

A Chemical and Genetic Approach Together Define the Biological Consequences of 3-Methyladenine Lesions in the Mammalian Genome*

(Received for publication, October 8, 1997, and in revised form, December 21, 1997)

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DNA-damaging agents produce a plethora of cellular responses that include p53 induction, cell cycle arrest, and apoptosis. It is generally assumed that it is the DNA damage produced by these agents that triggers such responses, but there is limited direct evidence to support this assumption. Here, we used DNA alkylation repair proficient and deficient isogenic mouse cell lines to demonstrate that the signal to trigger p53 induction, cell cycle arrest, and apoptosis in response to alkylating agents does emanate from DNA damage. Moreover, we established that 3-methyladenine, a relatively minor DNA lesion produced by most methylating agents (which form mainly 7-methylguanine), can specifically induce sister chromatid exchange, chromatid and chromosome gaps and breaks, S phase arrest, the accumulation of p53, and apoptosis. This study was made possible by the generation of 3-methyladenine DNA glycosylase null mutant cells by targeted homologous recombination and by the chemical synthesis of a methylating agent that almost exclusively produces 3-methyladenine DNA lesions. The combined use of these two experimental tools has defined the biological consequences of 3-methyladenine, a DNA lesion produced by endogenous cellular metabolites, environmental carcinogens, and chemotherapeutic alkylating agents.

Overlapping cellular responses protect mammals against DNA-damaging agents. The abilities to repair DNA damage, to stop cells with damaged genomes from cycling, and to eradicate cells bearing extensive DNA damage each contribute to that protection. Defects in cellular responses to DNA damage can result in cancer prone disease, as witnessed in individuals

suffering from hereditary nonpolyposis colorectal cancer, Li-Fraumeni syndrome, ataxia telangiectasia, and xeroderma pigmentosum (1).

Numerous mammalian proteins have been characterized for their roles as effectors of cell cycle arrest and apoptosis induced by DNA-damaging agents, most notably p53 (2). However, little is known about the specific types of DNA damage that can elicit these cellular responses or about the mechanisms for converting DNA damage into a signal for arrest or apoptosis. Indeed, direct evidence that DNA damage *per se* is the primary signal for the initiation of arrest and apoptosis is surprisingly limited. DNA double strand breaks are currently the only defined DNA lesion shown definitively to cause p53 induction (3) or to trigger cell cycle arrest (4), and indirect evidence suggests that they induce apoptosis (5); there is little evidence that more subtle forms of DNA damage can elicit these responses. In addition, it is quite clear that the cell nucleus is not required for the induction of apoptosis in response to some agents, such as ionizing radiation, thus excluding DNA damage as the trigger in these cases (6).

Alkylating agents are cytotoxic, mutagenic, carcinogenic, and teratogenic. These agents are present in our environment, they exist as normal cellular metabolites, and they are used for cancer chemotherapy (1). Alkylating agents can induce p53, cell cycle arrest, and apoptosis (7, 8), but it is unknown whether alkylated DNA elicits the signal to activate these responses and, if so, which specific alkylated DNA lesions are responsible. Most methylating agents transfer methyl groups to over a dozen nucleophilic nitrogens and oxygens in DNA (9), and so studies on the biological consequences of specific lesions have been difficult. One methyl lesion that has been studied extensively is *O*⁶-methylguanine (*O*⁶MeG).¹ Because this lesion is repaired by a highly specific DNA repair methyltransferase and cells deficient in this enzyme were readily available, the biological consequences of this toxic and mutagenic lesion have been explored in detail (1). The focus of this report is the *N*-alkylated DNA base lesion 3-methyladenine (3MeA), produced upon exposure of DNA to exogenous and endogenous methylating agents. 3MeA is excised from DNA by DNA glycosylases that initiate the base excision repair pathway (1); these glycosylases are present across species, from bacteria to humans, implicating 3MeA as a biologically important DNA lesion.

The mammalian 3MeA DNA glycosylase (named Aag for

* This work was supported by National Institutes of Health Grants RO1 CA55042 (to L. D. S.), PO1 ES03926 (to L. D. S.), and CA36727 (to B. G.) and by American Cancer Society Center Grant ACS SIG-16 (to B. G.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¶ Supported by a Pharmaceutical Manufacturers Association Foundation Advanced Predoctoral Fellowship in Pharmacology/Toxicology and by National Institutes of Health Toxicology Training Grant 5T32ES07155.

