<th>Mutagenic specificity</th>
<th>Genotoxicity</th>
<th>Repaired by prokaryotic enzymes/systems</th>
<th>Repaired by eukaryotic enzymes/systems</th>
</tr>
</thead>
<tbody>
<tr>
<td>O\textsuperscript{6}MeG</td>
<td>G → A</td>
<td>Toxic in presence of MMR</td>
<td>Ada, Ogt, UvrABC (NER), MMR</td>
<td>MGMT, NER, MMR</td>
</tr>
<tr>
<td>O\textsuperscript{6}EtG</td>
<td>G → A</td>
<td>Toxic in <em>Escherichia coli</em></td>
<td>Not repaired by Ada or Ogt</td>
<td>MGMT, NER, MMR</td>
</tr>
<tr>
<td>O\textsuperscript{6}MeC</td>
<td>T → C</td>
<td>Possibly toxic</td>
<td>Ada, Ogt</td>
<td>NER</td>
</tr>
<tr>
<td>O\textsuperscript{6}MeT</td>
<td>T → A in MMR-deficient cells</td>
<td>Possibly toxic</td>
<td>Ada</td>
<td>MGMT (minimal)</td>
</tr>
<tr>
<td>MePT</td>
<td>Not known</td>
<td>Not known</td>
<td>Ada</td>
<td></td>
</tr>
<tr>
<td>1MeA</td>
<td>A → T</td>
<td>Mutagenic and toxic to <em>E.coli</em> in the absence of AlkB</td>
<td>AlkB, AAG</td>
<td>AlkB, ABH2, ABH3</td>
</tr>
<tr>
<td>1EtA</td>
<td></td>
<td>Mutagenic and toxic to <em>E.coli</em> in the absence of AlkB</td>
<td>AlkB, AAG</td>
<td>AlkB, ABH2, ABH3</td>
</tr>
<tr>
<td>3MeA</td>
<td>A → T</td>
<td>Highly toxic</td>
<td>AlkA, Tag, UvrA</td>
<td></td>
</tr>
<tr>
<td>7MeA</td>
<td>Fapy-7MeA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1MeG</td>
<td>G → T in the absence of AlkB</td>
<td>AlkB, AAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3MeG</td>
<td>G → A</td>
<td>Possibly toxic</td>
<td>AlkA, Tag</td>
<td></td>
</tr>
<tr>
<td>7MeG</td>
<td>Fapy-7MeG</td>
<td>Toxic via formation of AP sites and Fapy-7MeG</td>
<td>AlkA, Fpg, hOGG1</td>
<td>AlkA, Fpg, hOGG1</td>
</tr>
<tr>
<td>3MeC</td>
<td>C → T in the absence of AlkB</td>
<td>AlkB, AAG</td>
<td>AlkB, ABH2, ABH3</td>
<td></td>
</tr>
<tr>
<td>3EtC</td>
<td>C → T</td>
<td>Toxic</td>
<td>AlkB</td>
<td></td>
</tr>
<tr>
<td>3MeT</td>
<td>AlkB-deficient cell</td>
<td>Strong block to replication</td>
<td>Weak substrate for AlkB</td>
<td>FTO</td>
</tr>
<tr>
<td>8MeG</td>
<td>G → C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>eA</td>
<td>AlkB-deficient cell A → T in the absence of AlkB</td>
<td>AlkB, AAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A → G</td>
<td></td>
<td>AlkB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EA</td>
<td>A → T (weak) in the absence of AlkB</td>
<td>AlkB, AAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A → C (weak)</td>
<td></td>
<td>Weak substrate for AlkA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A → G (weak)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1,2-eG</td>
<td>G → T</td>
<td>Mutagenic and causes frameshift</td>
<td>AlkB, AAG</td>
<td></td>
</tr>
<tr>
<td>G → C</td>
<td></td>
<td>AAG, MUG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2,3-eG</td>
<td>G → A</td>
<td>Mutagenic</td>
<td>AlkB, AAG</td>
<td></td>
</tr>
<tr>
<td>C → T</td>
<td>Mutagenic and toxic to <em>E.coli</em> in the absence of AlkB</td>
<td>AlkB, hTDG, dsUDG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1,2-eG, 1,N\textsuperscript{2}-Etheno-2,3-guanine; 2,3-eG, 3,N\textsuperscript{2}-etheno-2,3-guanine; Fpg, formamidopyrimidine-DNA-glycosylase; FTO, Fat mass and obesity associated protein; O\textsuperscript{6}MeC, O\textsuperscript{6}-methylcytosine; O\textsuperscript{6}MeT, O\textsuperscript{6}-methylthymine.

