Abstract

Purpose: Defining the cytotoxicity of individual adducts in DNA is necessary for mechanistic understanding of human brain tumor resistance to therapeutic alkylating agents and for design of DNA repair-related antiresistance strategies. Our purpose is to characterize the sensitivity of human glioma cells to methyl-lexitropsin (Me-lex), a sequence-specific alkylator that produces 3-methyladenine (3-meA) as the predominant (>90%) DNA lesion.

Experimental Design: We quantitated the Me-lex cytotoxicity of 10 human glioma cell lines that differ in O6-methylguanine (O6-meG)-DNA methyltransferase (MGMT) and mismatch repair activity. We used antisense suppression of alkyladenine DNA glycosylase (AAG) and Ape1 to assess the contribution of 3-meA and abasic sites to lethality and measured abasic site content.

Results: (a) The LD10 for Me-lex varied widely among the cell lines. (b) MGMT-proficient lines were more resistant than MGMT-deficient lines, an unexpected finding because Me-lex produces very little O6-meG. (c) Suppression of AAG increased Me-lex killing and reduced abasic site content. (d) Suppression of Ape1 increased Me-lex killing and increased abasic site content. (e) Ablation of MGMT had no effect on Me-lex cytotoxicity.

Conclusions: (a) Me-lex is cytotoxic in human glioma cells and AAG promotes resistance, indicating that 3-meA is a lethal lesion in these cells. (b) Abasic sites resulting from 3-meA repair are cytotoxic and Ape1 promotes resistance to these derivative lesions. (c) A factor(s) associated with MGMT expression, other than repair of O6-meG, contributes to Me-lex resistance. (d) Me-lex may have clinical utility in the adjuvant therapy of gliomas. (e) AAG and Ape1 inhibitors may be useful in targeting alkylating agent resistance.

Human Glioma Cell Sensitivity to the Sequence-Specific Alkylating Agent Methyl-Lexitropsin

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Many alkylating agents undergo chemical decomposition to produce highly reactive electrophiles (e.g., diazonium and carbonium ions) that readily transfer their alkyl moieties to multiple nucleophilic sites on purines and pyrimidines in DNA (reviewed in ref. 1). In dsDNA, reaction occurs preferentially at ring nitrogens of purines. The majority of methylation, including that produced by the chemotherapeutic agents procarbazine and temozolomide, occurs at N7 of guanine (70-80%) and N3 of adenine (~9%). Other N-methylpurines (e.g., 3-methylguanine, 1-methyladenine, and 7-methyladenine) constitute between 0.5% and 2.0% of total base adducts (1, 2). Work with repair-deficient bacteria (3, 4) indicates that 7-methylguanine is biologically innocuous and implicates 3-methyladenine (3-meA) as an important cytotoxic lesion. However, definitive elucidation of the contribution of individual N-methylpurines to cell killing has been hampered by the diversity of lesions produced by methylators, by the large biological effects of some quantitatively minor products [e.g., O6-methylguanine (O6-meG); refs. 5, 6], and by the instability of potentially cytotoxic lesions to manipulation in vitro. A novel approach that circumvents these problems exploited the sequence-specific binding properties of the antibiotics distamycin and netropsin, whose N-methylpyrrolecarboxamide moieties preferentially bind at A-T rich regions in the minor groove of DNA (reviewed in ref. 7). Gold et al. synthesized a series of compounds, designated lexitropsins, consisting of N-methylpyrrolecarboxamide subunits appended with methylating functionalities (8, 9). One derivative appended with an O-methyl sulfonate ester [1-methyl-4-(1-methyl-4-(3-methoxysulfonyl-propanamido)pyrrole-2-carboxamido) propane; methyl-lexitropsin (Me-lex)] methylates DNA almost exclusively at the N3 atom of adenine (8). Quantitation of Me-lex-induced adducts revealed that >90% of base lesions are 3-meA (8, 9).

The cytotoxicity of Me-lex has been documented in bacteria, yeast, and mammalian cells, strongly indicating that 3-meA is directly lethal or is converted into a lethal lesion (7). Available evidence indicates that cell killing by 3-meA is due, at least in

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part, to blockage of DNA replication as evidenced by arrest of S-phase progression in murine cells treated with Me-lex and by arrest of in vitro DNA synthesis by bacterial and phage DNA polymerases (reviewed in ref. 10). X-ray structures of bacterial and phage and also mammalian DNA polymerases bound to DNA have revealed contact between template-binding amino acid side chains and the N3 position of adenine. This interaction, which is sterically blocked by the methyl group in 3-meA, is apparently necessary for DNA synthesis. In light of the conservation of structure and mechanism among DNA polymerase families (11), the interaction is likely required for synthesis by mammalian polymerases as well, although direct demonstration is currently lacking. The lethality of 3-meA may thus reflect a crucial role of the N3 atom of adenine in stabilizing contact between DNA polymerases and the template strand required for insertion of the complementary deoxy nucleotide triphosphate.

