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Chemical Biology of Mutagenesis and DNA Repair: Cellular Responses to DNA Alkylation

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Abstract

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 (AID) and the apolipoprotein B editing complex (APOBEC) [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 B_1 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 to 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 alkylating 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 repairability, 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 comparatively weak mutagenic properties; however, it must be kept in mind that a lesion with a mutagenicity of only 0.1% creates mutations at a rate that is five orders of magnitude greater than the basal or spontaneous rate of mutagenesis. In the review, exocyclic mono-adducts 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*^{6}-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*^{6}-Ethylguanine (*O*^{6}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}-methylguanine-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 irreversibly 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^6AlkGua and O^4AlkThy are transferred to Cys 321 at the C-terminus, while those from a third substrate, methylphosphotriesters (MePT), are 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 of Cys-38 of Ada converts 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 1-2 molecules in an unadapted state to ~3000 molecules in a fully adapted cell [20;21]. It was initially found that Ada preferentially repairs O^6MeG as compared to O^4-methylthymine (O^4MeT) [22] but recent evidence suggests 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^4MeT 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 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^6MeG over O^4MeT [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^6MeG. Excision of O^6MeG on duplex substrates has been shown to occur *in vitro* [28] and *in vivo* [29]. When O^6MeG 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. found a 40 fold decrease in the G→A transition caused by an O^6MeG lesion introduced on a single-stranded *Φ*X174 genome in an NER deficient (*uvrA*) cell strain vs. wild type [31]. 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^6MeG in *Drosophila melanogaster* [32], and of O^6EtG in *D. melanogaster* [33] and mammalian cells [34].

The mismatch repair (MMR) pathway has also been implicated in the cellular response to O^6MeG [35]. O^6MeG can be processed by post-replicative mismatch repair 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^6MeG lesion, Rye et al. have shown that *dam*$^{-}$ and *mutH*$^{-}$ strains display the same mutation frequency as wild type, but *mutS*$^{-}$ and *mutL*$^{-}$ strains show a decrease [37]. This result suggests that MMR proteins may aid in the repair of O^6MeG in a cooperative fashion. While early work suggested that O^6EtG 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^6MeG in mammalian cells [39]. Nevertheless, in rat mammary cells, O^6EtG is repaired 20 times faster than O^6MeG by an unknown, MGMT-independent, mechanism [40]. In line with expectations based upon this finding, a G→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 (ENU) compared to tumors initiated by *N*-methyl-*N*-nitrosourea (MNU).

The toxicity of O^6MeG 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^6MeG is potently toxic in mammalian cells come from a number of studies, including those in MGMT knock-out mice, which display hypersensitivity to the lethal effects of alkylating agents that generate O^6MeG [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^6MeG in the synthetic oligonucleotide used as a template [42]. Also, human polymerase β, subcloned in an *E. coli* plasmid, is blocked by O^6MeG present on a single-stranded DNA

template [43]. The second mechanism leading to toxicity is that of futile cycling of the mismatch repair system at an O^6MeG: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^6MeG 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^6EtG is more toxic than O^6MeG [38] but the mechanism underlying this differential toxicity is unknown.

O^6MeG is known to be highly mutagenic. To study the mutations formed *in vivo*, Loechler *et al*. constructed single-stranded M13mp8 DNA containing O^6MeG 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 (MNNG; 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^6MeG, O^6EtG, introduced at a specific position in *Φ*X174 and transfected into *E. coli* produces higher mutation frequencies compared to O^6MeG in the same system [48;49]. O^6MeG and O^6EtG 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 [50].

A recent study used site-specific mutagenesis to generate single-stranded M13mp7 genomes containing O^6MeG in all sixteen 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^6MeG went from being 10% mutagenic in repair-proficient cells to 100% mutagenic in repair-deficient cells [30]. Moreover, it was found that DNA repair *in vivo* is sequence context-dependent.

With regard to effects on gene expression, O^6MeG can inhibit carbon-5 methylation of cytosines in CpG 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^6MeG 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.

