Cytotoxicity, DNA Damage, and Apoptosis Induced by New Fotemustine Analogs on Human Melanoma Cells in Relation to O\(^6\)-Methylguanine DNA-Methyltransferase Expression

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ABSTRACT

Fotemustine is a third generation chloroethylnitrosourea that has demonstrated significant antitumoral effects in malignant melanoma. However, its use is somewhat limited by its toxic side effects and chemoresistance caused by direct repair of O\(^6\)-alkyl groups by the enzyme O\(^6\)-methylguanine DNA-methyltransferase (MGMT). The aim of this work was to determine to what extent the expression of MGMT influences cytotoxicity, DNA damage, and apoptosis induced by new nitrososulfamide analogs of fotemustine (compounds 4 and 8), which have previously demonstrated interesting antiproliferative properties. We carried out complementary strategies that consisted of MGMT cDNA transfection in CAL77 Mer- melanoma cells and of MGMT inhibition with O\(^6\)-benzylguanine (BG) in A375 Mer+ melanoma cells. MGMT-transfected cells were 7 to 9 times less sensitive to fotemustine than parent cells, whereas no difference between the transfected and parent cells was observed for nitrososulfamide analogs. The cytotoxicity of these analogs vis-à-vis a MGMT-proficient A375 melanoma cell line was approximately 3 times greater than that of fotemustine. Coincubation of these cells with O\(^6\)-benzylguanine significantly increased the cytotoxicity of fotemustine and compound 8, whereas BG had little effect on the cytotoxicity of compound 4. Furthermore, DNA fragmentation determined by a comet assay was greater with nitrososulfamide analogs than with fotemustine. O\(^6\)-benzylguanine increased DNA fragmentation for fotemustine and compound 8, but not for compound 4, which induced comets with a typical apoptotic appearance. The ability of this compound to induce apoptosis in the absence of BG was confirmed by a specific enzyme-linked immunosorbent assay apoptotic assay using a single-stranded DNA monoclonal antibody.

Malignant melanoma (MM), which is frequently due to overexposure to the sun, is an incurable cancer that is becoming increasingly prevalent in industrialized countries. Despite recent advances in immunotherapy and vaccinotherapy, chemotherapy remains the standard therapeutic option for disseminated melanoma (Mackie, 2000). However, MM frequently displays primary chemoresistance, and only a few cytotoxic drugs have shown activity against this tumor. Higher remission rates are obtained with DNA-alkylating agents, including cisplatin, methylating agents such dacarbazine and temozolomide, or chloroethylating agents such 2-chloroethylnitrosoureas (CENU) (Daponte et al., 2000; Middleton et al., 2000a). Fotemustine is a third generation nitrosourea which is used both in melanoma and brain tumors (Merimsky et al., 1992). However, its clinical application is somewhat limited both by its toxicity and by the resistance of melanoma cells (Christmann et al., 2001).

It is known that nitrosoureas are subject to spontaneous decomposition to generate both electrophilic species, responsible for DNA alkylation and therapeutic efficacy, and isocyanates responsible for the carbamylation of proteins contributing substantially to toxic side effects of these compounds (Kroes et al., 1991; Brakenhoff et al., 1996; Vermeulen et al., 1998; Yin et al., 2001). In this context, a new family of alkylating agents named 2-chloroethylnitrososulfamides, structurally related to 2-chloroethylnitrosoureas but devoid of any carbamoylating activity were designed (Abd-aoui et al., 1996). We recently reported the synthesis of
nitrososulfamide analogs of fotemustine (Fig. 1) and their preliminary in vitro evaluation on two human melanoma cell lines (Winum et al., 2003). DNA damage and the resulting cytotoxicity of CENU's is based on their ability to form chloroethyl adducts on nucleic acids. Despite the fact that more than 14 DNA alkylation sites have been described, O⁶-chloroethylation of guanine is considered to be the most cytotoxic lesion through the secondary formation of a DNA-interstrand cross-link (Gnewuch and Sosnovsky, 1997).