Base excision repair is the primary defense against 3-meA in both prokaryotes and eukaryotes (12). In mammalian short patch base excision repair, removal of 3-meA is initiated by alkyladenine DNA glycosylase (AAG; also methylpurine-DNA glycosylase and 3-meA-DNA glycosylase), which cleaves the glycosylic linkage between the adducted base and deoxyribose, producing an abasic site (13). AAG remains bound to its abasic site product until displaced by Apel, an apurinic endonuclease (Ap endo) that incises the phosphodiester backbone 5’ to the baseless residue. The terminal 5’-deoxyribose phosphate is then excised by the 5’-deoxyribose phosphodiesterase activity of the 8-kDa NH2-terminal domain of DNA polymerase β. The resulting single nucleotide gap, with 3’-OH and 5’-phosphate at its margins, is filled and ligated by a complex of DNA polymerase β and DNA ligase III.

Bacterial and yeast mutants lacking DNA glycosylases homologous to AAG are hypersensitive to methylating agents (13), including Me-lex (14–16). In accord, Samson et al. have shown that embryonic stem cell lines derived from Aag null mice (17) are hypersensitive to killing by Me-lex (10). Deficiency of AAG resulted in elevated rates of sister chromatid exchange and chromosome aberration, induction of p53, and prolonged S-phase arrest with eventual exit into apoptosis. This constellation of findings, together with detection of 3-meA in Aag null but in not wild-type cells (18), represents the first documentation of the deleterious biological effects of unrepaired 3-meA in mammalian cells. Of significance is the observation that deletion of Ap endo in mice is filled and ligated by a complex of DNA polymerase β and DNA ligase III.

Me-lex survival. Me-lex (8) was dissolved and diluted in DMSO immediately before use so that a constant volume was added for all doses. No-drug controls received an equivalent volume of solvent. The final concentration of DMSO was 1%. Colony-forming ability

Materials and Methods

Cell culture. Cell lines were grown as adherent cultures at 37°C in 95%–5% humidified air/CO2 in DMEM/F12 supplemented with 2% iron-supplemented bovine calf serum (HyClone, Logan, UT), 100 units/ml penicillin, 100 mg/ml streptomycin, and 0.25 mg/ml amphotericin B (Invitrogen, Carlsbad, CA). Alkylating agent resistance and dependence of resistance on O6-meG-DNA methyltransferase (MGMT) of the glioblastoma-derived lines SNB19, SF763, SF767, T98G, UW18, and UW456 (21, 22) as well as of A1235 and its mismatch repair (MMR)-deficient derivative A1235-MR4 (hereafter MR4; ref. 23) have been described previously. Res256 was derived from a pediatric medulloblastoma (24). The MMR-deficient variant Res256MSh6− was isolated by serial selection for resistance to temozolomide; increased resistance was accompanied by loss of expression of MSH6 mRNA and MSH6 protein as revealed by reverse transcription-PCR and Western blotting, respectively.6 AAG and Ap endo activity. Subconfluent cultures were harvested by trypsinization, washed twice with PBS, flash frozen in liquid N2, and stored at −80°C. Extracts were prepared as described previously (25) and stored as multiple small aliquots. Aliquots could be flash frozen and thawed thrice without detectable loss of AAG or Ap endo activity. Activity was normalized to cell number by using DNA content of crude cell lysates estimated by the method of Burton (26), assuming a DNA content of 5 pg for a human cell.

AAG activity (pmol 3-meA released/106 cells/hour) was measured in an established assay (27, 28) that quantitates release of 3H-labeled N-methylpurines from a DNA substrate methylated with [3H]methyl-nitrosourea (13–20 Ci/mmol; Amershams, Indianapolis, IN). Each determination comprised measurement of radioactivity released as a function of time by three different amounts of extract. Rates for each amount were determined by regression analysis of points on the initial, linear portion of the curve. TLC of the released bases showed that ~90% of the counts comigrated with a 3-meA standard and 10% with a 7-methylguanine standard (data not shown), in accord with the observed substrate specificity of human AAG (27).

Ap endo activity (fmol abasic sites incised/cell/minute) was measured in a standard, highly sensitive assay that measures the conversion of plasmid DNA from supercoiled to relaxed form caused by incision at an abasic site (29). A detailed description of the procedure has been presented previously (30). Each determination comprised measurement of four different amounts of extract, each assayed in triplicate, and yielded activity calculated by regression analysis of points on the linear portion of the curve.