O\textsuperscript{6}MeG is known to be highly mutagenic. To study the mutations formed in *vivo*, Loechler et al. (7) constructed single-stranded M13mp8 DNA containing O\textsuperscript{6}MeG at a specific position and transfected the same into *E.coli*. It was found that the predominant mutation generated by this lesion was a G → A transition. In wild-type *E.coli*, the lesion was weakly mutagenic, but challenging the Ada and Ogt repair systems of the cell by treatment with N-methyl-N’-nitro-N-nitrosoguanidine (MNNNG; which forms alkyl adducts in the host genome) resulted in a robust, dose-dependent demonstration of the
mutagenic power of this adduct (7). This early study showed how significant even a few molecules per cell of a DNA repair protein could be as a protection against DNA damage. The ethyl homolog of \( O^6\)MeG, \( O^6\)EtG, introduced at a specific position in \( \phi X174 \) and transfected into \( E. coli \) produces higher mutation frequencies compared with \( O^6\)MeG in the same system (48,49). \( O^6\)MeG and \( O^6\)EtG also have been site-specifically incorporated in Chinese hamster ovary cells and are shown to have a mutation frequency of 19 and 11%, respectively, in cells lacking \( O^6\)-alkylguanine-DNA alkyltransferase (30).

A recent study used site-specific mutagenesis to generate single-stranded M13mp7 genomes containing \( O^6\)MeG in all 16 possible permutations and combinations of nearest neighbor sequence contexts. These genomes were then introduced into \( E. coli \) mutants of different repair backgrounds and the mutation frequencies were determined by a novel and very sensitive assay. It was found that \( O^6\)MeG went from being 10% mutagenic in repair-proficient cells to 100% mutagenic in repair-deficient cells (30). Moreover, it was found that DNA repair \textit{in vivo} is sequence context dependent.

With regard to effects on gene expression, \( O^6\)MeG can inhibit carbon-5 methylation of cytosines in 5'-deoxycytidine-deoxyguanosine-3' (dCpG) motifs by interfering with the binding of 5-methylcytosine DNA methyltransferases; eventually, this interference with natural methylation can lead to genome hypomethylation. The pairing of \( O^6\)MeG with thymine can also lead to DNA hypomethylation (51). By these mechanisms, the formation of this adduct could affect the epigenetic program of mammalian cells.

\textbf{4-Methylthymine}

\( O^4\)MeT is one of the mutagenic lesions formed concurrently with \( O^6\)MeG when DNA is exposed to alkylating agents that react with DNA by an \( S_21 \) mechanism. \( O^4\)MeT is formed at a much lower level than \( O^6\)MeG, for example, the thymine lesion was detected at a level 126 times lower than that of \( O^6\)MeG in calf thymus DNA treated with MNU (52). Although it is not an abundant lesion, \( O^4\)MeT can be very mutagenic. Using site-specific mutagenesis tools, it was shown that \( O^4\)MeT incorporated in single-stranded M13mp19 had a mutation frequency of 12% in repair-proficient \( E. coli \). \( O^4\)MeG gave a mutation frequency of <2% in the same repair-proficient system. Pre-treatment with MNNG to deplete or occupy endogenous DNA methyltransferases; eventually, this interference with natural methylation can lead to genome hypomethylation. The pairing of \( O^4\)MeG with thymine can also lead to DNA hypomethylation (51). By these mechanisms, the formation of this adduct could affect the epigenetic program of mammalian cells.

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\textbf{N1-Methyladenine and N1-Ethyladenine}

N1-methyladenine (1MeA) is formed by alkylating agents mainly in single-stranded DNA and has been detected \textit{in vitro} (62,63,64,65, 66,67,68) and \textit{in vivo} (64,69,70,71,72). \( S_2 \)G residues, such as methylthanesulfonate (MMS) and the naturally occurring methyl halides, can generate 1MeA (15); similarly, the ethyl homolog, N1-ethyladenine (1EtA), is formed by ethylating agents both \textit{in vitro} and \textit{in vivo} (64). The preference for formation in single-stranded DNA is owed to the extent of resistance to the cytotoxic effects of alkylating agents, similar sister chromatid exchange induction, as well as host-cell reaction of adenovirus (61). This observation suggests that MePT may not have cytotoxic effects in cells. The role of MePT seems to be a chemosensor for detection of methylation damage and induction of the adaptive response in \( E. coli \), but their role, if any, in eukaryotes is unknown.

\( O^6\)Methylguanine and 5-Methylthymine

5-Methylthymine can lead to genome hypomethylation. The pairing of \( O^6\)MeG with thymine can also lead to DNA hypomethylation (51). By these mechanisms, the formation of this adduct could affect the epigenetic program of mammalian cells.

