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The Biological Effects of N3-Methyladenine

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Abstract The targeting of damage to DNA remains an attractive strategy to kill tumor cells. One of the serious side effects of alkylating agents is that they create both toxic (desired) and mutagenic (undesired) lesions. The result is that patients successfully treated for a primary cancer are at significant risk to develop cancer related to their therapy. To address this issue we have prepared agents that selectively methylate DNA at the N3-position of adenine. The presence of this lesion in DNA is thought to halt DNA polymerase, and this then initiates a cascade of events including cell death. The toxicity and mutagenicity of the compound, Me-lex, used to generate N3-methyladenine is discussed in bacterial, yeast, and mammalian systems. Mechanisms are proposed to explain the biological activities of N3-methyladenine. J. Cell. Biochem. 91: 250–257, 2004. © 2003 Wiley-Liss, Inc.

Key words: DNA damage; toxicity; mutagenicity; base excision repair

Ed Bresnick was recruited to the University of Nebraska Medical Center as Director of the Eppley Institute for Research in Cancer in 1983 and it was a major turning point for me and the Eppley Institute. Having been trained as a chemist, my research focus was toward the chemical side of carcinogenesis. Ed's research group, which included at that time Alan Eastman (now at Dartmouth) and Ron Hines (now at The Medical College of Wisconsin) brought a molecular biology and biochemical focus, specifically in the area of carcinogen metabolism by P450's and the regulation of these processes, and DNA repair. In addition to Ed's group, there were a number of faculty that he recruited, and many of these people also had a significant impact on my research. Through his tenure as Director (1983–1989) Ed was able to obtain an NCI Cancer Center Support Grant, an NIH Training Grant, an American Cancer Society Institutional Grant, and an American Cancer Society Institutional Special Institutional Grant in Cancer Cause and Prevention. Fortunately, we have been able to sustain his successes, so his impact on UNMC is still evident. A lasting piece of advice that Ed instilled in me related to how he selected faculty recruits. His comment was, "remember Junior, you hire people not projects." I still remember the day that he told me that he was moving to Dartmouth: it was one of the saddest days of my life, that is until March when I heard he had passed away.

About 20 years ago, while Ed was still the Eppley Director, our laboratory embarked on an effort to understand the role of different DNA lesions in toxicity and mutagenicity. During the course of these studies we realized that this information could be useful in the design of DNA targeting antineoplastic agents that were not mutagenic. The reason that this is important is due to the significant incidence of secondary cancers, generally leukemia, caused by the successful treatment of cancers with alkylating anticancer drugs [Henry-Amar and Dietrich, 1993]. It also became evident that the ability to selectively control the type and level of specific DNA lesions in vivo would provide insight into the protective role of specific DNA repair proteins.

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DESIGN AND CHARACTERIZATION OF DNA AFFINITY BINDING ALKYLATING AGENTS

Since it had previously been shown by the groups of Arcamone, Dervan, and Lown, that *N*-methylpyrrolecarboxamide based peptides, similar to the natural products netropsin and distamycin A, could be used to target chemistry to the minor groove at A/T rich sequences [Sondhi et al., 1997], we decided to append simple alkylating functionalities on to the same class of small peptides. In all previous uses of these molecules, investigators employed peptides with high binding affinities. However, we intended that the peptides serve only as delivery agents to provide control of the position and sequence of DNA alkylation. In order to study the effects of DNA alkylation, the peptides could have no biological activity of their own. This meant that the binding affinity of the delivery peptide would have to be sufficiently high to sequence selectively bind to DNA but not high enough to interfere with normal DNA metabolism. The desired delivery molecule turned out to be a neutral dipeptide, whereas distamycin and netropsin have one and two cationic termini, respectively. After studying several potential models, we have focused on a methyl sulfonate ester as the alkylating functionality because of the bimolecular nature of the alkylation process and its conversion into a sulfonate anion once the methyl group has been transferred to DNA. The anionic group decreases the binding affinity due electrostatic repulsion with the polyanionic DNA. Isothermal titration calorimetry showed that the neutral lex peptide is a relatively weak equilibrium binder ($K_b = 10^5$) [Shah et al., 2001] that cannot be chemically footprinted on DNA [Church et al., 1990]. In contrast, distamycin and netropsin have K_b's above 10^7 [Shah et al., 2001] and are readily footprinted [Van Dyke et al., 1982].