Antisense suppression of AAG and Apel. Two antisense oligonucleotides (ASOs; Operon, Huntsville, AL) directed against human AAG, 5’-GCTGCCGCCCACCCCTGTAGCT-3’ and 5’-ATTACCTGTCCCA-GAAATGCC-3’ were used. The first targets nucleotides 405 to 426 of the mRNA (accession NM_002434.2), and the second targets the second exon-intron junction (nucleotides 2,435-2,456 of the genomic sequence; accession AF499437) and includes the TCCC motif that occurs in half of ASOs that suppress expression of tumor necrosis factor-β (31). Both have low potential for self-association. As a control for specificity, cells were concurrently transfected with oligomers complementary to the ASOs [i.e., sense oligonucleotides (SO)]. The sequences of SOs targeting Apel have been described previously (32). ASOs were introduced into cells by cationic lipid-mediated transfection as described previously (32). After 72 h of incubation, cells were plated for enzymatic assay and cytotoxicity determination and for abasic site measurement.

Me-lex survival. Me-lex (8) was dissolved and diluted in DMSO immediately before use so that a constant volume was added for all doses. No-drug controls received an equivalent volume of solvent. The final concentration of DMSO was 1%. Colony-forming ability

6 M. Bobola and J. R. Silber, unpublished data.
following a 2-h exposure to Me-lex was determined by clonogenic assay. Drug sensitivity was quantitated by analysis of survival curves (log surviving fraction versus dose) using standard methods we have described previously in detail (e.g., ref. 21). The survival variables LD₁₀ (the dose required to reduce overall survival to 10%), D₁ (the threshold dose below which cells are insensitive to alkylator cytotoxicity), and D₃₇ (the rate of killing as indicated by the slope of the linear portion of the survival curve) were derived by regression analysis of the linear portion of a composite kill curve derived from the replicate assays. The three survival variables are presented as mean ± SD and provide a complete description of alkylator sensitivity. Unless otherwise stated, each survival curve represents three to five independent experiments as indicated, in which every dose was assayed in triplicate (i.e., 9 to 15 determinations per drug concentration).

Quantitation of abasic sites. Abasic sites were quantitated by using a slot-blot technique (33) as we have described previously in detail (32), except that chemiluminescent bands were measured using phosphorimage analysis rather than autoradiography. Abasic site levels in cellular DNA were determined by comparison with a standard curve constructed with reference DNA containing 1,080 abasic sites per 10⁶ nucleotides, prepared as described previously (32).

Statistical analysis. Data analysis and statistical procedures were done using Microsoft Excel. Comparison of means was by Student’s t test assuming unequal variances. Relationships between continuous variables were assessed by regression analysis. Statistically significant relationships were determined at the 95% confidence level.

Results

Me-lex sensitivity of human glioma cells. In this work, we assessed the effect of 3-mea in DNA on survival of human glioma cells. To do so, we measured the cytotoxicity of Me-lex, a sequence-specific alkylator that introduces 3-meA as the predominant adduct (>90% of base lesions; ref. 9). We quantitated Me-lex cytotoxicity by clonogenic assay and analysis of survival curves in 10 human glioma lines, including lines that are either proficient or deficient in MGMT, the repair enzyme of 3-meA (12, 13), and 6-meG adducts from DNA (6). The survival variables D₁, D₃₇, and LD₁₀ that characterize Me-lex sensitivity of the lines are summarized in Table 1; examples of survival curves from which these variables were derived are shown in Fig. 1. The lines differed widely in sensitivity as evidenced by a 43-fold range in LD₁₀, a 40-fold range in D₃₇, and a >31-fold difference in D₁. Incubating two of the lines (SF767 and SNB19) with hydroxylated Me-lex did not reduce survival (data not shown), confirming that an active alkylating moiety is required for cell killing (10). Simultaneous incubation with Me-lex and a 5-fold molar excess of netropsin, an innocuous natural product that competes with Me-lex for binding to the minor groove in A-T rich regions of DNA (14), decreased cell killing 4-fold (data not shown). These control experiments indicate that Me-lex cytotoxicity was associated with methylation at minor groove A-T rich sequences in glioma cells.