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AlkB-proficient cells (75), underscoring the physiological relevance of the AlkB system for countering the toxicity of this base. While very toxic, as indicated above, 1MeA is at best weakly mutagenic. To the extent that it is mutagenic, 1MeA induces A to T mutations, which are enhanced following induction of the SOS polymerases. The base composition for A versus T was, respectively, 99 versus 0.61% in SOS/AlkB−/C0 cells, 99.7 versus 0.06% in SOS+/AlkB+ cells, and 98.6 versus 1.0% in SOS+/AlkB+ cells (75).

While the AlkB protein can repair the 1EtA lesion, it cannot repair 3-ethyladenine damage, which parallels AlkB’s activity on 1MeA but not on 3-methyladenine (76). AlkB repairs 1EtA somewhat less well than 1MeA.

N3-methyladenine

N3-methyladenine (3MeA) can be formed in DNA by methylating agents as well as non-enzymatically by intracellular SAM. In a mammalian cell, SAM or some other methylating agent reacts with DNA to generate an estimated 600 3MeA per day (77). The half-life of 3MeA in vivo is estimated to be between 4–24 h (78). While 3MeA is not particularly mutagenic, it is a cytotoxic DNA lesion by virtue of its ability to block replication or by virtue of its ability to give rise to a chemically or enzymatically generated abasic/apurinic site (AP site).

With regard to replication inhibition, it is thought that the methyl at the N3 position of purines sterically interferes with the required contact between the polymerase and minor groove on DNA (79). This property makes it essential for the cell to have in place defenses against this form of damage. 3MeA-DNA-glycosylases have evolved in both prokaryotic and eukaryotic systems to afford the efficient repair of this lesion. The prokaryotic system includes the highly selective and constitutive 3-methyladenine-DNA glycosylase I (TAG) protein and the inducible AlkA glycosylase with a broader specificity. The eukaryotic system is comprised of human 3MeA-DNA-glycosylase (AAG) and N-methylpurine-DNA glycosylase (MPG). AlkA and TAG repair 3MeA with equal efficiency on double-stranded DNA, but AlkA is 10- to 20-fold more efficient on single-stranded DNA (80). There is also evidence that UvrA, an ATPase and DNA-binding protein of the NER pathway, may be able to mitigate the cytotoxic effects of this lesion. One study used a neutral DNA equilibrium binding agent, Me-lex [N-methylpyrrolecarboxamide dipeptide (lex) modified with an O-methyl sulfonate ester functionality], to introduce selectively 3MeA lesions in the minor groove of DNA. It was shown that this agent shows increasing toxicity to E.coli mutants lacking one or more repair excision repair (BER) enzyme (AlkA), two BER enzymes (AlkA and TAG) or both BER and NER repair capabilities (AlkA, TAG and UvrA), in that order (81).

3MeA is not considered to be a seriously promutagenic lesion based upon work done in bacterial and in yeast systems. In 3MeA-DNA-glycosylase I (tag)-deficient E.coli mutants, treatment with MNU leads to a 5-fold increase in mutation frequency only under SOS-induced conditions. Furthermore, in repair-proficient cells, removal of 3MeA from the DNA does not show a significant difference in mutagenesis in SOS-induced versus SOS-uninduced cells (82). To study the mutational profile of 3MeA in eukaryotic cells, the p53 gene cDNA on a yeast expression vector was treated with Me-lex in vitro and transfected into a yeast strain containing the p53-dependent reporter ADE2 gene. The results show that Me-lex is a weak mutagen compared with MNU but that it induces A → T transversions as the most common genetic change (40% of all mutations) (83). Mutagenicity increased 2- to 3-fold in 3MeA-glycosylase-deficient strains, which suggests that the lesion driving the mutations is 3MeA (84).

Interestingly, the methylated adenosines in Me-lex-treated DNA give rise to mutations in a strictly sequence-specific manner. The cytotoxicity of 3MeA is well established in the literature. In vitro studies showing chain termination one nucleotide 3' to adenosines in methylated DNA templates pointed to 3MeA as a strong block to DNA replication. 3MeA in DNA has also been shown to be toxic in E.coli (81). Using Me-lex in combination with 3-methyladenine-DNA-glycosylase-proficient and -deficient cell lines, Engelward et al. (85) showed that 3MeA can cause p53 induction, S phase arrest, sister chromatid exchange, chromosome aberrations and apoptosis in mammalian cells. As with N7-methylguanine (7MeG), enhanced repair of 3MeA by DNA-glycosylases of the BER pathway can lead to a flood of AP sites that can also contribute to mutations and lethality (86).