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¹ The abbreviations used are: *O*⁶MeG, *O*⁶-methylguanine; Aag, alkyladenine DNA glycosylase; 3MeA, 3-methyladenine; 7MeG, 7-methylguanine; 3MeG, 3-methylguanine; Me-Lex, MeOSO₂(CH₂)₂-*N*-methylpyrrole dipeptide; MMS, methyl methanesulfonate; SCE, sister chromatid exchange; ES, embryonic stem.

alkyladenine DNA glycosylase) has a relatively broad substrate range, which includes 3MeA, 3-methylguanine (3MeG), and 7-methylguanine (7MeG), in addition to more complex alkyl lesions (10–12), but does not include O^6 MeG.² This broad substrate range, in conjunction with the fact that most simple methylating agents transfer methyl groups to numerous sites in DNA, has made it difficult to study the effects of 3MeA itself. Ideally, a method to specifically introduce 3MeA into DNA, used in conjunction with isogenic cell lines proficient and deficient in 3MeA repair, would provide the necessary tools for determining the biological effects of 3MeA. We therefore created 3MeA DNA glycosylase-deficient cells by targeted homologous recombination (13) and developed a methylsulfonate ester tethered lexitropsin compound (Me-Lex) that produces 3MeA DNA lesions almost exclusively (14, 15). Here we combine these tools to show that 3MeA in DNA elicits p53 induction, S phase arrest, sister chromatid exchange (SCE), chromosome aberrations, and apoptosis in mouse embryonic stem (ES) cells.

EXPERIMENTAL PROCEDURES

Drug Treatments—Log phase ES cells were cultured as described previously (13) and treated with methyl methanesulfonate (MMS) (Aldrich), Me-Lex, hydrolyzed Me-Lex (prereacted for 48 h in 10 mM Tris-HCl, 1 mM EDTA buffer at room temperature), ionizing radiation, or UV radiation. For treatments, cells were incubated in drug containing serum-free ES medium for 1 h at 37 °C and 5% CO₂. Excess drug was removed with PBS. For irradiation, cells were exposed to either a ⁶⁰Co source or a UV (254 nm) source.

Isolation of DNA Adducts from MMS and Me-Lex treated ES Cells—10⁷ *Aag*^{-/-} cells were treated with MMS, Me-Lex, or control medium. Cells were subsequently scraped from dishes, pelleted, and frozen in liquid N₂. Cells were suspended in 0.5 ml 10 mM Tris-HCl (pH 8), 100 mM NaCl, 1 mM EDTA, to which was added 0.05 volume of 10% SDS. After RNase treatment (100 µg/ml for 2.5 h at 37 °C), samples were treated with proteinase K (100 µg/ml overnight at 37 °C). DNA was isolated by phenol extraction (16) and dissolved in 10 mM Tris-HCl (pH 9.0); an aliquot was removed for DNA quantification (17). Samples were heated (75 °C for 8 h) to preferentially release 3- and 7-methylpurines from DNA (18). The partially apurinic DNA was precipitated at 0 °C by adding 0.1 volume of 0.1 N HCl (9). Supernatants were concentrated, resuspended in 110 µl of 10 mM Tris-HCl (pH 7.4), and analyzed by high performance liquid chromatography (25-cm Whatman SCX; 30-min linear gradient of 0–20% MeOH in 100 mM ammonium formate (pH 3.0) at 40 °C; 1 ml/min; detection, 273 nm and photodiode array). Adducts were quantified relative to a standard curve (linear from 20 to 150 pmol of adduct/injection) or by multicomponent analysis. The response of 7MeG was not affected by the amount of adenine in the sample.

SCEs and Chromosome Aberrations—SCEs were analyzed as described previously (13). For aberrations, cells were incubated in McCoy's 5A (Life Technologies, Inc.)-based ES medium supplemented with 10 µM 5-bromo-2'-deoxyuridine (Sigma) following treatment. Slides were prepared as for SCEs (13). For SCEs, 20 second division metaphase spreads were counted per data point. For chromosome aberrations, 50 first division metaphase spreads were counted per data point. Aberrations were scored blinded. Chromosome breaks were quantified by weighted scoring of each type of aberration as described previously (19), and the frequency of aberrations within a population of cells was calculated from the number of break events divided by the number of spreads examined.

Flow Cytometric Analysis of Cell Cycle Phase and Apoptosis—Following exposure to genotoxic agents, medium was reserved, cells were trypsinized, and trypsin was neutralized by replacing the reserved medium. Dispersed cells were pelleted, fixed in 70% ethanol, and stored at -20 °C. Pelleted cells were resuspended in staining solution (10 µg/ml RNase A (Sigma) and 40 µg/ml propidium iodide (Sigma) in PBS) and incubated at room temperature for at least 1 h prior to analysis. 10⁴ cells were analyzed on a fluorescence-activated cell sorter (Ortho) for relative DNA content.

Apoptosis Measurements—The apoptotic response was evaluated by three methods. (a) Sub-G₁ cells were quantified by flow cytometry as described previously (20). (b) A photometric enzyme immunoassay was

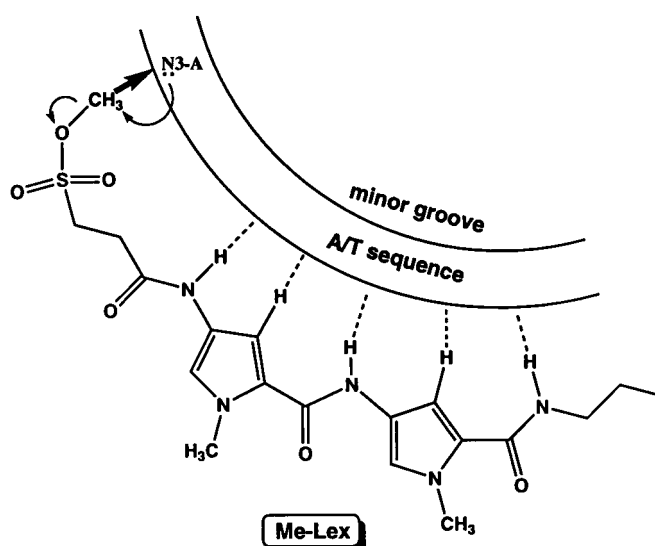


FIG. 1. Chemical structure of methyl-lexitropsin (Me-Lex) and its orientation when bound to an A:T rich sequence within the minor groove of DNA.