O^4-Methylthymine

O^4MeT is one of the mutagenic lesions formed concurrently with O^6MeG when DNA is exposed to alkylating agents that react with DNA by an S_N1 mechanism. O^4MeT is formed at a much lower level than O^6MeG; for example, the methylated thymine was detected at a level 126 times lower than that of O^6MeG in calf thymus DNA treated with MNU [52]. Although it is not

an abundant lesion, O^4MeT can be very mutagenic. Using site-specific mutagenesis tools, it was shown that O^4MeT incorporated in single-stranded M13mp19 had a mutation frequency of 12% in repair-proficient *E. coli*. O^6MeG gave a mutation frequency of less than 2% in the same repair-proficient system. Pretreatment with MNNG to deplete or occupy endogenous repair enzymes doubled this mutation frequency [53]. Similar results were obtained using double-stranded and gapped plasmids in *E. coli* (mutation frequency of 45% for O^4MeT vs. 6% for O^6MeG) leading to the conclusion that O^4MeT is much more mutagenic than O^6MeG [38] on a mole-per-mole basis under normal conditions of DNA repair proficiency in cells. O^4MeT mimics cytosine in structure and generates an overwhelming majority of T→C transitions [54]; it can also cause a small number of T→A transversions in MMR deficient cells [39]. O^4MeT has been examined as a site-specific adduct in mammalian vectors and again appears to be more mutagenic than O^6MeG in both repair-proficient and repair-deficient backgrounds [39;55]. In *E. coli*, O^4MeT is toxic but less so than O^6MeG and O^6EtG [38]. O^4MeT has been shown to be toxic to mammalian cells deficient in NER capability [56], suggesting a role for this repair pathway in the cellular defense against this adduct.

In *E. coli*, O^4MeT is repaired by the same alkyltransferases that repair O^6MeG. The Ogt protein from *E. coli* seems to have a preference for repair of O^4MeT over O^6MeG [25]. Ada repairs O^6MeG and O^4MeT with equal efficiency but human and rat alkyltransferases show a preference for O^6MeG repair [23;57]. Studies in mammalian cells have shown that the mutation frequency of O^4MeT does not vary significantly in the presence or absence of alkyltransferase, indicating that it is probably not repaired by MGMT [39;55]. In fact, mammalian alkyltransferases may actually inhibit repair of O^4MeT by the NER pathway by binding to and

shielding the lesion, as evidenced in *E. coli* in studies using plasmids expressing human and mouse methyltransferases [58]. A study done in human cells lines using site-specifically modified plasmids containing O^4MeT shows that repair is not influenced by the levels of alkyltransferase and that NER seems to be the most significant repair system for this lesion [56]. With regard to repair by MMR, one *in vitro* study found that *E. coli* MutS (a DNA mismatch repair binding protein) does not bind to oligonucleotide-duplexes containing a site-specifically incorporated O^4MeT:A base pair [35], while another shows that hMutSα, a protein of the MMR pathway in humans, recognizes and binds to a O^4MeT:A base pair quite well but very poorly to an O^4MeT:G base pair [59].

O^2-Methylcytosine and O^2-methylthymine

O^2-methylcytosine (O^2MeC) and O^2-methylthymine (O^2MeT) are minor reaction products formed by treatment of DNA with alkylating agents such as MNU or MNNG. Both lesions are repaired *in vitro* by *E. coli* AlkA [60]. O^2MeC and O^2MeT are predicted to interfere with minor groove contacts, yet there have been very few studies of these modifications, making the lesions good candidates for future study.