The dominating mechanism of chemoresistance to alkylating agents is the repair of DNA adducts by the enzyme O⁶-methylguanine DNA-methyltransferase (MGMT) (Pegg, 1990; Mineura et al., 1996; Margison and Santibanez-Koref, 2002). MGMT produces the transfer of O⁶-alkyl from DNA to the cytochrome 145 moiety of the enzyme in a stoichiometric manner, leading to protein degradation (Daniels and Tainer, 2000; Pegg, 2000). Expression levels of MGMT vary considerably from one cancer to another and tumors are usually classified as Mer+ or Mer− phenotypes in relation to their MGMT status. Several studies have demonstrated high expression levels in colon cancer, gliomas, lung cancer, and pancreatic carcinoma (for review, see Gerson, 2002). We recently demonstrated that in melanoma there is considerable variation in MGMT mRNA levels (up to 38-fold) using quantitative real-time PCR (Evvard et al., 2003). These findings are in agreement with previous immunohistochemistry studies (Ma et al., 2002). Clinical studies demonstrated that the Mer− phenotype is often associated to a more positive outcome in chemotherapy based on alkylating agents, and several attempts are currently being made to inhibit chemoresistance using MGMT suicide substrates such as O⁶-benzylguanine or O⁶-(4-bromoethyl) guanine (Dolan et al., 1991; Mineura et al., 1994; Middleton et al., 2000b).

The aim of this work was to determine to what extent the DNA repair enzyme MGMT could influence DNA damage, apoptosis, and cytotoxicity induced by two new nitrososulfamide analogs of fotemustine in melanoma cells. We therefore carried out complementary strategies that consisted of MGMT overexpression by cDNA transfection in Mer− melanoma cells, and of MGMT inhibition with O⁶-benzylguanine in Mer+ melanoma cells.

### Materials and Methods

#### Chemicals

Fotemustine was purchased from Servier (Neuilly-Sur-Seine, France), and nitrososulfamide analogs of fotemustine (compounds 4 and 8) were synthesized as described previously (Winum et al., 2003). DNA calf thymus, O⁶-benzylguanine, anti-actin antibody, and peroxidase substrate 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) were purchased from Sigma-Aldrich (St. Louis, MO). Anti-MGMT monoclonal MT3.1 and anti-seaDNA mAb F7-26 antibodies were from Chemicon International (Temecula, CA), whereas genetin and LipofectAMINE were obtained from Invitrogen (Carlsbad, CA). The chemiluminescence detection kit (ECL) and N-[H³]methyl-N-nitrosourea were purchased from Amersham Biosciences Inc. (Piscataway, NJ). Others standards reagents were from Sigma-Aldrich.

#### Cell Lines

The A375 human melanoma cell line was obtained from American Type Culture Collection (ATCC CRL-1619). CAL77, a Mer− (i.e., MGMT-deficient) melanoma cell line, was kindly provided by Prof. G. Milano (Centre Antoine Lacassagne, Nice, France). Both cell lines were checked for expression of standard melanoma antigens tyrosinase, melan-A, and NA-17 by RT-PCR analysis (data not shown). Cells were routinely maintained at 37°C in 5% CO₂ humidified atmosphere in Dulbecco’s modified essential medium supplemented with 10% fetal calf serum, 1% l-glutamine (2 mM), and 1% penicillin-streptomycin (50 U/ml).

#### Transfection of Human MGMT cDNA in CAL77 Cells

The full-length human MGMT cDNA (kindly provided by Dr. S. Mitra; University of Medical Branch, Galveston) was subcloned into the BamHI/NotI restriction sites of the mammalian expression vector pcDNA3 (Invitrogen) under the control of a cytomegalovirus promoter to yield pcMGMT. CAL77 cells were subjected to a stable transfection with pcMGMT using LipofectAMINE in accordance with the manufacturer’s instructions. Transfected cells were kept in a selective medium containing 1 mg/ml geneticin. Clones were randomly selected and controlled for MGMT expression by Western blot, RT-PCR, and MGMT activity analysis. Recombinant clones were then routinely cultured in a medium containing 500 µg/ml geneticin.