used for *in vitro* determination of cytoplasmic histone-associated DNA fragments (Boehringer Mannheim). Briefly, 3×10^6 cells were treated with genotoxic agents and harvested by trypsinization, and 3×10^4 cells were processed according to the manufacturer's instructions. (c) Apoptotic nuclei were identified by microscopy. Approximately 10⁴ cells fixed in 70% ethanol were dried onto microscope slides. Cells were stained in Hoechst 33258 buffer (0.5 µg/ml Hoechst 33258, 0.05% non-fat dried milk) for 20 min at room temperature. Apoptotic cells were identified by fluorescence microscopy. Each assay was repeated at least twice for each genotoxin, and representative graphs are shown.

Western Blot Analysis—Cells were boiled for 15 min in 1.7% SDS, 17% glycerol, 0.1 M DTT, 83 mM Tris-HCl (pH 6.8), 0.001% bromophenol blue at a density of 2.5×10^4 cells/µl before loading. Samples (3×10^5 cell equivalents/lane) were resolved by 12% SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes (Millipore), and probed with monoclonal p53 antibody (Pab 240) (Santa Cruz Biotechnology) followed by horseradish peroxidase-conjugated rabbit anti-mouse IgG (Amersham Corp.). Antibody binding was detected by enhanced chemiluminescence (Amersham Corp.). Equal loading was determined by Ponceau S staining.

RESULTS

3MeA Is the Major DNA Base Adduct Formed by *in Vivo* Me-Lex Exposures—To study the biological consequences of 3MeA, a synthetic compound was developed to target alkylation to the N3 position of adenine. Me-Lex is composed of an S_N2 methyl donating domain and the multicyclic lexitropsin peptide, which equilibrium binds the minor groove at A:T rich regions in double-stranded DNA (Fig. 1) (14, 15). Specific minor groove binding at A:T base pairs limits alkylation to the minor groove atoms and enhances alkylation at the nucleophilic N3 position of adenines that are in the Lex binding site. After *in vitro* DNA alkylation with Me-Lex, about 90% of the adducted bases are 3MeA (15). This represents a dramatic change from DNA alkylation by the S_N2 agent MMS, which produces about 80% 7MeG and only 10% 3MeA (9). However, *in vitro*, at a high molar ratio of Me-Lex relative to DNA, the specificity for alkylation at adenines decreases, because A:T rich Lex binding sites become saturated (15). It was therefore important to measure the relative abundance of 3MeA versus 7MeG after treatment of cells in culture with Me-Lex to determine whether Me-Lex retains its specificity for adduction at N3-adenine *in vivo*.

Aag^{-/-} cells are devoid of 3MeA DNA glycosylase activity (13). *Aag*^{-/-} ES cells were exposed to 1 mM Me-Lex (far greater than the doses used for subsequent experiments). As a control, we exposed cells to 1.3 mM MMS because the propor-

² L. D. Samson, unpublished observations.

TABLE I
Alkylation of *Aag*^{-/-} ES cells by Me-Lex

Aag^{-/-} murine ES cells were treated with either MMS or Me-Lex, and DNA was subsequently assayed for 3MeA and 7MeG as described under "Experimental Procedures." All values are the mean and standard deviation.

| Treatment | 3MeA | | 7MeG | |
|-------------------|--------------------------|--|--------------------------|--|
| | pmol / μ mol DNA | | | |
| Control (no drug) | ND ^a (n = 3) | | 44.2 \pm 7.9 (n = 3) | |
| 1.0 mM Me-Lex | 369.3 \pm 32.3 (n = 2) | | 21.5 ^b | |
| 1.3 mM MMS | 12.9 \pm 1.0 (n = 3) | | 131.7 \pm 27.7 (n = 4) | |

^a ND, not detected.

^b 7MeG levels were only detected in one of three samples.

tions of DNA lesions produced by MMS are well established (9). As expected, MMS induced a significant increase in both 3MeA and 7MeG lesions, at a ratio of ~1:7 (after background correction) (Table I). However, although Me-Lex produced a dramatic increase in 3MeA lesions, it did not produce any measurable increase in 7MeG lesions (Table I). Similar results were observed in two independent experiments.³ Thus, Me-Lex clearly maintains its alkylation specificity when used to treat cells in culture. Because *in vitro* studies indicate that Me-Lex induces at least 1000-fold more 3MeA than O⁶MeG (15) and at least 75-fold more 3MeA than 3MeG³, we infer that 3MeAs make up the vast majority of alkylated DNA bases produced in mouse ES cells exposed to Me-Lex.