The molecule that we synthesized, Me-lex (Fig. 1), was reacted with end-labeled DNA to determine where and how well it methylated DNA [Zhang et al., 1993]. To summarize these studies: (i) the molecule selectively and efficiently methylated DNA in the minor groove at A/T rich sequences; (ii) the predominant product was N3-methyladenine (3-MeA) that formed in >90% yield relative to other lesions; and (iii) the alkylation could be competitively inhibited by the co-addition of netropsin or distamycin [Zhang et al., 1993; Encell et al., 1996; Kelly et al., 1999].

TOXICITY OF N3-METHYLADENINE

The first effort to characterize Me-lex in *E. coli* was done in WT and base excision repair (BER) defective cells. In *E. coli*, there are two glycosylases (i.e., Tag and AlkA) that excise 3-MeA lesions as part of the BER system. The Tag

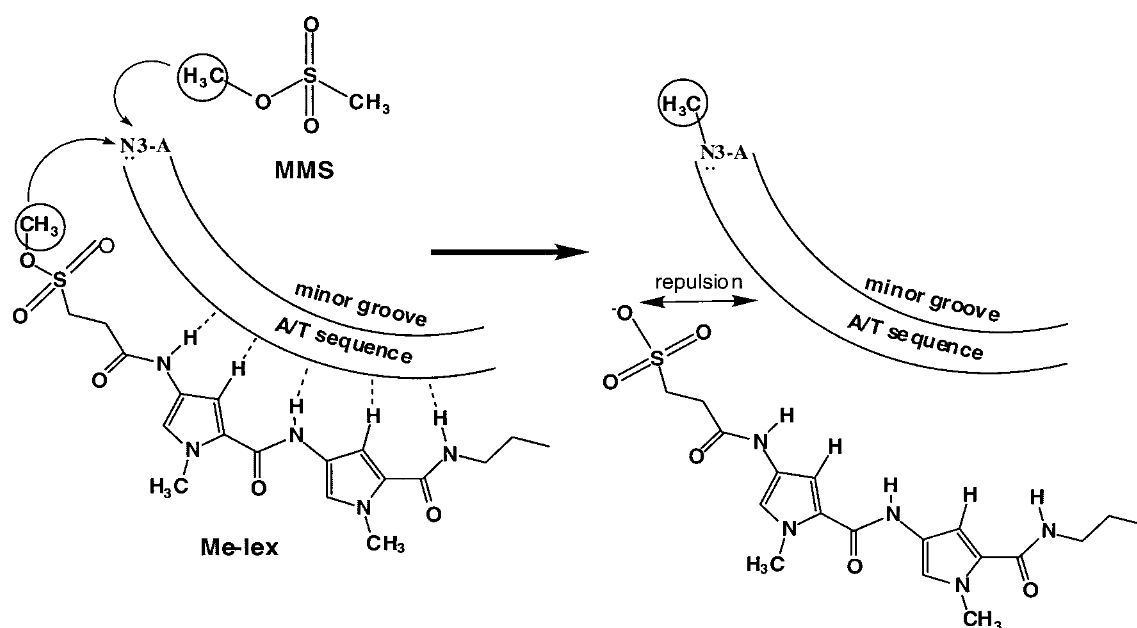

Fig. 1. Structures of MMS and Me-lex, and the mechanism for the transfer of the methyl group to DNA.

protein is highly selective for 3-MeA and is constitutively expressed, while AlkA, which has a broader substrate specificity, is constitutively expressed at low levels [Samson and Cairns, 1977; Karran et al., 1982]. In mammalian systems, only one glycosylase (AAG/APNG/MPG) that removes 3-MeA has been identified and it is homologous to AlkA in its glycosylase activity [O'Connor and Laval, 1990; Chakravarti et al., 1991]: both AlkA and AAG excise a wide variety of lesions, e.g., 3-MeA, N3-methylguanine, 1,N^6-ethenoadenine, etc. [Dosanjh et al., 1994; Hang et al., 1997]. In order to complete the repair process, the abasic site created by the glycosylase must be sequentially and coordinately processed by AP endonuclease, DNA polymerase, and DNA ligase [Seeberg et al., 1995]. Accordingly, cells that cannot efficiently perform one or more of the steps in the BER pathway accumulate repair intermediates and are sensitive to the toxic and/or mutagenic activity of many DNA alkylating agents.