AAG, Ap endo, and MGMT activities. We measured the activity of AAG, the base excision repair enzyme that excises 3-meA from DNA, as well as Ap endo activity, which cleaves the DNA backbone adjacent to the resulting abasic site (Table 1). In contrast to Me-lex cytotoxicity, AAG and Ap endo activity varied little among the cell lines, suggesting that additional mechanisms may contribute to sensitivity. Regression analysis revealed no significant relationship between the two activities. In addition, univariate and multivariate regression analyses showed no association between AAG or Ap endo activity and either LD₁₀, D₁, or D₃₇ for Me-lex. Nevertheless, because correlative data cannot establish the presence or absence of biochemical function, we used antisense suppression to directly examine the contribution to Me-lex resistance of both AAG and Ape1, the predominant Ap endonuclease activity in human cells (34), as presented below.

We also measured the activity of MGMT. We observed that, on average, MGMT-proficient lines were more resistant to Me-lex than MGMT-deficient lines as judged by a 7-fold greater LD₁₀ (Table 1). We did not expect this result because the yield of O⁶-meG adducts introduced by Me-lex is very low (0.07%; ref. 9) and, we believe, insufficient to confer demonstrable cytotoxicity. We examine this result in relation to additional findings in a later section.

Suppression of AAG activity increases Me-lex cytotoxicity. AAG is the only known enzyme in mammalian cells that catalyzes excision of 3-meA (12, 13), and Aag null mouse embryonic stem cells and fibroblasts are hypersensitive to Me-lex (17, 27).

<table>
<thead>
<tr>
<th>Table 1. Me-lex sensitivity of human brain tumor lines</th>
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<tbody>
<tr>
<td>Line</td>
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<tr>
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</tr>
<tr>
<td>MGMT⁺</td>
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*fmol [³H]CH₃ groups removed/10⁶ cells: the lower limit of detection is 0.25.
⁻ pmol 3-meA released/10⁶ cells/hour.
⁻ fmol abasic sites incised/cell/minute.
To examine the contribution of AAG to Me-lex resistance in human glioma cells, we used ASOs to suppress activity in SF767 (MGMT+/MMR+), SNB19 (MGMT+/MMR+), and MR4 (MGMT−/MMR−). As illustrated in Fig. 2, ASO treatment reduced AAG activity and resistance to Me-lex in the three lines. Resistance was uniformly reduced within each of the ASO-treated lines as indicated by the constant slope of the final linear portion of the survival curves, suggesting that all cells were similarly affected. As summarized in Table 2, ASO treatment reduced AAG activity 1.6- to 1.8-fold, and LD10 1.4- to 1.5-fold, relative to SO-treated cells, differences that were statistically significant in every case. Greater sensitivity to Me-lex reflected statistically significant reduction in both D10 and D50. The enhancement in glioma cell killing accompanying suppression of AAG was comparable in magnitude with the difference in sensitivity between wild-type and Aag−/− mouse embryonic cells (17, 27).

Multiple control experiments provide evidence that increased Me-lex sensitivity reflected reduction of AAG activity rather than nonspecific effects of transfection or ASOs on cell physiology or DNA repair capacity. First, ASO- and SO-treated cells did not differ in plating efficiency (SF767, 28 ± 6% versus 30 ± 7%; SNB19, 27 ± 5% versus 29 ± 5%; MR4, 27 ± 10% versus 30 ± 10%). Second, treatment with cationic lipid alone had no effect on alkylator sensitivity in SNB19 (32) or on sensitivity to hydroxyurea and 4-NQO in SF767 (25). Third, the AAG activity and Me-lex sensitivity of SO-treated cells did not differ from that of untransfected cells (data not shown; Table 1). Fourth, ASOs and SOs had no effect on Ap endo activity in the three lines or on MGMT activity in SF767 (37). As illustrated in Fig. 4, ASO treatment reduced Ap endo activity and resistance to Me-lex in the three lines. In all cases, resistance was uniformly reduced in the ASO-treated lines as indicated by the constant slope of the final linear portion of the survival curves, suggesting that all cells in each line were similarly affected. As summarized in Table 2, ASO treatment reduced Ap endo activity 1.9-fold, and LD10 1.4- to 1.5-fold, relative to SO-treated cells, differences that were statistically significant in every case. Greater sensitivity to Me-lex reflected significant reduction in both D10 and D50.