3MeA DNA Lesions Induce SCEs and Chromosome Aberrations—O⁶MeG DNA lesions are known to induce SCEs (21). We investigated the possibility that 3MeA DNA lesions can also induce SCEs. 3MeA DNA glycosylase deficient *Aag*^{-/-} cells are much more sensitive than wild type cells to SCEs induced by Me-Lex (Fig. 2A). *Aag*^{-/-} cells are also more sensitive to MMS-induced SCEs (Fig. 2B), as expected (13). In contrast, *Aag* status does not affect SCEs induced by UV (Fig. 2C), indicating that the targeted *Aag* mutations do not produce a general defect resulting in increased SCEs induced by any type of DNA damage, but rather produce a sensitivity that is alkylation-specific. Furthermore, we infer that the SCEs observed in Me-Lex treated *Aag*^{-/-} cells were induced by 3MeA DNA lesions. The concentration of Me-Lex required to induce one SCE per chromosome in the *Aag*^{-/-} cells (0.04 mM) is significantly lower than the concentration of MMS required to give the same response (0.6 mM). This is consistent with the ability of Me-Lex to induce more 3MeA than MMS at approximately equimolar concentrations (Table I) and provides further evidence that 3MeA is the primary lesion responsible for SCE induction by Me-Lex. Moreover, because O⁶MeG, 7MeG, and 3MeG are all produced at a lower relative frequency by Me-Lex compared with MMS (9, 14, 15), then a higher concentration of Me-Lex relative to MMS would be required for O⁶MeG, 7MeG, and 3MeG to induce SCE to the same level. The lower Me-Lex concentration relative to MMS required to induce a similar response was consistently observed for all end points studied (see Figs. 2–6). However, although it is clear that 3MeA is the lesion primarily responsible for SCE induction by Me-Lex, we acknowledge that there may be a minor contribution from other lesions.

Alkylating agents are potent inducers of chromosome aberrations (22), but apart from O⁶MeG, little is known about the specific types of DNA damage that trigger them. Cells lacking O⁶MeG DNA methyltransferase are sensitive to chromosome aberrations following treatment with methylating agents, but methyltransferase overexpression does not completely suppress these aberrations (23), suggesting that lesions other than

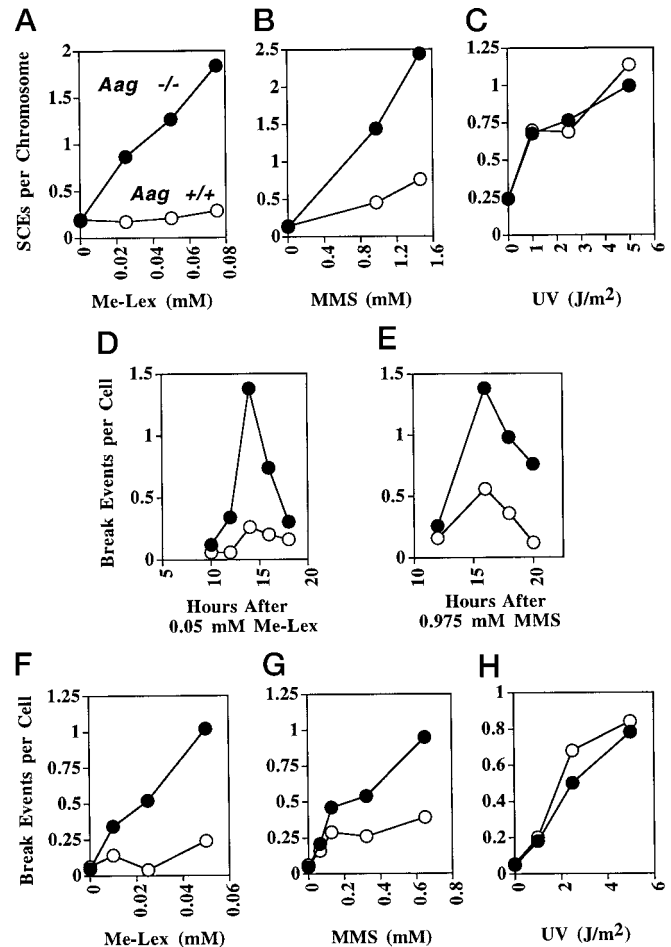


FIG. 2. SCE induction and chromosome aberration induction in *Aag*^{+/+} (open circles) and *Aag*^{-/-} (filled circles) murine ES cells. Shown are SCEs in response to Me-Lex (A), MMS (B), and UV (C) and chromosome aberrations induced by Me-Lex (D and F), MMS (E and G), and UV (H) over time (D and E) or 14–16 h post-treatment (F–H).

O⁶MeG induce chromosome aberrations. Here we show that 3MeA is a clastogenic DNA lesion.