Methylphosphotriesters

Methylation damage can occur on the DNA sugar-phosphate backbone to form methylphosphotriesters (MePT). The physical accessibility and negative charge of the phosphate oxygens makes them a favorable site for chemical reaction. When double-stranded DNA is treated with MNU, 17% of the total methylation occurs on the backbone to yield methylphosphotriesters [13]. These adducts react with water and other nucleophiles much faster

than the common diester form of phosphate linking adjacent nucleosides, leading to facile cleavage of the backbone. Of the two diastereomers formed, only the S_p-MePT is repaired by the Cys-38 residue in the N-terminal domain of Ada (N-Ada). This selective repair results because the oxygen atom on the phosphate in the S_p diastereomer is only 3.5Å away from the acceptor cysteine residue, vs. 5Å in the R_p configuration [18]. As discussed earlier, N-Ada has an inherent electrostatic switch that works in a methylation-dependent fashion to modulate its affinity for DNA and ability to act as a transcription activator. There is no known homolog of N-Ada in eukaryotes, thus making the repair of MePT in mammalian cells uncertain.

In vivo studies using wild-type Ada and truncated Ada (lacking MePT repair capability) transfected into HeLa cells showed the same extent of resistance to the cytotoxic effects of alkylating agents, similar sister-chromatid exchange induction, as well as host-cell reactivation 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.

N1-Methyladenine and N1-ethyladenine

N1-Methyladenine (1MeA) is formed by alkylating agents mainly in single-stranded DNA and has been detected *in vitro* [62-68] and *in vivo* [64;69-72]. S_N2 agents, such as methylmethanesulfonate (MMS) and the naturally occurring methyl halides can generate 1MeA [15]; similarly, the ethyl homolog, N1-ethyladenine (1EtA), is formed by ethylating agents both *in vitro* and in *vivo* [64]. The preference for formation in single-stranded DNA is owed to location of the N1 atom of adenine at a site usually protected by base pairing in double-stranded

DNA [73]. 1MeA is cytotoxic because it disturbs DNA replication [15]. 1-Methyldeoxyadenosine is known to be unstable due to a base-catalyzed Dimroth rearrangement, a complex mechanism, the net result of which is the migration of the N1 methyl group to the exocyclic N6 position of adenine [74].

A specialized DNA repair system protects cells from N1-substituted DNA lesions. The AlkB enzyme of the adaptive response repairs 1MeA both *in vitro* and *in vivo* [75] in an oxidative reaction that liberates formaldehyde from the methylated base, affording complete reversal of the damage. The role of AlkB in the repair of 1MeA seems to be the prevention of genotoxicity, because this very toxic adduct is only weakly mutagenic in cells. AlkB and its human homologs, ABH2 and ABH3, also repair 1EtA residues in DNA, with the release of acetaldehyde as the repair product [76]. Studies of 1MeA *in vivo* reveal that the lesion severely blocks DNA replication, but the replication block can be partially overcome by the induction of SOS bypass polymerases. The 1MeA blockade is completely removed in 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 vs. T was respectively 99% vs. 0.61% in $SOS^{-}/AlkB^{-}$ cells, 99.7% vs. 0.06% in $SOS^{-}/AlkB^{+}$ cells, and 98.6% vs. 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 this lesion. The prokaryotic system includes the highly selective and constitutive TAG protein, and the inducible AlkA glycosylase with a broader specificity. The eukaryotic system is comprised of alkylpurine-DNA-N-glycosylases (APNG) and human 3MeA-DNA-glycosylase (AAG/ MPG). AlkA and TAG repair 3MeA with equal efficiency on double-stranded DNA, but AlkA is 10-20 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 base excision repair (BER) repair 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 vs. 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 2-3 fold in 3MeA-glycosylase deficient strains, which suggests that the lesion driving the mutations is 3MeA [84]. Interestingly, the methylated adenines 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 adenines 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.* showed that 3MeA can cause p53 induction, S phase arrest, sister chromatid exchange (SCE), chromosome aberrations, and apoptosis in mammalian cells [85]. As with 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 derivative, Fapy-7MeA; this latter hydrolysis reaction is especially favored for the 7MeA in RNA [88], which has a stabilized glycosidic bond as compared to 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 ^{o}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 (DMS) treated DNA was compared before and after treatment with alkali, which hydrolyzed the imidazole rings of N7-methylated adenines and guanines, forming the Fapy derivatives. DMS and alkali treated DNA was 60-fold more mutagenic than DNA treated with DMS 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 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 to 4 hours [89]. It has been shown that *E. coli alkA* 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 GC rich double-stranded DNA sequence, albeit with an efficiency 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-position of guanine, respectively [13]. Within the cell, N7-methylguanine (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 during repair 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-5N-methyl-formamidopyrimidine (Fapy-7MeG). While this lesion does not cause