N7-methyladenine

N7-methyladenine (7MeA) is a minor lesion formed at a level 40-fold below that of 7MeG, which is typically the most abundant lesion in alkylated DNA (87). Like 7MeG, 7MeA possesses a cationic imidazole ring, which facilitates depurination and, alternatively, can favor hydrolysis of the five-membered ring to form the formamidopyrimidine (Fapy) derivative. Fapy-7MeA; this latter hydrolysis reaction is especially favored for the 7MeA in RNA (88), which has a stabilized glycosidic bond as compared with DNA. The half-life of 7MeA in DNA in vivo is only 2–3 hours, which is similar to its half-life in vitro at pH 7.2, 37°C (89). Fapy-7MeA is a mutagenic lesion displaying A → G transitions in single-stranded M13mp18 DNA transfected into SOS-induced E.coli (87,90). In these studies, dimethylsulfate-treated DNA was compared before and after treatment with alkali, which hydrolyzed the imidazole rings of N7-methylated adenosines and guanines, forming the Fapy derivatives. Dimethylsulfate- and alkali-treated DNA was 60-fold more mutagenic than DNA treated with dimethylsulfate alone and showed mutations primarily at A:T sites.

N1-methylguanine

N1-methylguanine (1MeG) has been found both in vitro (67) and in vivo (91). With regard to biological relevance, the AlkB protein can repair 1MeG both in vitro and in vivo (75, 92). The glycosylase AAG, which repairs 3MeA and a range of other lesions, is also active against 1MeG in vitro (93) but the in vivo relevance of AAG against this adduct has not been established as yet. 1MeG is a very strong block to replication, which can be partially overcome when the DNA lesion is partially repaired by AlkB; lesion bypass of 1MeG in vivo increases 8-fold from 2% in AlkB- cells to 16% in AlkB+ cells. Similarly, AlkB causes a reduction in the mutagenicity of 1MeG from a very high frequency of 80% in AlkB- cells to 4% in AlkB+ cells. Taken together, these data indicate that AlkB is a powerful protection against the mutagenic activity of this dangerous alkylated base. The mutational fingerprint of 1MeG reveals G → T (57% of all progeny), G → A (17%) and G → C (6%) mutations. In many instances, the induction of the SOS bypass polymerases results in increased bypass of a given lesion at the expense of reduced fidelity at the site of damage; however, the SOS polymerases are somewhat anti-mutagenic when they bypass this modified base (75).

N3-methylguanine

N3-methylguanine (3MeG) is thought to block replication in the same way as 3MeA does, but it is formed in DNA at a 15-fold lower level. The half-life of 3MeG in vivo has been shown to be 3–4 hours (89). It has been shown that E.coli alkB mutants are sensitive to alkylating agents even though they express Tag (94), which repairs 3MeA (a known cytotoxic lesion) as efficiently as AlkA on double-stranded DNA (80). This result suggests that 3MeG contributes to the toxic effects of alkylation seen in these cells.

Using cell extracts from adapted E.coli, it was shown that the AlkA protein can repair 3MeG present on methylated DNA in vitro. The same study also shows persistence of this adduct in unadapted E.coli 30 min after exposure to MNNG (95). A second in vitro study has shown that Tag also repairs 3MeG present on a synthetic G:C-rich double-stranded DNA sequence, albeit with an efficiency of only 1/70th that of AlkA (96).

N7-methylguanine and its degradation products

The N7 atom of guanine is the most chemically vulnerable site to attack by alkylating electrophiles as it has the highest negative electrostatic potential of all the other atoms within the DNA bases (97). This property also makes it a highly reactive ligand for metal ions such as platinum (98). When double-stranded DNA is treated with MMS or MNNG, 82 and 67% of the methylation occurs on the N7
in vitro and the naturally occurring methyl halides (15) preferentially in lesions to a striking 70%. Although investigations involving replication polymerases in the absence of AlkB increases the mutagenicity of both /3MeC and 3EtC are bypass polymerases. With regard to mutagenic potential, if a cell has a portion of the toxicity can also be overcome by induction of the SOS gene are still under investigation.

From the stand-point of its potential to induce genetic change, 3MeT is ~60% mutagenic in SOS /AlkB cells, providing mostly T → A (47%) and T → C (9%) mutations. Studies performed in vitro also show that 3MeT is a strong block to the Klenow fragment of DNA polymerase I, which slightly increases deoxothymidine triphosphate (dTPP) incorporation on a poly(dC-d3MeT) template (117); interestingly, T is exclusively incorporated opposite the analogous 3-ethyldeoxothymidine adduct in one study (118), whereas A is exclusively incorporated in another (119).