We first determined whether *Aag* status alters the time of peak aberration induction after exposure to alkylating agent. Chromosome aberrations in cells undergoing their first metaphase post-Me-Lex or MMS exposure peaked at around 14–16 h for both *Aag*^{+/+} and *Aag*^{-/-} cells (Fig. 2, D and E). During the time course, or when cells were treated with a range of MMS or Me-Lex doses, *Aag*^{-/-} cells were consistently more sensitive to aberration induction than *Aag*^{+/+} cells (Fig. 2, D–G). In contrast, *Aag* status had no influence on UV-induced chromosome aberrations (Fig. 2H). Thus, 3MeAs are clearly clastogenic lesions. The average number of each type of aberration per cell was calculated from the combined data of 850 chromosome spreads of cells treated with Me-Lex or MMS and from 150 chromosome spreads of cells exposed to UV. Compared with wild type cells, *Aag*^{-/-} cells are particularly sensitive to chromatid and chromosome breaks and gaps induced by Me-Lex and only modestly sensitive to other types of aberrations (Fig. 3A). This bias was diminished for MMS-treated cells and absent for UV-treated cells (Fig. 3, B and C).

3MeA DNA Lesions Induce S Phase Arrest—MMS is known to induce an S phase arrest in mammalian cells (24), and here we show that 3MeA DNA lesions contribute to that arrest. Cell cycle distribution was measured by flow cytometry according to DNA content. Untreated, exponentially growing, asynchronous *Aag*^{+/+} and *Aag*^{-/-} cells had similar cell cycle distributions

³ J. D. Kelly and B. Gold, unpublished observations.

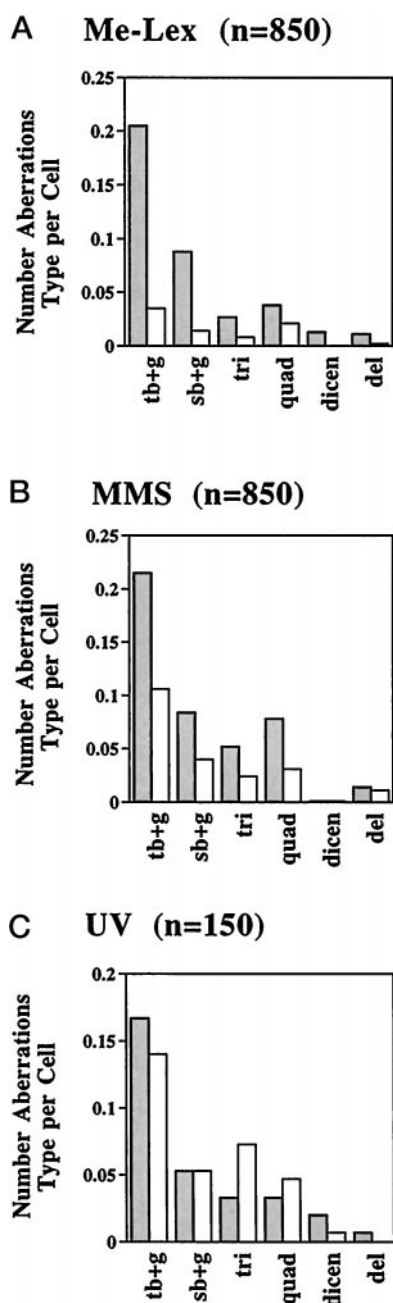


FIG. 3. Qualitative analysis of chromosome aberrations induced in *Aag* +/+ (open columns) and *Aag* -/- (gray columns) murine ES cells. Chromosome aberrations were measured in response to 0.05–0.1 mM Me-Lex (A), 0.05–0.5 mM MMS (B), and 1–5 J/m² UV (C). The number of metaphase chromosome spreads used for analysis is indicated by *n*, and equal numbers of spreads from the various doses were used for *Aag* +/+ and *Aag* -/-. The following aberrations were monitored: chromatid breaks and gaps (*tb+g*), chromosome breaks and gaps (*sb+g*), triradial chromosomes (*tri*), quadriradial chromosomes (*quad*), dicentric chromosomes (*dicen*), and chromosome deletions (*del*).

(Fig. 4, 0 Hrs), and estimates of the duration of G₁, S, and G₂/M agree with published reports (25).⁴ MMS and Me-Lex exposure caused wild type cells to accumulate in early S phase (Fig. 4, A and B), indicating a robust S phase arrest that was most pronounced at 10 h for MMS and 6 h for Me-Lex. The arrested *Aag* +/+ cells emerged from S phase by 20 h post-MMS treatment (Fig. 4A) or 10 h post-Me-Lex treatment (Fig. 4B). In stark contrast, *Aag* -/- cells suffer a much more prolonged S

phase arrest; at 20 h post-MMS treatment (Fig. 4A) and 14 h post-Me-Lex treatment (Fig. 4B), a significant portion of cells remained arrested in the early stages of S phase. This delayed subpopulation of *Aag* -/- cells eventually moved through S phase, but at a significantly reduced rate relative to *Aag* +/+ cells. Taken together, these data indicate that DNA alkylation damage, and more specifically 3MeA DNA lesions, can elicit an S phase arrest.

Ionizing radiation causes DNA damage of a kind that is not thought to be repaired by *Aag*. When cells were exposed to ionizing radiation, there was no significant difference in the cell cycle response of the *Aag* +/+ cells and *Aag* -/- cells (Fig. 4C).