misparing with dAMP or dTMP, *in vitro* experiments using *E. coli* DNA polymerase I and poly (dGC) templates [102], or Klenow fragment and M13mp8 template DNA [103] show that Fapy-7MeG blocks DNA chain elongation. Fapy-7MeG lesions present on M13mp8 phage template DNA also leads to a 2-3 fold increase in G→C and G→T transversions when transfected into SOS induced *E. coli* [87]. However, DNA polymerase I preferentially incorporates dCMP opposite Fapy-7MeG and a Fapy-7MeG: C pair is extended most efficiently compared to 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* (formamidopyrimidine-DNA-glycosylase (Fpg)) and mammalian cells (human 7,8-dihydro-8-oxoguanine DNA glycosylase (hOGG1)) [105] that remove Fapy-7MeG lesions. *E. 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 overexpression 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 overexpression of MPG, but in combination they can overwhelm the cell with AP sites leading to cytotoxicity. Rinne *et al*. 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 [106].

N3-Methylcytosine and N3-ethylcytosine

N3-Methylcytosine (3MeC) is formed by S_N2 agents such as MMS and the naturally occurring methyl halides [107] preferentially in single-stranded DNA. It has been detected both

in vitro [62;64-68;108;109] and *in vivo* [64;70;71;91;109]. 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;110]. As with the 1-alkyladenines, these lesions likely exist only or predominantly in single-stranded DNA because this site of modification is normally protected by base pairing [73]. 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 N1-methylguanine (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 [108;111;112]. However, some adduct bypass occurs with the incorporation of dAMP and dTMP opposite 3MeC [108]. Therefore, the rules for misreplication of the 3-alkylcytosine 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 vitro* [75;108].

N3-Methylthymine

N3-Methylthymine (3MeT) has been found both *in vitro* [64;65;67;68;113] and in *vivo* [113;114] and is formed through the reaction of DNA with S_N2 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, FTO (fat mass and obesity associated) protein has been shown as a 2-oxoglutarate-dependent demethylase for nucleic acid [115;116]. 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 [115;117]. While there are numerous epidemiological studies associating the FTO gene with obesity, the biological basis for metabolic effects of this gene are still under investigation.

From the standpoint of its potential to induce genetic change, 3MeT is approximately 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 dTTP incorporation on a poly(dC-d3MeT) template [118]; interestingly, T is exclusively incorporated opposite the analogous 3-ethyldeoxythymidine adduct in one study [119], while A is exclusively incorporated in another [120].

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 [121] and DNA [122] by methyl radicals generated by oxidation of 1,2-dimethylhydrazine and methylhydrazine respectively. Proof of *in vivo* DNA alkylation by carbon-centered radicals was given by Netto *et al*. who detected 8MeG in DNA isolated from the liver and colon of rats administered 1,2-dimethylhydrazine [123]. Other studies have shown that this lesion can also be produced *in vitro* and *in vivo* by genotoxic agents such as *tert*-butylhydroperoxide, diazoquinones and arenediazonium ions [124]. These findings are significant as they suggest a possible contribution of 8MeG in the carcinogenic effects of these agents, especially 1, 2-dimethylhydrazine, which induces adenocarcinomas of the colon in rodents.

Site-specific studies using 8MedG-containing-oligonucleotides prepared by phosphoramidite synthesis have explored the mutagenecity 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 [125]. 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 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 [125]. These numbers mirror the thermodynamic stability of the 8MeG:dNMP base pair, decreasing in the order dCMP > dGMP > dAMP >> dTMP. 1,2-Dimethylhydrazine induces both O^6MeG and 8MeG in similar amounts in the DNA of rats [123]. However, the mutation frequencies of 8MeG are two orders of

magnitude less than those of O^6MeG [126]. Therefore, we may conclude that 8MeG is a weakly mutagenic lesion that in principle can contribute to G →C transversions in cells.