While methyl methanesulfonate (MMS) has little effect on WT bacteria even at concentrations as high as 10 mM, 250 μM Me-lex causes a 2-log decrease in survival in WT and *alkA* cells. The *apn* mutant shows a 2-log increase in sensitivity to Me-lex relative to WT. The *alkA*/*tag* double mutant is an additional 4-logs more sensitive to Me-lex than the *apn* mutant (at the 50 μM dose). The difference between the *alkA*, *apn*, and *alkA*/*tag* mutants dramatizes the protective role of the constitutively expressed Tag glycosylase and indicates that 3-MeA is more cytotoxic than an abasic site in *E. coli* [Dinglay et al., 1998; Shah et al., 2001]. We measured the levels of 3-MeA in the cells to determine whether there is a relationship between toxicity and adduct level (Table I) [unpublished]. Me-lex efficiently generates 3-MeA in bacterial cells, which is consistent with its potent toxicity in *E. coli* as compared to other non-equilibrium binding methylating agents such as MMS [Dinglay et al., 1998; Shah et al., 2001]. A direct comparison of 3-MeA levels in cells that cannot repair this lesion shows that for an equimolar amount of Me-lex and MMS there is >1,000-fold more 3-MeA formed from Me-lex. For example, 50 μM Me-lex yields 55 μmol 3-MeA/mol DNA while a 5 mM dose of MMS yields approximately 1 μmol 3-MeA/mol DNA (5,500-fold difference). Similar ratios are seen at higher doses of Me-lex. This difference in adduct levels between the two methylating agents roughly correlates to the toxicity of the two compounds: 5 mM MMS generates 1.3 μmol 3-MeA/mol DNA and this

TABLE I. In Vivo Adduct Levels (μmol/mol DNA) Produced by Me-lex and MMS in Wild Type and *alkA*/*tag* Mutant *E. coli* and In Vitro With Calf Thymus DNA

			3-MeA		7-MeG
Compound	Dose (μM)	Netropsin[b] (μM)	Wild type	*alkA*/*tag*	*alkA*/*tag*
In vivo Me-lex[a]	5	—	n.d.[c]	4.88 ± 1.36	n.d.
	5	25	n.d.	n.d.	n.d.
	10	—	n.d.	11.30 ± 0.76	n.d.
	10	25	n.d.	n.d.	n.d.
	25	—	n.d.	29.36 ± 10.87	n.d.
	25	25	n.d.	n.d.	n.d.
	50	—	n.d.	54.90 ± 16.50	n.d.
	50	25	n.d.	4.05 ± 1.44	n.d.
	100	—	51.37 ± 8.22	136.77 ± 15.01	n.d.
	100	25	n.d.	1.69 ± 0.31	n.d.
	150	—	81.08 ± 8.41	171.14 ± 17.56	n.d.
	150	25	n.d.	2.43 ± 0.13	n.d.
	250	—	122.10 ± 9.68	n.d.	n.d.
	250	25	n.d.	n.d.	n.d.
In vivo MMS[a]	5,000	—	n.d.	1.28 ± 0.18	<10[d]
	5,000	25	n.d.	3.48 ± 2.09	<10[d]
	10,000	—	—	3.30 ± 0.42	43.10 ± 39.30
	10,000	25	—	5.35 ± 1.88	40.95 ± 15.29
In vitro Me-lex[e]	300	—	5,657 ± 1,521	—	437 ± 84
	300	150	60 ± 14	—	483 ± 272
	900	—	7,232 ± 1,356	—	769 ± 183
	900	150	1,554 ± 346	—	3,721 ± 651

[a]The bacteria (3×10^8) were treated with Me-lex for 1 h.
[b]Netropsin or solvent (control) was added to the bacteria for 30 min prior to the addition of Me-lex.
[c]n.d., not determined.
[d]Limit of detection was ≤10 μmol adduct/mol DNA.
[e]DNA (200 μM) incubated with Me-lex for 1 h at 37°C.

causes a 3–4 log decrease in survival, while 5 μM Me-lex yields 4.9 μmol adduct/mol DNA and a similar decrease in survival. Thus, despite the very different array of adducts produced by MMS [Beranek et al., 1980] and Me-lex [Zhang et al., 1993; Encell et al., 1996; Kelly et al., 1999], the results show that at approximately the same level of 3-MeA both methylating agents induce close to the same level of toxicity in the *alkA*/*tag* mutant cells. It should be noted that at high, but non-cytotoxic, concentrations of MMS, significant amounts of 7-MeG are formed. This lesion, which is not removed by the Tag protein and is inefficiently removed by AlkA, can persist in the genome after cell division and is not considered cytotoxic [Lawley and Warren, 1976; Karran et al., 1980]. Our results are consistent with the toxicity of MMS and Me-lex being mainly due to the formation of 3-MeA.