#### MGMT Activity Assay

MGMT activity in melanoma cells lysates was verified by measuring the transfer of H⁵-methyl groups from the DNA substrate (obtained by alkylation of calf thymus DNA with N-[H³]methyl-N-nitrosourea) to the MGMT protein as described previously (Baer et al., 1993). Cells were homogenized on ice in 100 µl of a lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 100 µg/ml phenylmethylsulfonfyl fluoride, 1 µg/ml aprotinin, and 1% Triton X-100). The mixture was centrifuged at 20,000g for 30 min at 4°C, and the protein content of the supernatants was determined by the Bradford assay (Bradford, 1976). One hundred micrograms of protein extracts in a final volume of 500 µl of Tris-EDTA (pH 8) were incubated at 37°C for 1 h with H⁵-methylated DNA substrate in a final volume of 500 µl of Tris-EDTA (pH 8). The proteins were then precipitated by adding 30 µl of trichloroacetic acid and incubated for 30 min at 80°C. After centrifugation at 14,000 rpm/15 min/4°C, the protein pellets were washed with 30% trichloroacetic acid and redissolved in 200 µl of 10 mM NaOH. The [H³]MGMT content was then quantified by liquid scintillation counting.

#### Western Blot Analysis

Twenty micrograms of cell lysates, obtained as described above, were subjected to electrophoresis on a 12% SDS-polyacrylamide gel.
Proteins were transferred onto a nitrocellulose membrane at room temperature using a semidry electrophoretic transfer device. The membrane was allowed to soak overnight at 4°C in PBS-Tween containing 1% full-fat dried milk. The next day, the membrane was incubated for 1 h with an anti-MGMT monoclonal antibody MT3:1 (1/500) and an anti-actin monoclonal antibody as a loading control. It was then incubated with a peroxidase-conjugated anti-mouse IgG (1/2000). Antibody binding was detected using a chemiluminescence detection kit (ECL).

**Cell Sensitivity Assay**

**Neutral Red Assay.** This assay was carried out as described previously (Evrard et al., 1999). Briefly, cells were seeded in 96-well microtiter plates at 5 x 10^3 cells/well for A375 and 10 x 10^3 cells/well for CAL77. After 24-h incubation, cells were treated for 1 h with increasing concentrations (0–1000 μM) of fotemustine or nitrosourea analogs (150 μl in fresh medium per well; six wells per concentration level). The drug-containing medium was then replaced by fresh medium, and the cells were allowed to grow for 72 h. Thereafter, the cells were washed with PBS, and 150 μl of neutral red solution (40 μg/ml) was added. After 3 h at 37°C, the cells were washed with PBS and destained with 150 μl of 1% acetic acid/50% ethanol (v/v). The absorbance was read at 540 nm (A540) was measured using a microplate reader (Multiscan MS; Labsystem, Helsinki, Finland). The effect of the drugs on cell survival was expressed as the percentage of cell viability in relation to that of untreated cells, and IC50 values (concentrations inhibiting growth by 50%) were obtained from cytotoxicity curves.

**Clonogenic Assay.** This assay was carried out as described previously (Domoradzki et al., 1984). A375 and CAL77 cells were plated at a density of 1.4 x 10^6 cells in 75-cm² flasks. After 24-h incubation, the cells were treated with various concentrations (0, 50, 100, and 200 μM) of fotemustine or nitrosourea analogs for 1 h. The drug-containing medium was then replaced by fresh medium, and cells were allowed to grow for 16 h. Then cells were harvested by trypsinization, counted, and resuspended at 10 x 10^5 cells/60 cm² in a Petri dish. After 10 to 12 days’ incubation, cell colonies were stained with Giemsa and counted automatically using BioID image analysis software (Vilber Lourmat, Marne-La-Vallée, France). The loss of colony-forming ability was determined for each drug concentration and was expressed as a percentage of the concentration required to inhibit the growth of untreated cells.