The repair of 8MeG has been studied *in vitro* by Gasparutto *et al*. 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) [124].

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 [127]. This lesion is also used as a chemical modification to stabilize qaudruplex 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 [128]. The wide range of potential biological activities of this lesion makes it a prime target for future investigations.

1,N^6-Ethenoadenine and 1,N^6-ethanoadenine

The formation of 1,N^6-ethenoadenine (eA) results from the reaction of adenine with products of unsaturated lipid peroxidation [129-132]. This bifunctional DNA lesion arises endogenously under normal physiological conditions in both rodents and humans [133;134]. 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 [135]. Oxidative stress associated with inflammation is increasingly being linked to neurological disease, cancer promotion and accelerated aging [136].

In duplex DNA, eA can be repaired *in vitro* by glycosylases of the BER pathway [93;137]. Mammalian cells can also repair etheno lesions by this route *in vivo* [138;139]. 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. By 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 [140]. Early genetic studies on the mutagenicity of eA in *E. coli* reinforced this conundrum [141]. 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* [142;143]. AlkB uses a unique iron-mediated biochemical reaction involving α-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, glyoxal. 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 deoxyadenosine monophosphate opposite to eA [142].

1,N^6-Ethanoadenine (EA) is the chemically reduced form of eA and forms through the reaction of adenine with the antitumor drug *bis*-chloroethylnitrosourea (BCNU). EA can be weakly repaired by the *E. coli* enzyme AlkA [144] and the corresponding human enzyme AAG [93;145], which suggested that BER is a means of repair of this adduct. Recent work, however, by Frick et. al. show that the direct-reversal repair enzyme AlkB easily alleviates the toxicity of EA in *E. coli in vivo* [146]. In an AlkB-proficient cell, EA is almost nontoxic (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 [146].

1,N^2-Ethenoguanine and 3,N^2-ethenoguanine

1,N^2-Ethenoguanine (1,2-eG) and its isomer 3,N^2-ethenoguanine (2,3-eG) are cyclic DNA adducts formed, as with eA, by reagents such as chloroacetylaldehyde [147] or 4-hydroperoxy-2-nonenal (HPNE) [148]. Significantly, the former has been found in the liver DNA of rodents exposed to vinyl chloride [147]. 1,2-eG can moderately block DNA polymerase and cause G→T and G→C base substitutions, as well as frameshift mutations [149]. It can be repaired by mammalian uracil-DNA-glycosylase (MUG) and AAG [93;150]. 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 [151]. 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 [147]. Nevertheless, Loeb and colleagues successfully determined the mutation frequency of the lesion to be approximately 13% in *E. coli,* where it primarily induces G →A transitions.

3,N^4-Ethenocytosine

3,N^4-Ethenocytosine (eC) is produced from the same precursors and by the same pathways that generate eA in DNA [129-131;152]. 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 [138;152]. 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 [142]. 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 adduction 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 footsteps on the path between normalcy and malignancy [153]. 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 [154;155]. 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 occurs when a polymerase tries to copy past a mutagenic DNA lesion such as those described in this 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 [156]. Answers may come from studies of virally induced diseases, such as HIV and hepatitis, where recent work has discovered enzymatic DNA-targeted base deamination systems that cause a high density of mutations within a genome [157]. Answers might also come from the field of immunology where enzymes such as AID cause, once again, a high density of mutations in a localized stretch of DNA [158;159].