Since we knew that netropsin, which has the identical dipeptide core as Me-lex, could effectively block DNA methylation by Me-lex in vitro, it was of interest to determine if it would have the same effect in vivo and thereby inhibit toxicity. The effect of 25 μM netropsin, which was determined to be a non-toxic concentration in *E. coli*, on the toxicity of Me-lex is dramatic with quantitative inhibition of toxicity induced by concentrations as high as 250 μM Me-lex. This corresponds to almost 4-log protection. In contrast, no inhibition of toxicity by netropsin is seen with MMS in the *alkA*/*tag* cells. Adduct levels were also determined in experiments where netropsin was co-incubated with the *alkA*/*tag* bacteria for 30 min prior to the addition of Me-lex or MMS. The effect of netropsin is very different for the two methylating agents (Table I). Netropsin causes an increase, which is not statistically different in the amount of 3-MeA formed from MMS in the *alkA*/*tag* mutant cells. In contrast, the 3-MeA levels generated by Me-lex in the presence of netropsin are significantly reduced by >90% in the *alkA*/*tag* mutant. Similar to the results for 3-MeA, netropsin had no effect on 7-MeG levels for MMS. The observation that both the level of 3-MeA and the cytotoxicity in MMS treated bacteria are not sensitive to the presence of netropsin is consistent with the dominant biological role of 3-MeA in MMS toxicity. The reason that 3-MeA is not affected by netropsin is because MMS randomly methylates the DNA in both the major (7-MeG) and minor (3-MeA and 3-MeG) grooves. Since minor groove methylation by MMS is not restricted to A/T rich sequences, as is the case for Me-lex, MMS is not sensitive to the occupation of minor groove A/T sites by netropsin. It was recently suggested that the toxicity of MMS in mammalian cells was not due to 3-MeA, but rather to the formation of 7-MeG, because the authors did not observe a decrease in toxicity upon the co-incubation of netropsin with MMS [Horton et al., 2003]. This interpretation assumed that netropsin, which sequence selectively binds to DNA, universally blocks minor groove methylation by the non-sequence specific methylating agent. This is not the case as shown in vitro [Wurdeman and Gold, 1998] and in vivo (unpublished results). Our results do not rule out the involvement of other lesions (e.g., 3-MeG) in the toxicity of MMS, but they do indicate that 3-MeA plays a dominant role.

Me-lex is also highly cytotoxic in murine cells, and cells that cannot remove the 3-MeA lesion ($Aag^{-/-}$) are significantly more sensitive than wild type cells [Engelward et al., 1996]. By combining a chemical and a genetic approach it was shown that unrepaired 3-MeA has the potential to induce sister chromatid exchanges, chromosome aberrations, cell cycle arrest at the G1/S boundary, p53 induction, and apoptosis [Engelward et al., 1998], but exactly how this is achieved is not yet clear. Me-lex is also highly cytotoxic in human cells [Tentori et al., 2000] and it was shown the mechanism of toxicity generally requires DNA synthesis, and is related to both the DNA repair status and the dose of Me-lex [Tentori et al., 2001]. Specifically, it was observed in wild type MT-1 and Jurkat cells that the predominant pathway for toxicity involved necrosis. The interpretation is that in WT cells the high level of 3-MeA formed from Me-lex is processed by BER which results in DNA nicks that trigger PARP activation and depletion of NAD^+. Depending on the level of nicks, the consequence is exhaustion of cellular ATP and cell death by necrosis [Tentori et al., 2000, 2001]. Consistent with this interpretation is the observation that PARP inhibitors protect the cells from Me-lex induced necrosis, but now cell death is via apoptosis [Tentori et al., 2001]. In a remarkable study in WT and $Aag^{-/-}$ mice, it was shown that the same switch in the mechanism of cell death occurs as a consequence of the BER status in islet cells treated with the β-cell toxin, streptozotocin, which also methylates

DNA [Cardinal et al., 2001]. In this case, the $Aag^{-/-}$ mice are spared from the normally rapid onset of diabetes by necrosis and instead experience a moderate wave of apoptosis in the pancreas but do not become diabetic. Equally fascinating is that the mice acquire a delayed autoimmune response to β-cells and develop diabetes at approximately 8 months after treatment. Obviously, the nature of cell death in tissues by necrosis and/or apoptosis can have a significant effect on the long-term biological consequences.