The effect of O6-benzylationoune on the cytotoxicity of alkylating agents was measured both by neutral red and clonogenic assays. A375 cells were incubated in a culture medium containing 100 μM BG 1 h before treatment with different concentrations of fotemustine analogs as described above; also for 1 h during the treatment, the cells were co-incubated with the drugs and 100 μM BG.

**Single Cell Gel Electrophoresis (Comet) Assay.** The Comet assay was performed as described previously (Yusuf et al., 2000). Conventional microscope slides were coated with 1% normal melting point agarose dissolved in PBS and allowed to solidify overnight at room temperature. The next day, A375 cells were treated as described above; thereafter, they were harvested by trypsinization, washed in PBS, and resuspended in a standard medium at 10^6 cells/ml. Ten microliters of cellular suspension was mixed with 75 μl of low melting point agarose and spread onto a microscope slide and then cooled to 4°C for 5 min with a coverslip to allow the slide to solidify. After removing the coverslip, a second aliquot of low melting point agarose was dropped onto the slides, and they were again cooled to 4°C for 5 min. Cells were then placed for 1 h in a lysis buffer containing 10 mM Tris base, 2.5 M NaCl, 100 mM EDTA, and 1% Triton X-100 (pH 10). The slides were subsequently incubated in an electrophoretic solution (300 mM NaOH and 1 mM EDTA, pH 13) for 40 min at 4°C. Electrophoresis was carried out at 25 V for 20 min. Slides were then washed three times with 0.05 M Tris-HCl neutralization buffer (pH 7.5) for 5 min, and fixed in methanol and allowed to dry at room temperature. After staining with 10^-2 SYBR gold fluorescent dilution dye, DNA damage was determined on 50 cells/slide using Komet 4.0 analysis system (Kinetic Imaging Ltd., Liverpool, UK). Results were expressed as the percentage of DNA in the comet tail, and as the tail extent moment, i.e., (tail%DNA × tail length)/100.

**Enzyme-Linked Immunosorbent Assay (ELISA).** To quantify the extent of apoptosis after drug treatment, we used an anti-ssDNA monoclonal antibody that allows specific detection of apoptotic cells as described previously (Frankfurt and Krishan, 2001). Cells were plated in 96-well microtiter plates at a density of 10 x 10^3 cells/well for A375, and at 20 x 10^3 cells/well for CAL77. After 24-h incubation, the cells were treated with fotemustine and analogs as described above. Plates were centrifuged at 200g for 5 min, and the culture medium was replaced by 200 μl of 80% methanol in PBS. After 30-min incubation at room temperature, the plates were centrifuged again and the methanol was removed. The plates were incubated for 20 min at 37°C. DNA was denatured with 50 μl of 100% formamide in a water bath at 75°C for 10 min, and plates were then cooled at 4°C for 5 min. After removing the formamide, the plates were incubated for 1 h with 100 μl of 3% nonfat dried milk to block nonspecific binding sites. The plates were incubated at room temperature for 30 min with 100 μl of a mixture antibodies containing equal volumes of primary anti-ssDNA mAb F7-26 (5 μg/ml PBS supplemented with 5% fetal bovine serum) and secondary peroxidase-conjugated anti-mouse IgM antibodies (1/100). After washing three times with PBS, the plates were incubated for 1 h with the peroxidase substrate 2,2’-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), and the absorbance was read at 405 nm using a microplate reader (Multiscan MS; Labsystem). Results were expressed as a percentage of the control absorbance [A_control - A_reagent/A_control] x 100.