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 [142] or that express specialized polymerases [160] 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 [136]. 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 … RNA repair. Some mRNA species are so long that it takes a day to transcribe them [161-163]. These important molecules not only need to have their informational integrity protected, but the energy used in their synthesis is large and would be wasted if a single lesion, for example an eA residue, made them un-readable. 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 [164], which induces C to T transitions, were the starting point for a novel application in the development of antiviral agents [165]. 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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Tables

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

Lesion	Mutagenic specificity	Genotoxicity	Repaired by prokaryotic enzymes/systems	Repaired by eukaryotic enzymes/systems
O^6MeG	G →A	Toxic in presence of MMR	Ada, Ogt UvrABC (NER) MMR	MGMT NER MMR
O^6EtG	G →A	Toxic in *E.coli*	Not repaired by Ada or Ogt	MGMT NER MMR
O^2MeC		Possibly toxic		
O^2MeT		Possibly toxic		
O^4-MeT	T →C T →A in MMR-deficient cells	Toxic, but less than O^6MeG and O^6EtG in *E.coli*	Ada, Ogt	NER MGMT (minimal)
MePT	None known	None known	Ada	
1MeA	A →T	Mutagenic and toxic to *E. coli* in the absence of AlkB	AlkB	AAG ABH2, ABH3
1EtA		Mutagenic and toxic to *E. coli* in the absence of AlkB	AlkB	ABH2, ABH3
3MeA	A →T	Highly toxic	AlkA Tag UvrA	AAG/ APNG/ MPG
7MeA	Fapy-7MeA A →G			
1MeG	G →T G →A G →C	Mutagenic and toxic to *E. coli* in the absence of AlkB	AlkB	AAG
3MeG		Possibly toxic	AlkA Tag	
7MeG	Fapy-7MeG G →C G →T	Toxic via formation of AP sites and Fapy-7MeG	AlkA Fpg	AAG/ MPG hOGG1
3MeC	C →T C →A	Toxic	AlkB	AAG ABH2, ABH3
3EtC	C →T C →A	Toxic	AlkB	
3MeT	AlkB deficient cell T →A	Strong block to replication	Weak substrate for AlkB	FTO

	T →C			
8MeG	G →C		AlkA	Not repaired by known enzymes
eA	AlkB deficient cell A →T A →G A →C	Mutagenic and toxic to *E. coli* in the absence of AlkB	AlkA AlkB	AAG
EA	A →T (weak) A →C (weak) A →G (weak)	Toxic to *E. coli* in the absence of AlkB	AlkB Weak substrate for AlkA	AAG
1,2-eG	G →T G →C	Mutagenic and causes frameshift	MUG	AAG
2,3-eG	G →A	Mutagenic	AlkA	
eC	C →A C →T	Mutagenic and toxic to *E. coli* in the absence of AlkB	AlkB dsUDG	hTDG