8-Methylguanine
C8-alkylated DNA bases exist but have not been reported extensively in the literature. Recent studies have suggested that carbon-centered radicals can be a source of C8-alkylated lesions. 8-Methylguanine (8MeG) was shown to be produced in vitro in RNA (120) and DNA (121) by methyl radicals generated by oxidation of 1,2-dimethylyhydrazine and methylhydrazine, respectively. Proof of in vivo DNA alkylation by carbon-centered radicals was given by Bernardi et al. (122) who detected 8MeG in DNA isolated from the liver and colon of rats administered 1,2-dimethylyhydrazine. Other studies have shown that this lesion can also be produced in vitro and in vivo by genotoxic agents such as tert-butylhydroperoxide, diazooquinones and arenediazonium ions (123). These findings are significant as they suggest a possible contribution of 8MeG in the carcinogenic effects of these agents, especially 1,2-dimethylyhydrazine, which induces adenocarcinomas of the colon in rodents.

Site-specific studies using 8MeG-containing oligonucleotides prepared by phosphoramidite synthesis have explored the mutagenicity and toxicity of this lesion. It was found that 8MeG on the template strand blocks in vitro extension of DNA by mammalian polymerase α, but not by the E.coli Klenow fragment (124). The products from the primer extension reaction were then analyzed for mutations. 8MeG was found to direct exo-Klenow fragment-based incorporation of dCMP most of the time (77%) but also paired occasionally with deoxyguanosine monophosphate (dGMP) (1.1%) and dAMP (0.41%). Similar numbers were obtained for extension assays with mammalian polymerase α. Replication with the Klenow fragment also introduced small amounts of one (0.38%) and two (0.81%) base-pair deletions (124). These numbers mirror the thermodynamic stability of the 8MeG-deoxynucleoside monophosphate base (dNMP) pair, decreasing in the order dCMP > dGMP > dAMP >> dTMP. 1,2-Dimethylyhydrazine induces both O6MeG and 8MeG in similar amounts in the DNA of rats (122). However, the mutation frequencies of 8MeG are two orders of magnitude less than those of O6MeG (125). Therefore, we may conclude that 8MeG is a weakly mutagenic lesion that in principle can contribute to G → C transversions in cells.

position of guanine, respectively (13). Within the cell, 7MeG is produced at the rate of 4000 residues/human genome/day by the non-enzymatic reaction of SAM with DNA (77), and its steady-state level in repair-proficient cells is estimated to be 3000 bases (99). 7MeG has been detected in human DNA at the level of a few adducts per 10^7 bases (100). 7MeG by itself does not have any major mutagenic or cytotoxic effects. However, methylation at the N7 position destabilizes the N-glycosidic bond leading to spontaneous depurination of this lesion (101) and the resulting AP sites are toxic. AP sites can also be formed duringrepair of 7MeG by N-alkylpurine DNA-glycosylases, which are part of the BER pathway. Although not examined directly in the context of alkylation, the mutagenic and toxic properties of AP sites have been thoroughly investigated (86).

In addition to its role as a source of AP sites, 7MeG can manifest toxicity by converting to its imidazole ring-opened form. Hydrolysis of the imidazole ring of 7MeG forms 2,6-diamino-4-hydroxy-5-N3-methylformamidopyrimidine (Fapy-7MeG). Whereas this lesion does not cause mispairing with deoxyadenosine monophosphate (dAMP) or deoxythymidine monophosphate (dTMP), in vitro experiments using E.coli DNA polymerase I and poly[5'-deoxyguanosine-deoxycytidine-3' (dGpC)] templates (102) or Klenow fragment and M13mp18 template DNA (103) show that Fapy-7MeG blocks DNA chain elongation. Fapy-7MeG lesions present on M13mp18 phage template DNA also lead to a 2- to 3-fold increase in G → C and G → T transversions when transferred into SOS-induced E.coli (87). However, DNA polymerase I preferentially incorporates deoxythymidine monophosphate (dCMP) opposite Fapy-7MeG and a Fapy-7MeG:C pair is extended most efficiently compared with other possibilities. This property makes Fapy-7MeG a lesion with weak mutagenic potential (88).

In E.coli, AlkA is known to excise 7MeG from methylated DNA (95). In humans, this reaction is carried out by AAG/MPG (104). There exist specific DNA-glycosylases in E.coli [formamido-pyrimidine-DNA-glycosylase (Fpg)] and mammalian cells [human 7,8-dihydro-8-oxoguanine DNA glycosylase (hOGG1)] (105) that remove Fapy-7MeG lesions. Escherichia coli Fpg repairs 7MeG very efficiently, with a K_m in the nanomolar range (88). It has been shown in a mammalian cell line by site-directed mutagenesis that over-expression of MPG sensitizes cells to alkylation damage by converting 7MeG into toxic AP sites, which lead to strand breaks. 7MeG by itself is not toxic to cells, nor is over-expression of MPG, but in combination, they can overwhelm the cell with AP sites leading to cytotoxicity. Rimne et al. (106) propose that these two aspects combined with appropriate delivery systems could be exploited for the selective targeting of tumor cells, thereby reducing the peripheral effects of DNA damage by drugs.