**Suppression of Ape1 increases Me-lex cytotoxicity.** Concur- rent deletion of the genes for the two known Ap endo activities in the yeast *Saccharomyces cerevisiae* increases Me-lex killing (15, 16). To examine the contribution of Ap endo activity to Me-lex resistance in human glioma cells, we used ASO against Ape1 in SF767, SNB19, and MR4. Ape1 is the predominant Ap endo activity in human cells, catalyzing >95% of abasic site cleavage (34). As illustrated in Fig. 4, ASO treatment reduced Ap endo activity and resistance to Me-lex in the three lines. In all cases, resistance was uniformly reduced in the ASO-treated lines as indicated by the constant slope of the final linear portion of the survival curves, suggesting that all cells in each line were similarly affected. As summarized in Table 2, ASO treatment reduced Ap endo activity 1.9-fold, and LD10 1.4- to 1.5-fold, relative to SO-treated cells, differences that were statistically significant in every case. Greater sensitivity to Me-lex reflected significant reduction in both D10 and D50. We have previously shown that suppression of the Ap endo activity of Ape1 increases the level of temozolomide-induced and 1,3-bis(2-chloroethyl)-1-nitrosourea–induced abasic sites in SNB19 (32). In accord, abasic site content was 1.6-fold greater in ASO-treated compared with SO-treated SNB19 (68 ± 5.4% versus 41 ± 2.4% Ap sites/108 nucleotides) 2 h after exposure to 40 μmol/L Me-lex (Fig. 3B). The 1.5-fold reduction in LD10 that accompanied the increase in abasic sites suggests that unrepaird abasic sites

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**Fig. 1.** Me-lex cytotoxicity in human glioma-derived cell lines. Me-lex sensitivity was measured by clonogenic assay in the MGMT+/MMR− cell lines SF763 (¶), UW456 (▲), UW18 (●), T98G (○), A1235 (♦), and SF767 (□; B); the MGMT−/MMR− lines A1235 (♦) and SNB19 (○; B); and the MGMT−/MMR− line MR4 (○; B). Points, mean of 9 to 15 determinations (i.e., triplicate determinations at each drug dose in three to five independent experiments); bars, SD. The final linear portion of the curves was constructed by linear regression analysis. Where error bars are not shown, the SD is too small to be indicated. The survival variables LD10, D, and D50 derived from the curves are compiled in Table 1.
derived from AAG-catalyzed excision of 3-meA mediate Me-lex cytotoxicity.

A factor(s) associated with expression of the MGMT gene promotes Me-lex resistance. Five of the 10 lines we examined are MGMT+ and another 3 are MGMT-; as indicated in Table 1, these 8 lines are proficient in MMR. We also measured Me-lex cytotoxicity in MMR-deficient derivatives of two of the MGMT- lines. Loss of MMR in MGMT- cells permits tolerance of O6-meG lesions without lethality (5).

We have previously shown that, among the eight MMR+ lines, MGMT proficiency is accompanied by greater resistance to clinical and laboratory methylators, such as temozolomide and N-methyl-N’-nitro-N-nitrosoguanidine (data not shown; refs. 21, 22). We did not anticipate a comparable result for Me-lex because the yield of O6-meG adducts is ~100-fold lower (approximately 0.07% versus 6-12%; ref. 9). Yet, we observed a 7-fold higher mean LD10 for the five MGMT+ versus five MGMT- lines (200 ± 152 versus 27 ± 6 μmol/L; P ≤ 0.035), as well as a significant correlation between MGMT activity and LD10 (P ≤ 0.006); for the latter calculation, we assumed a value of 0.25 fmol/106 cells, our lower limit of detection (35). Four observations indicate that a factor(s) associated with expression of the MGMT gene, rather than the repair activity of the protein, is the major factor responsible for increased Me-lex resistance in the MGMT+ lines. First, O6-meG accounts for <0.1% of Me-lex-induced base adducts, whereas 3-meA accounts for >90% (9); this 1,000-fold difference would likely obscure any contribution of O6-meG to cell killing in a clonogenic assay. Second, one of the MGMT+ lines, SF767, was more sensitive than all other lines (Table 1), and a second, SF763, displayed no detectable resistance to low Me-lex doses (D1 = 0; Table 1), suggesting that factors associated with MGMT expression, rather than or in addition to MGMT activity, may contribute to Me-lex resistance. Third, loss of MMR had no effect on Me-lex sensitivity as evidenced by unchanged LD10 for the MMR- derivatives MR4 and Res256MSH6- relative to the parental MMR+ lines A1235 and Res256, respectively (Table 1). In contrast, loss of MMR had profound effects on sensitivity to other methylators: the LD10 for N-methyl-N’-nitro-N-nitrosoguanidine is 40-fold greater in MR4 than in A1235 (5.9 versus 0.15 μmol/L) and the LD10 for temozolomide is 10-fold greater in Res256MSH6- than in Res256 (200 versus 20 μmol/L). Fourth, ablation of MGMT with the substrate analogue inhibitor O6-benzylguanine had no effect on Me-lex...
cytotoxicity in SF767 (10.7 versus 10.4 μmol/L), SF763 (409 versus 398 μmol/L), and T98G (145 versus 144 μmol/L) cells. In contrast, the LD_{10} for temozolomide was reduced 2- to 20-fold in the three lines (22). We conclude that expression of the MGMT gene in our glioma lines is associated with additional phenomena that confer resistance to 3-meA.