**Results**

**Expression of MGMT in Melanoma Cells.** Human melanoma CAL77 cells were transfected with the pcMGMT expression vector and stable transfectants were selected for genetin resistance. The growth rate of transfected CAL77 cells did not significantly vary from that of wild-type or pcDNA3-transfected cells (data not shown). Clones of pc-MGMT-transfected cells (c1 to c13) were randomly selected and analyzed for MGMT expression and enzyme activity. Levels of enzyme activity were closely related to protein expression assessed by Western blot experiments and measurements of enzyme activity (1298 ± 13 and 1508 ± 65 dpm, respectively). These clones were subsequently used to evaluate the effect of MGMT on the cytotoxicity of fotemustine analogs. Western blot experiments and measurements of enzyme activity showed that A375 cells displayed constitutive MGMT expression at a lower rate (626 ± 42 dpm) than CAL77 MGMT-transfected cells. Treatment with 100 μM BG during 2 h led to a nearly complete inhibition of MGMT in A375 cells.

**Cytotoxicity of Fotemustine Analogs on MGMT-Transfected Cells.** The effect of MGMT overexpression on the sensitivity of melanoma cells to fotemustine analogs was evaluated by neutral red and clonogenic assays. In the neutral red assay, cells were treated with increasing concentrations of drug being tested to determine the IC_{50} values, which represent the dose required to inhibit 50% of cell growth. The clonogenic assay was used to determine the loss of colony-
forming ability, which is expressed in percentage terms relative to untreated cells. A combination of both assays permits a distinction to be made between cytotoxic (cell kill) and cytostatic (decreased growth rate) effects.

As shown in Fig. 3A, the levels of response determined by neutral red assay were dose-dependent in parent and transfected cells for all drugs tested. After treatment with fotemustine analogs, cell viability of parent cells was compared with that of transfected cells. For fotemustine, the IC$_{50}$ values calculated from survival curves of wild-type cells was 90$\mu$M, whereas the corresponding values for transfected cells were 82$\mu$M for clone 2 (9.1 times that of wild-type cells) and 70$\mu$M for clone 7 (7.8 times). This difference in cell viability between wild-type and transfected cells was highly significant (Student's $t$ test; $p < 0.01$). On the other hand, the cytotoxicity curves for nitrososulfamide analogs do not show a significant difference between transfected (clones 2 and 7) and parent cells; IC$_{50}$ values were 200 and 90$\mu$M for compounds 4 and 8, respectively.

Results were similar for both clone 2 and clone 7, which displayed comparable MGMT activity. Thus, in the CAL77 human melanoma model, the cytotoxic effects of nitrososulfamide analogs, evaluated by both neutral red and clonogenic assays, seemed to be independent of MGMT expression.

**Effect of BG on Cell Sensitivity to Fotemustine Analogs.** The ATCC-referenced A375 human melanoma cell line, which constitutively expresses MGMT protein, was chosen to evaluate the effect of BG on the cytotoxicity of fotemustine analogs (Fig. 4). The fotemustine cytotoxicity curves showed an IC$_{50}$ shift from 250 to 30$\mu$M (8.3-fold) when cells were pretreated and coincubated with 100$\mu$M BG. Compounds 4 and 8 displayed higher cytotoxic effects than fotemustine with IC$_{50}$ values of about 75$\mu$M for both analogs in absence of BG. As shown in Fig. 4A, a difference was observed between the effects of compounds 4 and 8 when BG was added. Indeed, whereas BG had little effect on the cytotoxicity of compound 4 (IC$_{50}$ value reduced from 75 to 35$\mu$M), compound 8, like fotemustine, was more cytotoxic in the presence of BG (IC$_{50}$ value reduced from 75 to 2$\mu$M).