3MeA DNA Lesions Trigger Apoptosis—To investigate whether simple alkylated bases can induce apoptosis, we compared MMS- and Me-Lex-induced apoptosis in *Aag* +/+ and *Aag* -/- mouse ES cells. The apoptotic response was measured by accumulation of DNA ends and histone-associated DNA fragments (Fig. 5A), by the appearance of apoptotic nuclei by DNA fragmentation as measured by Hoechst staining (Fig. 5B), and by sub-G₁ DNA content by flow cytometry (Fig. 5C). *Aag* -/- cells were consistently more susceptible to both MMS- and Me-Lex-induced apoptosis. Because there was no significant difference between *Aag* +/+ and *Aag* -/- cells in their sensitivity to γ -ray-induced apoptosis (Fig. 5, A–C), we conclude that the sensitivity of *Aag* -/- cells to apoptosis is alkylation-specific. Moreover, we infer that 3MeA lesions can trigger apoptosis.

3MeA DNA Lesions Signal p53 Induction—Apoptosis can be induced by p53-dependent and p53-independent pathways (26), and so we determined whether the induction of apoptosis by a simple methylated base like 3MeA was accompanied by p53 induction. *Aag* +/+ and *Aag* -/- cells were exposed to MMS, Me-Lex, or ionizing radiation. For MMS and Me-Lex, there was a much greater induction of p53 in the *Aag* -/- cells than in the *Aag* +/+ cells (Fig. 6, A and B), indicating that alkylated DNA bases, including 3MeA, can signal p53 induction. In contrast, ionizing radiation induced similar amounts of p53 in both cell types (Fig. 6C), indicating that *Aag* -/- cells do not have a general increase in their p53 induction response to DNA damage.

The Biological Effects of Me-Lex Are Dependent upon Methylation—To confirm that the observed effects of Me-Lex are due to its methylating activity, we determined whether the parent lexitropsin compound was able to induce any biological effects under the conditions used in our studies. Hydrolyzed Me-Lex (which can no longer alkylate DNA) did not result in significant levels of cell killing, SCE induction, chromosome aberrations, S phase arrest, or apoptosis.⁵ We therefore conclude that the biological activity of Me-Lex is dependent upon its ability to produce 3MeA DNA lesions and is independent of the DNA binding properties of lexitropsin.

DISCUSSION

3MeA DNA lesions have long been considered replication-blocking lesions because a bacterial and a viral DNA polymerase were shown to terminate at adenine residues in a methylated DNA template *in vitro* (27). Although these studies were indirect, and although such studies have not yet been reported for any mammalian DNA polymerase, recent structural information on DNA polymerases provides a plausible mechanism for the inhibitory effect of 3MeA. For four DNA polymerases (namely mammalian polymerase β , *Thermus aquaticus* *Taq* polymerase, T7 bacteriophage DNA polymerase, and *Esche-*

⁴ J. M. Allan and L. D. Samson, unpublished observations.

⁵ B. P. Engelward, A. J. Dreslin, and L. D. Samson, unpublished observations.

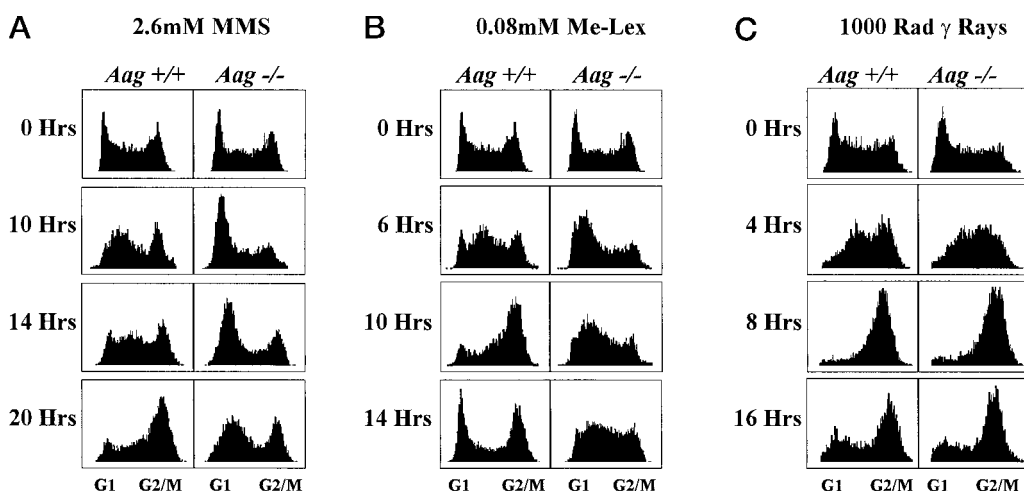


FIG. 4. Flow cytometric analysis of cell cycle phase by DNA content in *Aag +/+* and *Aag -/-* murine ES cells. Cell cycle distribution was measured in response to 2.6 mM MMS (A), 0.08 mM Me-Lex (B), and 1000 rad γ -rays (C). 10,000 cells were analyzed per histogram. Cells with sub-G₁ DNA content were not included in the analysis.