**Discussion**

Knowledge of the mechanisms responsible for resistance of human primary brain tumors to therapeutic alkylating agents is limited by our inability to assess the cytotoxicity of individual alkyl base adducts in DNA. The multiplicity of adducts induced by most alkylators (1, 2) and the numerous, often redundant repair and tolerance pathways that process DNA lesions (e.g., refs. 12, 36) pose major impediments to understanding. The adducts O^6^-meG and 3-meA are exceptions to this dilemma. Demonstration of the lethality of O^6^-meG was made possible by creation of knockout mice lacking AAG (17, 27), the enzyme that catalyzes initial removal of 3-meA in base excision repair of this lesion (13), and synthesis of the sequence-specific methylator Me-lex that induces 3-meA as the predominant adduct (8, 9). Although cell killing mediated by 3-meA has been documented in mouse embryonic stem cells, fibroblasts, and neurons (7, 37), the role of 3-meA in alkylator cytotoxicity in human brain tumor cells has yet to be established.

3-meA is cytotoxic in human glioma-derived cell lines. Here, we show that Me-lex is cytotoxic in 10 human glioma lines and that antisense suppression of AAG activity potentiates Me-lex killing. These results are in accord with the Me-lex hypersensitivity of embryonic stem cells derived from Aag knockout mice (17, 27). Suppression of AAG in glioma cells was

### Table 2. Antisense suppression of AAG and Ape1 increases Me-lex cytotoxicity in human glioma cells

<table>
<thead>
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<th>Line</th>
<th>AAG Activity</th>
<th>Ape1 Activity</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Sense</td>
<td>ASO</td>
</tr>
<tr>
<td>SF767</td>
<td></td>
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</tr>
<tr>
<td>Activity*</td>
<td>3.3 ± 0.9</td>
<td>1.8 ± 0.6</td>
</tr>
<tr>
<td>LD_{10} (μmol/L)</td>
<td>10 ± 1.3</td>
<td>6.6 ± 0.9</td>
</tr>
<tr>
<td>D_T (μmol/L)</td>
<td>1.4 ± 0.3</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>D_{37} (μmol/L)</td>
<td>4.5 ± 0.5</td>
<td>2.8 ± 0.7</td>
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<table>
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<tr>
<th>Line</th>
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<td>SNB19</td>
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<tr>
<td>Activity</td>
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<td>LD_{10} (μmol/L)</td>
<td>22 ± 2.0</td>
</tr>
<tr>
<td>D_T (μmol/L)</td>
<td>3.6 ± 1.1</td>
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<tr>
<td>D_{37} (μmol/L)</td>
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<tr>
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<th>Ape1 Activity</th>
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<tbody>
<tr>
<td>MR4</td>
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<tr>
<td>Activity</td>
<td>4.2 ± 0.8</td>
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<tr>
<td>LD_{10} (μmol/L)</td>
<td>31 ± 0.9</td>
</tr>
<tr>
<td>D_T (μmol/L)</td>
<td>3.7 ± 0.5</td>
</tr>
<tr>
<td>D_{37} (μmol/L)</td>
<td>14 ± 2.8</td>
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*AAG activity is expressed as pmol 3-meA released/10^6 cells/hour and Ape1 as fmol abasic sites incised/cell/minute.

Fig. 3. Abasic site content of Me-lex-treated glioma cells following antisense-mediated suppression of AAG (A) and Ape1 (B) activity. SF767 cells were treated with 8 μmol/L Me-lex (A) and SNB19 cells with 40 μmol/L (B). Abasic sites in ASO-treated (x-x) and SO-treated (o-o) cells were measured in a slot-blot assay using an aldehyde-reactive probe (33). & inset, example of a standard curve constructed with calf thymus DNA containing increasing amounts of abasic sites prepared as described in Silber et al. (32).
accompanied by a decrease in the level of abasic sites; abasic sites are the product of AAG-mediated excision of 3-meA and are themselves cytotoxic. These results provide the first direct evidence that unrepaired 3-meA in DNA is a lethal lesion in human brain tumor cells. We also showed that antisense suppression of Ape1 increases the sensitivity of glioma cells to Me-lex; Ape1 is the major mammalian activity that incises abasic sites and is the second enzyme in base excision repair of 3-meA. Suppression of Ape1 was accompanied by an increase in abasic site abundance. Taken together, the foregoing experiments provide strong biochemical evidence that (a) 3-meA is cytotoxic in human glioma cells and that AAG promotes resistance to this adduct and (b) abasic sites resulting from 3-meA repair are cytotoxic and that Ape1 promotes resistance to these derivative lesions.