MUTAGENICITY OF N3-METHYLADENINE

A main reason behind the design of Me-lex was to create a DNA damaging agent that is not mutagenic. The question remained as to whether 3-MeA was mutagenic in cells. To answer this question we did some work in bacteria and were not able to detect any sign of mutagenicity. To evaluate Me-lex in an eukaryotic system, we turned to a yeast shuttle vector assay. In this assay, mutations in the human p53 cDNA can be selected and sequenced [Ishioka et al., 1993]. The initial experiments were done in WT *S. cerevisiae*. The sites of 3-MeA formation from Me-lex were mapped in a p53 cDNA target in order to understand the relationship between alkylation and mutations that have been genetically and functionally studied in human cancers. As expected, Me-lex selectively methylates a subset of A's that are within A/T rich regions. The mutagenicity results showed that only at very high concentrations of Me-lex were mutations observed. However, the effect of the repair status of the yeast cells had a major impact on the quantitative and qualitative nature of the mutations observed. While the bottom line is that Me-lex is very weakly mutagenic in WT cells, the characterization of mutations that were observed has opened up another interesting avenue of research.

The toxicity and mutagenicity of Me-lex in WT, Δ*mag*, Δ*apn1apn2*, and Δ*magapn1apn2, S. cervisaie* strains were determined [Kelly et al., 1999; Monti et al., 2002]. MAG is the yeast homologue of AAG and alkA, and APN1 and APN2 (homologue of human HAP1) are the yeast proteins with the major AP endonuclease activity. The sensitivity of the strains to the toxicity of Me-lex is: Δ*magapn1apn2*- $\sim$ Δ*apn1apn2* $\gg$ Δ*mag* $>$ Δ*apn1* $\sim$ Δ*apn2* $\sim$ WT [Kelly et al., 1999; Monti et al., 2002]. The similar toxicity of Δ*magapn1apn2* and Δ*apn1apn2* mutants suggests that 3-MeA and AP sites are equally cytotoxic in yeast, which contrasts to the much higher sensitivity of bacteria to the 3-MeA lesion [Dinglay et al., 1998; Shah et al., 2001]. We have obtained preliminary data in yeast indicating that nucleotide excision repair (NER) can also play a role in the detoxification of 3-MeA (manuscript submitted). Recently, it was shown that Me-lex induced alkali-labile lesions appear more persistent in the BER/NER background than in BER null mouse cells [Plosky et al., 2002].

As mentioned above, the mutagenicity of Me-lex is low even at very high, cytotoxic concentrations. As might be expected from the alkylation pattern of Me-lex, the majority of mutations are A/T targeted in WT and in BER mutant strains of yeast. Of the A/T mutations, AT > TA transversions are the most common in WT yeast, while in the BER mutants AT > GC transitions become equally frequent with a concomitant reduction in AT > CG transversions. Although the mutation pattern in the WT cells is different from the repair mutants, the pattern for the different repair mutants is indistinguishable [Kelly et al., 1999; Monti et al., 2002]. The mutagenicity results showed that in the transcribed (non-coding) strand; 50-CAA(A402)(A403)C in the transcribed (non-coding) strand; and 50-G(A773)AG in the non-transcribed (coding) strand (Fig. 2). In the WT cells, only the first site is a hotspot for mutations. Since there is a difference in the mutation pattern between the BER null and WT cells, 3-MeA must be more efficiently bypassed by trans-lesion polymerases or the adduct hydrolyses to an abasic site which is actually responsible for the miscoding during replication.