N3-methylcytosine and N3-ethylcytosine
N3-methylcytosine (3MeC) is formed by S92 agents such as MMS and the naturally occurring methyl halides (15) preferentially in single-stranded DNA. It has been detected both in vitro (62,64–68,107,108) and in vivo (64,70,71,91,108). The corresponding ethyl homolog, N3-ethylcytosine (3EtC), is formed by ethylating agents in single-stranded DNA and also has been detected in vitro (64,65) and in vivo (64,109). As with the 1-alkyladenines, these lesions probably exist only or predominantly in single-stranded DNA because this site of modification is normally protected by base pairing (75). 3MeC stalls DNA synthesis and is likely to be toxic (15).

In E.coli, the AlkB protein has good activity against 3MeC and 3EtC both in vitro and in vivo (62,63,75). The appreciable mutagenesis and toxicity of the 3-alkylcytosines in vivo is decimated by AlkB, although a portion of the toxicity can also be overcome by induction of the SOS bypass polymerases. With regard to mutagenic potential, if a cell has no AlkB and uninduced SOS bypass polymerases, 3MeC and 3EtC are 30% mutagenic, with the predominant mutations being C → T and C → A. Basal expression of AlkB of a few molecules per cell abrogates the mutagenicity of 3MeC and 3EtC, whereas expression of SOS bypass polymerases in the absence of AlkB increases the mutagenicity of both lesions to a striking 70%. Although investigations involving replication past 1MeA and 1MeG, which similarly have a blocked Watson–Crick hydrogen bonding face, by DNA polymerases in vitro are lacking, it is known from in vitro studies that 3MeC inhibits replication by DNA polymerase I and does not cause mutation (107,110,111). However, some adduct bypass occurs with the incorporation of dAMP and dTMP opposite 3MeC (107). Therefore, the rules for misreplication of the 3-alkylcytosines lesions are the same both in vitro and in vivo, although the replicative system in cells is capable of a much higher mutation rate than is achieved in vivo (75,107).

N3-methylthymine
N3-methylthymine (3MeT) has been found both in vitro (64,65,67,68,112) and in vivo (112,113) and is formed through the reaction of DNA with S92 alkylating agents such as MMS. This adduct is a very weak substrate for AlkB, and it is a strong block to replication in vivo, which can be only slightly overcome by SOS bypass polymerase induction (75). Recently, fat mass obesity associated protein (FTO) has been shown as a 2-oxoglutarate-dependent demethylase for nucleic acid (114,115). FTO can efficiently repair 3MeT in single-stranded DNA but not in double-stranded DNA; it also shows strong activity on the demethylation of 3-methyluracil in single-stranded RNA (114,116). While there are numerous epidemiological studies associating the FTO gene with obesity, the biological basis for metabolic effects of this gene is still under investigation.

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The repair of 8MeG has been studied in vitro by Gasparutto et al. (123). In this study, the authors incorporated 8MeG site specifically into oligonucleotides and probed the ability of bacterial, yeast and mammalian glycosylases to repair this lesion. Of the extensive list of enzymes evaluated, only AlkA was able to excise 8MeG. Human MPG did not repair 8MeG, nor did any of the glycosylases involved in repair of oxidative damage (Fpg, Nth of E.coli; Ntg1, Ntg2, Ogg1 of Saccharomyces cerevisiae and human Ogg1) (123).

8MeG has been shown to stabilize the Z-conformation of DNA in short oligonucleotides even in low salt concentrations. This property may be relevant in vivo as Z-DNA is thought to have a role in the regulation of DNA supercoiling (126). This lesion is also used as a chemical modification to stabilize quadruplex structures of G-rich sequences of DNA, which are proposed to have a role in telomeric DNA stability and in repression of transcription at the c-myc promoter (127). The wide range of potential biological activities of this lesion makes it a prime target for future investigations.


1,N\textsuperscript{6}-ethenoadenine and 1,N\textsuperscript{6}-ethanoadenine

The formation of 1,N\textsuperscript{6}-ethenoadenine (eA) results from the reaction of adenine with products of unsaturated lipid peroxidation (128–131). This bifunctional DNA lesion arises endogenously under normal physiological conditions in both rodents and humans (132,133). Of great toxicological concern is the observation that eA is induced by common industrial agent vinyl chloride and its metabolites, such as chloroacetaldehyde. eA also occurs in chronically inflamed human and rodent tissues (134). Oxidative stress associated with inflammation is increasingly being linked to neurological disease, cancer promotion and accelerated aging (135).