The clonogenic assays (Fig. 4B) demonstrated that treatment of cells with 50$\mu$M fotemustine, or fotemustine analogs without BG, lead to a comparable decrease in colony-forming ability (about 30%). When the cells were pretreated and coincubated with 100$\mu$M BG, a total loss in colony-forming ability was observed with 50$\mu$M fotemustine, whereas a 53% survival rate was observed with 50$\mu$M compound 4. Concerning compound 8, BG induced an enhanced decrease of colonies with only 8% survival at 50$\mu$M. Thus, results of clonogenic and neutral red cytotoxicity assays concur and demonstrate that MGMT inhibition by BG significantly increases the cytotoxicity of compound 8 and fotemustine but sensitizes A375 cells to compound 4 to a lesser extent.
DNA Damage Induced by Fotemustine Analogs. We assessed DNA fragmentation induced by fotemustine and fotemustine analogs both with and without BG using the alkaline comet assay on A375 cells. The results are summarized in Table 1, and representative examples of comets are shown in Fig. 5. We first observed that in the absence of BG, both fotemustine analogs produced more damage, in terms of the percentage of DNA in the comet tail, compared with fotemustine. However, when results are expressed as tail extent moment, the values were greater for compound 4, and compound 8 displayed tail extent moment values close to those for fotemustine. Thus, the mean tail length was significantly higher for compound 4 than for compound 8 (4.7 ± 0.15 versus 3.5 ± 0.39 μm). This implies that compound 4 produces a greater mean tail length than compound 8 and therefore the DNA fragments are smaller for the former than for the latter.

With the addition of BG, DNA damage induced by fotemustine was significantly increased (up to 3.7-fold when results are expressed as percentage DNA in the comet tail). Different effects were observed between the two fotemustine analogs, as was previously seen in cell viability assays. Indeed, whereas BG had very little effect on compound 4-induced damage, compound 8 produced significantly greater DNA damage at 10 μM but not at 100 μM. No DNA fragmentation was observed in control cells either with or without BG. Furthermore, in absence of BG, we noticed a high percentage (>10%) of typical apoptotic comets with compound 4, whereas compound 8 and fotemustine induced a very few apoptotic comets (<2%). These results are consistent with cytotoxicity studies and suggest that, in A375 melanoma cells, O6-alkylation of guanine residues is not the predominant lesion induced by compound 4.

**Table 1**

<table>
<thead>
<tr>
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<th>Percentage of DNA in Tail</th>
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<tbody>
<tr>
<td></td>
<td>10 μM</td>
<td>100 μM</td>
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<tr>
<td></td>
<td>BG−</td>
<td>BG+</td>
</tr>
<tr>
<td>Fotemustine</td>
<td>7.79 ± 1.54</td>
<td>28.78 ± 0.47</td>
</tr>
<tr>
<td>Compound 4</td>
<td>34.34 ± 0.81</td>
<td>44.01 ± 3.36</td>
</tr>
<tr>
<td>Compound 8</td>
<td>18.11 ± 0.34</td>
<td>29.21 ± 2.94</td>
</tr>
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</table>

Tail Extent Moment (μm)

|                      | 10 μM                     | 100 μM                    |
|                      | BG−                        | BG+                        | Fold          | BG−                        | BG+                        | Fold          |
| Fotemustine          | 0.55 ± 0.14               | 1.42 ± 0.03               | 2.57**        | 1.62 ± 0.08                | 2.59 ± 0.29               | 1.6**         |
| Compound 4           | 1.57 ± 0.22               | 2.06 ± 0.25               | 1.31          | 3.38 ± 0.14                | 3.28 ± 0.14               | 0.97          |
| Compound 8           | 0.44 ± 0.15               | 1.13 ± 0.15               | 2.56**        | 1.16 ± 0.02                | 1.63 ± 0.20               | 1.41          |

**p < 0.01 according to Student’s t test comparing values obtained in the presence of BG with those obtained without BG.
Quantitation of Apoptosis by ELISA. To distinguish between DNA fragmentation due to chemo-induced lesions and DNA fragmentation due to nucleases during the apoptosis process, we carried out specific detection of apoptosis by ELISA using an anti-ssDNA Ab as described previously (Frankfurt and Krishan, 2001). This assay is based on the way in which DNA is specifically denatured in apoptotic cells reflecting changes associated with chromatin condensation. As shown in Fig. 6, we observed that although fotemustine and compound 8 produced a slight increase in apoptosis (8 and 8.9%, respectively), compound 4 caused significant apoptosis (51%) even in the absence of BG. When BG was added, apoptosis was increased for fotemustine and compound 8, but no effect was noted for compound 4. This result demonstrates that apoptosis induced by compound 4 is not linked to MGMT expression.