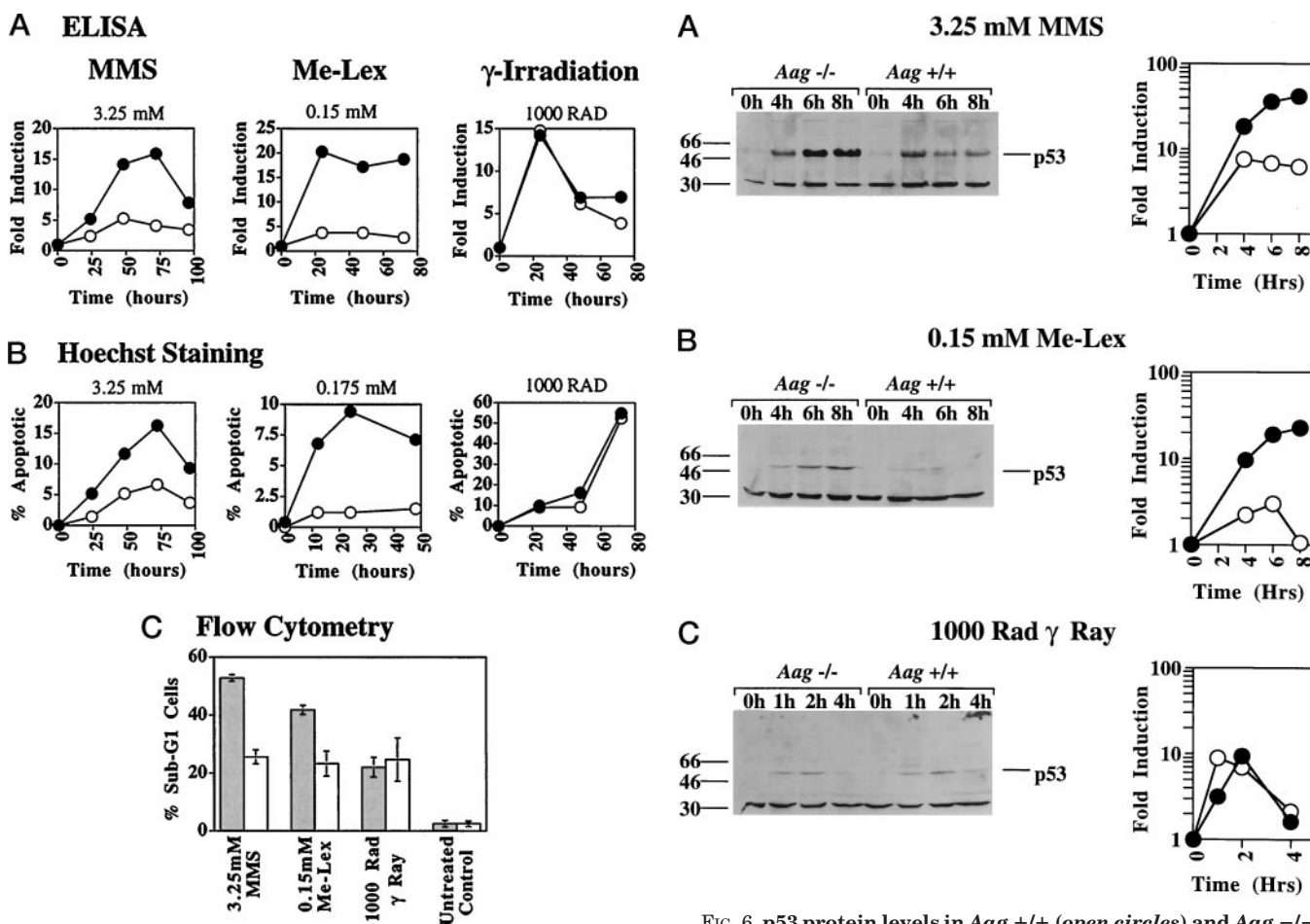


FIG. 5. Apoptosis in *Aag +/+* (open circles and columns) and *Aag -/-* (filled circles and columns) murine ES cells in response to MMS, Me-Lex, and γ -rays. Apoptosis was measured using enzyme-linked immunosorbent assay (ELISA) (A), Hoechst analysis (B), and flow cytometry (C). Histograms in C are the mean \pm S. D. from three independent experiments in which 10,000 cells were analyzed per sample.

richia coli polymerase I), contacts between key amino acid residues and the N3 atom of purines in the template strand have been established (28–31), and for polymerase β , this contact was shown to be crucial for catalytic activity (32). Our observations that 3MeAs specifically cause SCEs, S phase ar-

rest, and chromosome aberrations are consistent with an inhibitory effect of 3MeA on mammalian DNA replication, as discussed below.