It should be pointed out that only a small subset of methylated A's induce mutations and the mutation pattern is dependent on the neighboring bases. For example, 5′-GAAAAG and 5′-CAAAT sequences are extensively methylated but do not show mutations in contrast to the 5′-CAA $\underline{A}^{402}\underline{A}^{403}$ C sequences that contains two hotspots. This sequence specificity could be related to the effect of sequence on the rate of repair and there are reports that this can occur [Ye et al., 1998]. It is also possible that the flanking bases effect the efficiency of adduct by-pass by trans-lesion polymerases [Yang, 2003]: more efficient by-pass will afford more

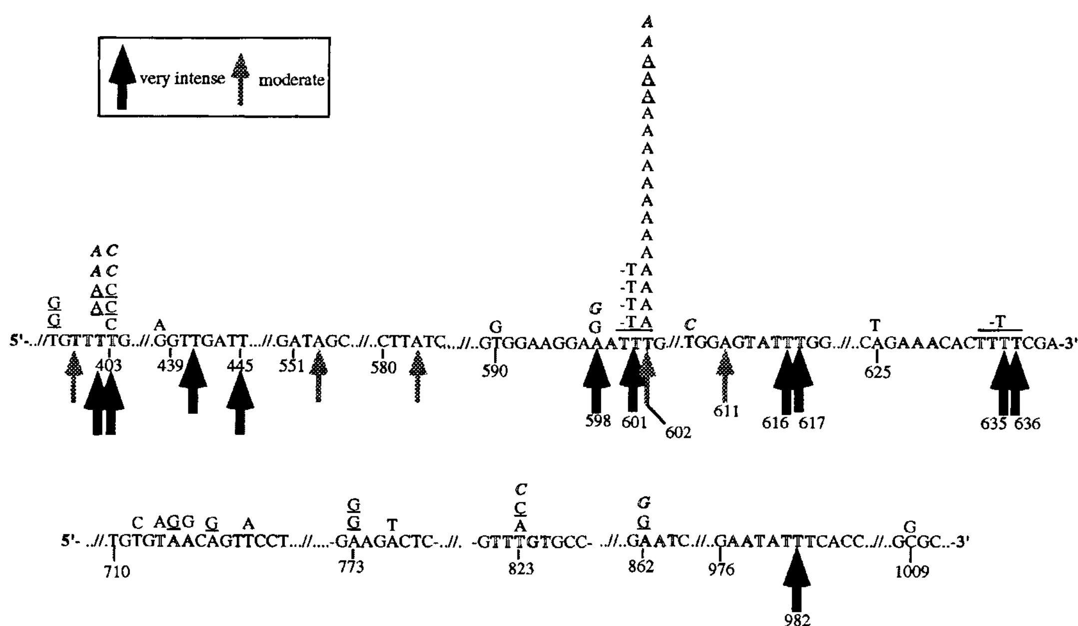

Fig. 2. Alkylation and mutation pattern induced by Me-lex in WT, Δ*mag1* (underlined mutations) and Δ*apn1apn2* (italicized mutations) *S. cerevisiae*: the darker the arrow the more intense the methylation. Only the nontranscribed (coding) strand is shown.

mutations. A final explanation for the mutation pattern relates to selection: not all mutations will affect the transcriptional function of p53 and, therefore, will be silent in our assay. It is very likely that all three factors may be involved.

HOW DOES N3-METHYLADENINE LEAD TO MUTATIONS?

DNA polymerases have a conserved arginine residue that is critical for contact between the protein and the N3-position of purines in the template strand [Dublie et al., 1998]. Any interference with this contact reduces the efficiency of elongation and causes the polymerase to become less proccessive. Accordingly, mutating the arginine residue inhibits polymerase activity. While it has not yet been proven, placing a methyl group at the N3-position of adenine (or guanine) will sterically block the required contact between the minor groove and the polymerase, and should have the same effect as mutating the arginine residue. For a DNA polymerase blocking lesion such as 3-MeA to induce mutations it must be directly or indirectly by-passed by DNA polymerase(s). The inability of DNA polymerase to by-pass a lesion will surely lead to cell death and would explain why 3-MeA is so toxic to the cells. The indirect route involves the depurination of 3-MeA into an abasic site, which is known to be by-passed by polδ insertion opposite the lesion and polζ extension pass it [Haracska et al., 2001]. The formation of abasic sites by enzyme and/or non-enzymatic pathways from 3-MeA would also account for the protective effect of NER in mouse cells that have defective BER [Plosky et al., 2002] since there are several reports that AP sites can be repaired by NER. We propose the pathways detailed in Figure 3. In cells with functional glycosylase, 3-MeA lesions are rapidly and efficiently converted into AP sites that are further processed by AP endonucleases. In the absence of glycosylase activity, the lesions persist in double-stranded DNA until replication is initiated. 3-MeA, which blocks DNA polymerase, causes stalled replication forks so the lesion is now in a single- or pseudo-single stranded region of DNA. Since the hydrolytic stability of 3-MeA in single-stranded DNA is 40-fold lower than in double-stranded DNA, there will be significant depurination to afford AP sites. AP sites in single-stranded regions, i.e., stalled replication forks, are not substrates for the AP endonucleases and will eventually yield lethal double strand breaks or be by-passed by polymerases (polζ and polδ) that