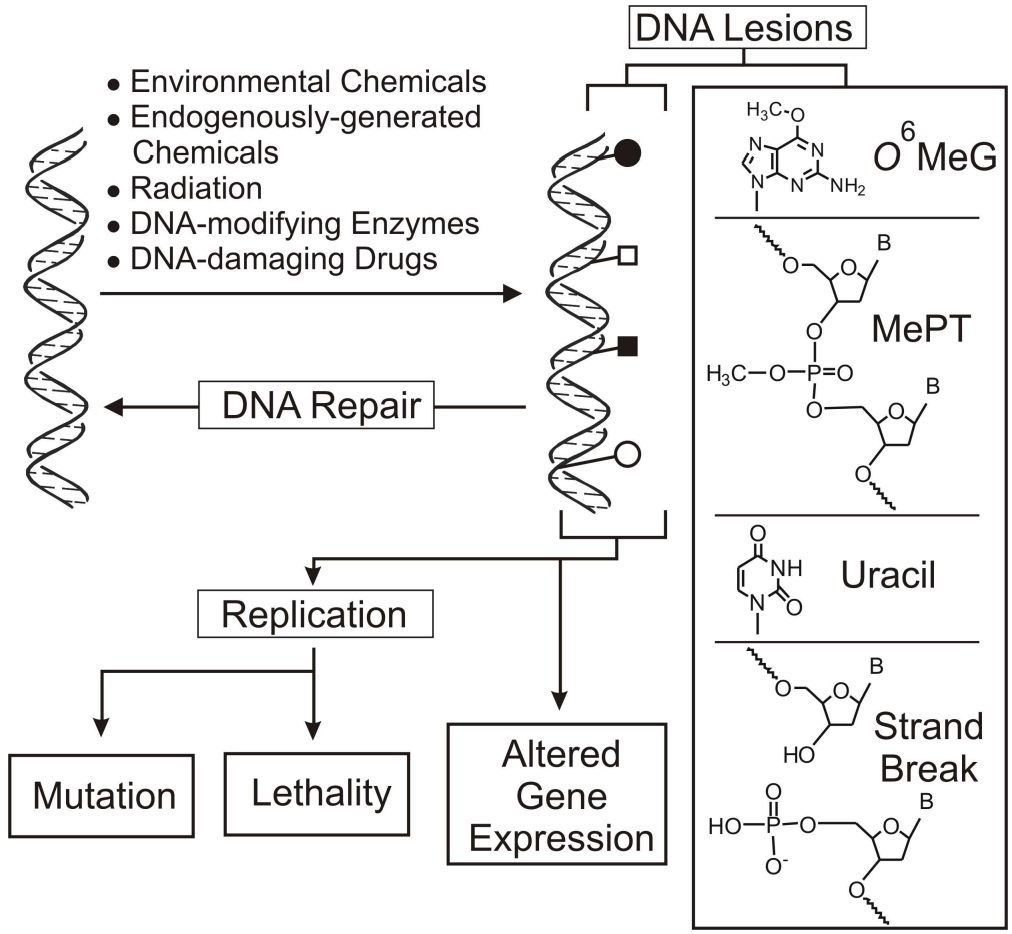
Legends to Figures

Fig. 1. Pathways by which DNA damaging agents induce biologically relevant events. Agents from the environment, chemically reactive natural species generated within cells, and the misdirected action of natural intracellular enzymatic systems can result in the formation of a collection of DNA lesions (symbols attached to the helix). These lesions can be formal chemical-DNA adducts, such as O^6MeG, which is a miscoding lesion during replication. They can be modifications of the sugar-phosphate backbone, such as MePT, which triggers a change in gene expression. They can be bases such as uracil, which can appear as the enzymatic deamination product of cytosine. Or, they can be lethal strand breaks, as would form after treatment of a cell with ionizing radiation or certain anticancer agents. Finding the relationships between the structures of each lesion and the biological endpoints of mutation, lethality and gene expression is the subject of this review.

Fig. 2. A. Methods to evaluate the biological relevance of DNA damage. 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 forms 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.

Fig. 3. Structures of DNA alkylation lesions.



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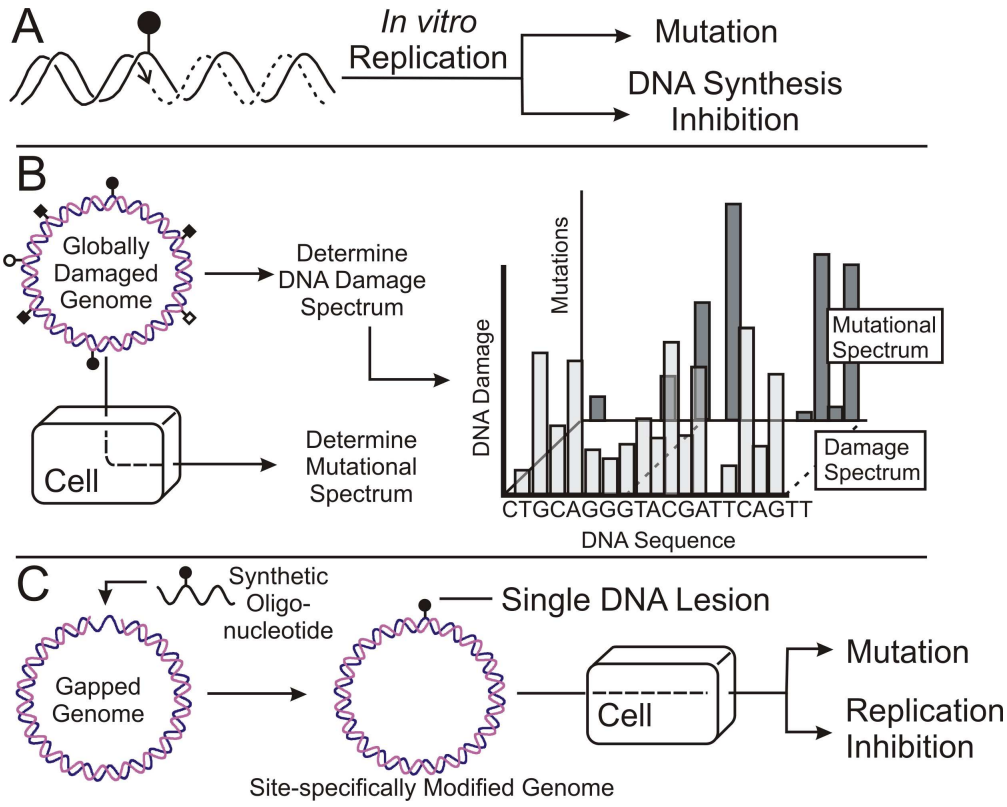