SCEs are often regarded as the outcome of recombination between sister chromatids, and so one might infer from our results that 3MeA DNA lesions stimulate DNA recombination between sister chromatids. However, it has also been proposed that SCEs result from strand switching at stalled replication

FIG. 6. p53 protein levels in *Aag +/+* (open circles) and *Aag -/-* (filled circles) murine ES cells. p53 protein levels were measured in response to 3.25 mM MMS (A), 0.15 mM Me-Lex (B), and 1000 rad γ -rays (C). Western blots are representative examples. Fold induction was calculated from the mean of three or four independent experiments.

forks for the following reasons: (i) cells that sustain damage in G₂ do not form SCEs, and (ii) DNA damage must be present during S phase to induce SCEs (33–35). Indeed, it was reported that the lowest dose of MMS that results in S phase arrest coincides with the lowest dose required for SCE induction (24). Our data support this model in that a more robust 3MeA-induced S phase arrest coincides with increased SCE induction. We therefore suggest that the ability of 3MeA to inhibit DNA replication is responsible for SCE induction.

The induction of chromosome aberrations by 3MeA DNA lesions is mainly accounted for by increased chromatid and chromosome breaks/gaps, which are also thought to be stimulated by blocked replication forks (36). If replication is blocked in one of the growing strands, a short unreplicated region could remain in that sister chromatid, and this region may or may not be converted to a DNA double strand break. The single-stranded DNA region (or the double strand DNA break) is thought to disrupt chromatin condensation so that a chromatid break/gap is visible at metaphase (36). The mechanism by which breaks/gaps are simultaneously produced in both sister chromatids (chromosome breaks/gaps) is not yet known. It has been proposed that unreplicated regions arise when two forks are unable to meet each other because of intervening DNA lesions (37). We propose that these unreplicated regions may segregate to each sister chromatid and appear as symmetric breaks/gaps in both sister chromatids. Our results support these models in that the increased S phase arrest induced by 3MeA lesions coincides with increased numbers of chromatid and chromosome breaks/gaps. Further, Me-Lex induction of chromosome aberrations shows induction kinetics that are typical of S phase-dependent clastogens (peaking 12–16 h post-treatment) (36, 38). We therefore suggest that the ability of 3MeA to inhibit DNA replication is responsible for the induction of chromosome aberrations.

S phase arrest can be separated into two parts: inhibition of DNA chain elongation and inhibition of replicon firing (39, 40). The two components of S phase arrest are, to some extent, independent because low doses of ionizing radiation inhibit replicon firing without affecting chain elongation (41). In fact, such inhibition of replicon firing causes a subpopulation of cells to accumulate in early S phase, and with time, this cohort of cells can be seen to move as a bolus through S phase (42). This pattern is reminiscent of the kind of S phase arrest we observed in response to 3MeA DNA lesions, raising the possibility that 3MeAs can inhibit replicon firing, in addition to chain elongation. Furthermore, it has been shown that several checkpoint genes are required for *Saccharomyces cerevisiae* to experience an S phase delay during low level MMS exposure (43, 44). Studies are currently under way to determine whether 3MeA-induced S phase arrest requires the participation of cell cycle checkpoint and other proteins. 3MeA is clearly a strong inhibitor of S phase progression. Further studies are necessary to determine whether this lesion also affects other cell cycle phases.

The fact that many DNA-damaging agents induce apoptosis has led to the assumption that DNA damage *per se* elicits the initial inducing signal to trigger this programmed cell death pathway. However, for some agents, apoptosis can be induced in a DNA damage-independent manner (6, 45), and there is limited formal evidence to support the assumption that DNA lesions actually trigger the response (46–48). Indeed, several studies clearly demonstrate that certain DNA-damaging agents elicit signals at the cell membrane that trigger apoptosis (45) and that trigger the activation of transcription factors implicated in apoptosis (49, 50). It has also been assumed that DNA damage *per se* signals the stabilization of p53, thus in-

ducing p53 levels (stabilization is thought to be achieved by the specific phosphorylation of p53 in response to DNA-damaging agents). Again, apart from the case of double strand DNA breaks, there is little formal evidence to support the assumption that DNA lesions themselves elicit the signal to stabilize p53 or to induce apoptosis. Here, we demonstrate that a particular damaged DNA base, 3MeA, can trigger both apoptosis and p53 induction, but exactly how this is achieved and whether p53 induction is required for the observed apoptosis are not yet clear. It has been suggested that the single strand DNA breaks that are inevitably generated during the excision repair of DNA damage could serve as the signal for p53 induction (3). However, our results provide strong evidence against this mechanism, because it is the cells *lacking* Aag-initiated base excision repair that experience increased p53 and apoptosis. Although we may eliminate the base excision repair pathway as being responsible for eliciting the damage response pathway, we cannot exclude nucleotide excision repair as having an effect. However, it seems likely that unrepaired 3MeA lesions are responsible for eliciting these responses in the *Aag*^{-/-} cells.

In summary, we have shown that unrepaired 3MeA DNA lesions have the potential to induce SCEs, chromosome aberrations, S phase arrest, p53 induction, and apoptosis. The importance of the N3 atom of the purine ring for stabilizing the contact between DNA polymerase and its template (28–32) is likely responsible for the extremely toxic nature of this rather subtly modified DNA base.

Acknowledgment—We gratefully acknowledge the expert technical assistance of Amy Imrich, Harvard School of Public Health.

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