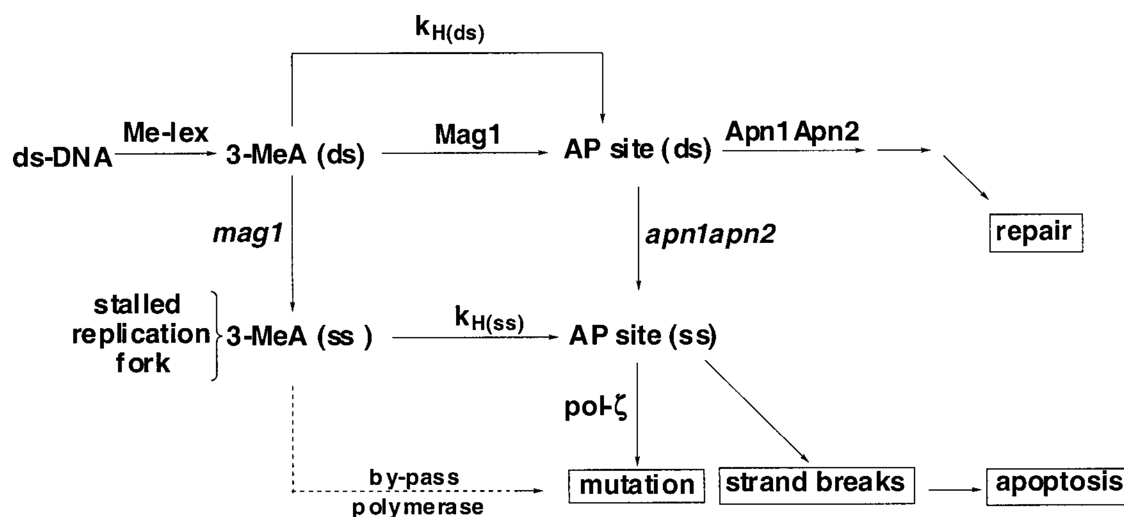

Fig. 3. Scheme to explain the toxicity and mutagenicity of N3-methyladenine.

introduce errors (i.e., T is not inserted opposite 3-MeA). In fact, the AT > GC mutation that we see in the BER mutants is exactly what is observed in yeast when AP sites are present on a plasmid [Gibbs and Lawrence, 1995]. It is of interest that the type of mutation is dependent on whether the AP site is in a plasmid versus genomic DNA. At this time we do not know if the 3-MeA adduct can be by-passed, but an in vitro model has been developed to study this question.

As a result of the studies summarized above, we embarked on collaborative studies to determine if Me-lex can be clinically useful as an anticancer drug. In an NCI screen, Me-lex showed good activity against three cell lines: SNB-19 (glioma), NCI H460 (non-small cell lung cancer), MAMLE-3M (melanoma). The origin of this sensitivity is unclear, but the lines are mutant for p53 [O'Connor et al., 1997]. The greater toxicity of Me-lex in MT-1 versus Jurkat cells correlates with their AAG activity [Tentori et al., 2000], but it is unlikely that BER activity will always predict sensitivity. Because one of the NCI lines that was particularly sensitive to Me-lex is from a glioma and because of the dire prognosis for people with this type of cancer, we are planning to look at Me-lex and analogues in an in vitro brain tumor model. Whether a useful drug can be developed is not clear, but the resistance to currently used antineoplastic agents, e.g., temozolomide and BCNU, in the treatment of brain cancer involve over-expression of alkylguanine DNA-alkyltransferase and loss of mismatch repair. Me-lex will not be effected by these resistance pathways. The potential use of Me-lex in combination with PARP inhibitors is also under investigation.

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