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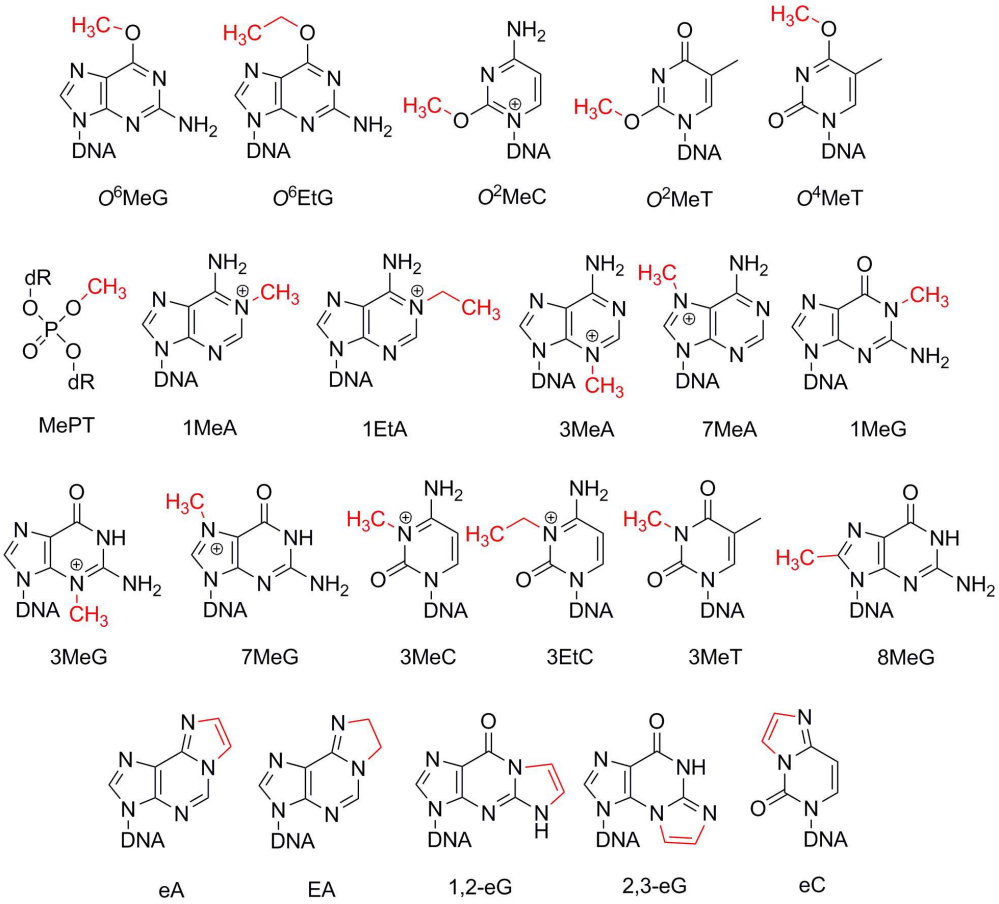

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