Resistance to Me-lex is multifactorial. Our data also indicate that as yet uncharacterized determinants in addition to AAG and Ape1 promote resistance to 3-meA in human glioma cells. This conclusion is consistent with the lack of correlation of Me-lex sensitivity with either AAG or Ap endo activity. The lack of association is exemplified by SF767, which has AAG and Ap endo activity comparable with that of nine other lines but is more sensitive to Me-lex as evidenced by 2- to 40-fold lower LD10 (Table 1). Our data show that one or more additional determinants of Me-lex resistance are associated with expression of the MGMT gene. We observed that, with the exception of SF767, MGMT-proficient lines are more resistant to Me-lex than MGMT-deficient lines (Table 1) and also that MGMT activity is correlated with survival. Yet, MGMT-mediated repair of O6-meG is not the mechanism underlying these observations or at least is not the primary mechanism. Two types of direct evidence support this conclusion. First, loss of MMR in two MGMT−/− lines (A1235 and Res256) had no effect on Me-lex sensitivity (Table 1), whereas N-methyl-N′-nitro-N-nitrosoguanidine cytotoxicity was potentiated 40-fold for MR4 relative to the parental A1235 and temozolomide cytotoxicity was potentiated 10-fold greater for Res256MSH6−/− relative to the parental Res256.6 Because loss of MMR negates the lethality of O6-meG (5, 36), these findings indicate that the small proportion of O6-meG adducts (0.07%; ref. 9) caused by Me-lex was not responsible for detectable levels of killing. Hence, lack of repair by MGMT could not be responsible for the diminished survival of MGMT-deficient lines. Moreover, ablating MGMT with O6-benzylguanine did not change the Me-lex sensitivity of MGMT-proficient SF767, SF763, or T98G cells. We think it is probable that loss of MGMT expression, believed to be due to transcriptional silencing (6), is but one manifestation of a wider sensitivity to DNA alkylation damage.

![Fig. 4. ASOs against Ape1 reduce Ap endo activity (A-C) and increase Me-lex cytotoxicity (D-F) in MGMT+/− MMR+ (SF767), MGMT−/− MMR+ (SNB19), and MGMT−/− MMR− (MR4) human glioma cell lines. Ap endo activity and Me-lex sensitivity in ASO-treated (+) and SO-treated (○) cells were measured by biochemical and clonogenic assay, respectively. Points, mean of 9 to 15 determinations (i.e., triplicate determinations at each drug dose in three to five independent experiments); bars, SD. The final linear portion was constructed by linear regression analysis. Where error bars are not shown, the SD is too small to be indicated. Mean Ap endo activities, and survival variables derived from the curves, are compiled in Table 2. We have previously shown that ASO-mediated suppression of Ap endo activity is accompanied by a comparable reduction of Ape1 protein content (e.g., ref. 32).](www.aacrjournals.org)
mediated by epigenetic changes in gene expression. In accord with this hypothesis are reports that loss of MGMT expression in human primary brain tumors is accompanied by epigenetic silencing of other genes, including some that promote DNA repair (e.g., MLH1), damage response (e.g., p16INK4a and p14ARF), and cell survival (e.g., p53 and p73; refs. 38–40). Epigenetic changes include not only decreases but also increases in levels of gene expression (41), some of which may affect Me-lex sensitivity as well.

Yet other likely determinants of Me-lex sensitivity have been identified. We have shown that suppression of the Werner syndrome protein WRN, a DNA helicase/exonuclease, increases Me-lex killing in SF767 and SNB19 (25). WRN promotes genomic stability, in part by mediating resumption of DNA replication at replication forks stalled by DNA damage (42), probably including 3-meA. In addition, the slow but measurable removal of 3-meA from Me-lex-treated Aug knockout mouse embryonic fibroblasts is completely ablated when the cells are also defective in nucleotide excision repair (43), implicating nucleotide excision repair in Me-lex resistance.

A hypersensitive cell line yields possible clues to mechanisms of Me-lex cytotoxicity. The extreme sensitivity of SF767 to Me-lex may afford clues to the mechanisms of Me-lex lethality. SF767 becomes more sensitive than other glioma lines to clinical and may afford clues to the mechanisms of Me-lex lethality. SF767 Me-lex cytotoxicity.