In duplex DNA, eA can be repaired in vitro by glycosylases of the BER pathway (93,136). Mammalian cells can also repair etheno lesions by this route in vivo (137,138). Indeed, the BER enzyme AAG and its homologs are likely to be the primary vehicles of repair of eA in the duplex genomes of eukaryotes. In contrast, the in vivo repair of etheno adducts in E.coli was not clearly understood until recently; for example, one early study showed that neither BER nor NER figures prominently in etheno lesion repair (139). Early genetic studies on the mutagenicity of eA in E.coli reinforced this conundrum (140). The eA adduct was neither toxic nor mutagenic despite the fact that the base lacks any structural possibility of Watson–Crick complementarity. The issues raised in these studies were resolved in 2005 when biochemical studies provided the possibility that the direct reversal enzyme, AlkB, may play a significant role in the defense of cells against this type of bifunctional DNA damage. These biochemical studies showed that AlkB and its human homolog ABH3 can efficiently repair eA in vitro (141,142). AlkB uses a unique iron-mediated chemical reaction involving 2-ketoglutarate as a cofactor to putatively epoxidize the exocyclic double bond of eA. An epoxide may be hydrolyzed to a glycol with the glycol moiety being liberated as the dialdehyde, glyxal. The direct reversal mechanism is also likely to be operative in vivo, as evidenced by genetic studies in which a single-stranded vector containing a single eA was replicated in AlkB-proficient and -deficient E.coli cells. In AlkB-deficient cells, eA is 35% mutagenic, yielding 25% A → T, 5% A → G and 5% A → C mutations. SOS induction causes an increased incorporation of dAMP opposite to eA (141).

1,N\textsuperscript{6}-ethanoadenine (EA) is the chemically reduced form of eA and forms through the reaction of adenine with the antitumor drug bis-chloroethyliminosourea. EA can be weakly repaired by the E.coli enzyme AlkA (143) and the corresponding human enzyme AAG (93,144), which suggested that BER is a means of repair of this adduct. Recent work, however, by Frick et al. shows that the direct reversal repair enzyme AlkB easily alleviates the toxicity of EA in E.coli in vivo (145). In an AlkB-proficient cell, EA is almost non-toxic (i.e. easily bypassed) and not significantly mutagenic. However, in AlkB-deficient cells, EA is extremely toxic, showing an 86% reduction in replication. The adduct is weakly mutagenic causing A → C (2%), A → G (1%) and A → T (1%) mutations (145).

1,N\textsuperscript{2}-etheno guanine and 3,N\textsuperscript{2}-etheno guanine

1,N\textsuperscript{2}-etheno guanine (1,2-eG) and its isomer 3,N\textsuperscript{2}-etheno guanine (2,3-eG) are cyclic DNA adducts formed, as with eA, by reagents such as chloroacetaldehyde (146) or 4-hydroperoxy-2-nonenal (147). Significantly, the former has been found in the liver DNA of rodents exposed to vinyl chloride (146). 1,2-eG can moderately block DNA polymerase and cause G → T and G → C base substitutions, as well as frameshift mutations (148). It can be repaired by mammalian uracil-DNA glycosylase (MUG) and AAG (93,149). In recent work, 1,2-eG was shown to be repaired, albeit weakly, by BER using a truncated form of the AAG enzyme (93). The AlkA protein can release 2,3-eG from DNA (150). The glycosidic bond of 2,3-eG is extremely labile, a property that has made assessment of biological significance of this modified base a difficult task (146). Nevertheless, Loeb and colleagues successfully determined the mutation frequency of the lesion to be ~13% in E.coli, where it primarily induces G → A transitions.

3,N\textsuperscript{2}-ethenocytosine

3,N\textsuperscript{2}-ethenocytosine (eC) is produced from the same precursors and by the same pathways that generate eA in DNA (128–130,151). As with eA, the BER pathway ([human thymine-DNA-glycosylase (hTDG)] in human and [double-stranded uracil-DNA-glycosylase (dsUDG)] in E.coli) is an established strategy used by nature to suppress the biological effects of this adduct (137,151). The cellular defense network against eC additionally involves the AlkB pathway, at least in E.coli., which should be mechanistically similar to that of eA repair by AlkB (141). In E.coli, AlkB has a modest effect on eC toxicity but reduces the mutation rate of the adduct by about two-thirds from 82% in AlkB-deficient cells to 37% in AlkB-proficient hosts, implying incomplete conversion to cytosine prior to polymerase traversal. The mutations of eC in AlkB-deficient and -proficient cells are C → A and C → T, which are of approximately equal abundance in each cellular background.

Perspective

Thirty years ago, when Carcinogenesis was a new Journal, the field of cancer research looked very different from the way it looks today. The field was richly populated by chemists who identified carcinogens and studied the molecular transformations whereby those agents damaged DNA. The work described in this review started shortly after the Journal began when the complexities of DNA addition confounded attempts to relate specific types of DNA damage with genetic changes that, presumably, attend the conversion of a normal cell into a fully malignant one. From that time to the present, much has been learned. Many oncogenes and tumor-suppressor genes have been discovered and placed like footstep on the path between normality and malignancy (152). More recently, linkages have been made between the genetic events of oncogene activation and tumor-suppressor gene inactivation and parallel disruptions in biochemical networks. These studies are revealing the secrets of how cancer cells obtain the energy and the raw materials to finance their growth into a tumor (153,154). One revelation to come out of the last few decades is that the number of mutations in cancers is far in excess of the number one would expect on the basis of normal replication errors or perhaps even the enhanced rate of replication errors that occur when a polymerase tries to copy past a mutagenic DNA lesion such as those described in the manuscript. While it seems likely that genetic changes induced by carcinogens are an important step in the early stage of malignant transformation, it now seems clear that we need to find other chemical or biochemical events that underpin the ‘mutator phenotype’ of tumors (155). Answers may come from studies of virally induced diseases, such as human immunodeficiency virus and hepatitis, where recent work has discovered enzymatic DNA-targeted base deamination systems that cause a high density of mutations within a genome (156). Answers might also come from the field of immunology where enzymes such as activation-induced deaminase cause, once again, a high density of mutations in a localized stretch of DNA (157,158).
One of the most important contributions of work on the chemical biology of mutagenesis has been the collateral impact of this field on the nearby field of DNA repair. It is now common for workers in the repair field to use oligonucleotides with single lesions, originally made for studies of mutagenesis, to characterize the detailed biochemical mechanisms by which repair enzymes or complexes reverse the damage. Moreover, studies of mutagenesis done using cells that are defective in a specific repair enzyme (141) or that express specialized polymerases (159) have provided high-quality data that have established the physiological relevance of specific enzymes as protectors from damage or as the vehicles by which damage is processed into events with disastrous consequences for the cell and organism. Looking ahead, there is much to do. To give one example, the process of inflammation is clearly associated with cancer development (135). The range of DNA damages created by inflammation-generated reactive oxygen and nitrogen species is vast, and the task will be a large one to determine how each of these lesions contributes to the biological endpoints downstream of an inflammatory event. As a second example, workers will soon develop modified versions of the tools described herein to probe what may become a new field … DNA repair. Some mRNA species are so long that it takes a day to transcribe them (160–162). These important molecules not only need to have their informational integrity protected but also the energy used in their synthesis is large and would be wasted if a single lesion, for example, an eA residue, made them unreadable. Finally, while this review focuses on only one class of lesion, the small alkylated bases, it illustrates how much can be learned about the chemical rules of mutagenesis. Ten years ago, studies of the mutagenic properties of 5-hydroxycytosine (163), which induces C to T transitions, were the starting point for a novel application in the development of anti-viral agents (164). It is expected that additional examples of this nature, in which basic studies of mutagenesis drive clinical development, will help propel this field into a robust future.

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References

55. Shah,D. et al. (2001) Evidence in Escherichia coli that N3-methyladenine lesions induced by a minor groove binding methyl sulfonate ester can be processed by both base and nucleotide excision repair. Biochemistry, 40, 1806–1803.
108. Mele, A. et al. (2008) New immunofluorescence-LC-MS/MS methodology reveals that Aag null mice are deficient in their ability to clear 1,N6-etheno-deoxyadenosine DNA lesions from lung and liver in vivo. DNA Repair (Amst.), 7, 257–265.
123. Augusto,O. et al. (2004) New immunoaffinity-LC-MS/MS methodology reveals that Aag null mice are deficient in their ability to clear 1,N6-etheno-deoxyadenosine DNA lesions from lung and liver in vivo. DNA Repair (Amst.), 3, 257–265.
124. Augusto,O. et al. (2004) New immunoaffinity-LC-MS/MS methodology reveals that Aag null mice are deficient in their ability to clear 1,N6-etheno-deoxyadenosine DNA lesions from lung and liver in vivo. DNA Repair (Amst.), 3, 257–265.


148. Langouet, S. *et al.* (1997) Misincorporation of dNTPs opposite 1,N2-ethenoguanine and 5,6,7,9-tetrahydro-7-hydroxy-9-oximidazo[1,2-


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