Repair (43), implicating nucleotide excision repair in Me-lex ablated when the cells are also defective in nucleotide excision knockout mouse embryonic fibroblasts is completely ablated when the cells are also defective in nucleotide excision repair (43), implicating nucleotide excision repair in Me-lex resistance.

A hypersensitive cell line yields possible clues to mechanisms of Me-lex cytotoxicity. The extreme sensitivity of SF767 to Me-lex may afford clues to the mechanisms of Me-lex lethality. SF767 becomes more sensitive than other glioma lines to clinical and laboratory methylators when MGMT is ablated with O6-benzylguanine (21, 22), consistent with deficiency in resistance to 3-meA. As ~10% of alkyl base adducts induced by temozolomide and procarbazine are 3-meA, elucidation of the sources of SF767 hypersensitivity could provide new insights into the mechanisms of glioma resistance to clinically used methylating agents. The source(s) of SF767 hypersensitivity to Me-lex does not include a defect in the initial steps of base excision repair because AAG and Ap endo levels are representative (Table 1), and suppression of either activity significantly increases sensitivity (Table 2). SF767 is not hypersensitive to UV light or 137Cs γ-rays, suggesting that it is not defective in nucleotide excision repair or in the major pathways of single-strand and double-strand break repair. In contrast, the LD10 for bleomycin is 3- to 12-fold lower in SF767 than in seven of eight glioma lines we have examined, including six used in this study. Approximately 40% of bleomycin-induced lesions are C4'-oxidized abasic sites, which, when opposite or closely opposed to a single-strand break, produce a double-strand lesion that cannot be resolved by base excision and strand break repair pathways (44). Such clustered lesions are strong blocks to DNA replication and promote double-strand break formation (45), raising the possibility that SF767 is deficient in a mechanism(s) that promotes recovery from stalled replication forks. The binding of Me-lex at A-T rich regions (4) may promote formation of clustered 3-meA adducts akin to the case of clustered bleomycin damage. These clustered lesions may tax mechanisms for recovery from stalled or collapsed forks, one or more of which mechanisms could be deficient in SF767 cells. As noted above, our evidence suggests that WRN is not a candidate (25).

Clinical implications. Our data suggest that Me-lex, either as a single agent or in combination with temozolomide, may have utility in the adjuvant therapy of malignant brain tumors. We have previously reported that 25% of adult malignant gliomas lack detectable MGMT activity (35, 46), suggesting that at least some of these tumors may be intrinsically sensitive to Me-lex due to factors associated with MGMT deficiency. Regression analysis of survival data for the lines used in this study showed no association between the LD10 for Me-lex and temozolomide (r = 0.26; P = 0.52; Table 1; ref. 22), a drug that is increasingly used in the chemotherapy of malignant brain tumors (20). Me-lex might serve as an alternative chemotherapeutic agent for tumors that are intrinsically resistant to temozolomide or that develop resistance during treatment. The lack of correlation between the cytotoxicity of the two methylators reflects in part the insensitivity of Me-lex cell killing to loss of MMR, a loss that greatly increases the temozolomide resistance of glioma cells by rendering them insensitive to O6-meG (36). A small study of adult glioblastomas has suggested that less favorable response to temozolomide is associated with an decreasing fraction of MMR protein-immunopositive cells (47). The clinical applicability of Me-lex awaits evaluation of toxicity and permeability of the blood brain barrier in animal models.

Our data also suggest suppression of either AAG or Ap endo as a complement to ablation of MGMT in overcoming temozolomide resistance in primary human brain tumors. We have previously shown that antisense suppression of the Ap endo activity of Ap endo activity of Ap endo increases temozolomide cytotoxicity in an MGMT adult glioma line (32) and in an MGMT pediatric medulloblastoma line (24). Our demonstration of the cytotoxicity of 3-meA and derivative abasic sites indicates that AAG and Ap endo are promising targets for antiresistance therapies and strengthens the rationale for developing pharmacologic inhibitors. The development of inhibitors for both AAG and Ap endo is ongoing in several laboratories (48, 49). Importantly, incomplete ablation of either AAG or Ap endo yields statistically significant reduction in 3-meA resistance in all the glioma lines we examined, including those insensitive to unrepaired O6-meG due to loss of MMR. The efficacy of partial suppression contrasts with the need to completely ablate MGMT activity for prolonged periods to increase methylator cytotoxicity in human primary brain tumor cell lines (e.g., ref. 24). These observations suggest that inhibiting repair of 3-meA or abasic sites may be a more readily achievable approach to suppression of glioma methylator resistance.

References


Human Glioma Cell Sensitivity to the Sequence-Specific Alkylating Agent Methyl-Lexitropsin