Discussion

Despite the emergence of new immunotherapy or vaccino-therapy strategies, chemotherapy with alkylating agents such CENUs remains the standard therapeutic option for disseminated melanoma. The major drawbacks of chemotherapy are toxic side effects and chemoresistance. It has been reported that the toxicity of CENUs is essentially due to carbamoylation of proteins by highly toxic isocyanate residues arising from the metabolism of nitrosoureas (Kroes et al., 1991; Brakenhoff et al., 1996; Yin et al., 2001). It is in that way that the third generation nitrosourea fotemustine lead to the formation of diethyl-ethylphosphonate-isocyanate, which is responsible for the leakage of lactate dehydrogenase, depletion of glutathione, and lipid peroxidation (Vermeulen et al., 1998). We previously reported the synthesis and preliminary pharmacological evaluation of new CENU analogs, the chloroethylnitrosouramides (Winum et al., 2003). Replacement of the carbonyl group by a sulfonyl group preserves the electrophilic effect but prevents the release of carbamoylating species. These new analogs demonstrated interesting antitumor effects on several cancer cell lines, including colon (A549) and breast (MCF7) cancers and melanoma (A375).

Nitrosoureas produce DNA alkylation essentially through an SN2 mechanism and occasionally through an SN1 process. More than 14 different types of DNA alkylations were described, leading essentially to the formation of N2-, N3-, and N7-alkyladenines or to N7- and N2-O'-alkylguanines (Gnewuch and Sosnovsky, 1997). However, O6-alkylguanine is considered to be the most mutagenic and cytotoxic lesion. This lesion can be directly repaired by O6-methylguanine DNA-methyltransferase, which causes the direct transfer of alkyl groups to an internal cysteine residue, resulting in protein degradation (Daniels and Tainer, 2000; Pegg, 2000). MGMT is a key DNA repair protein whose physiological role is to protect cells against the mutagenic effects of environmental carcinogens. In cancer chemotherapy, it has been extensively reported that expression of MGMT in tumor cells often leads to chemoresistance to nitrosoureas and to other alkylating agents such as dacarbazine, temozolomide, or cyclophosphamide (Esteller et al., 2000). Expression levels of MGMT may vary extensively between cancers, and several studies demonstrated that it would be useful to verify the MGMT status of tumors to predict the outcome of chemotherapy (Gerson, 2002). Malignant melanomas classically display high primary chemoresistance. However, acquired resistance to fotemustine, caused by reactivation of the MGMT gene through hypermethylation of the CpG island in the body of the gene, was also described in melanoma cells (Christmann et al., 2001).

In this report, we investigated the impact of MGMT expression on the sensitivity of human melanoma cells to new nitrososulfamide analogs of fotemustine. We first subcloned full-length human MGMT cDNA into the mammalian expression vector pcDNA3 (pcMGMT). Human melanoma CAL77 cells displaying neither detectable MGMT protein nor MGMT activity were chosen to establish stable transfectants. Transfection of pcMGMT into melanoma CAL77 cells induced the expression of immunodetectable human MGMT with significantly increased MGMT activity. Clones 2 and 7, which exhibited the greatest MGMT activity, were used to evaluate the effect of MGMT overexpression on the cellular response to fotemustine analogs. Results showed a significant decrease of fotemustine cytotoxicity after MGMT gene transfer into human melanoma cells, whereas the sensitivity of transfected cells to nitrososulfamide analogs did not differ from that of parent cells. This result strongly suggests that nitrososulfamide analogs of fotemustine lead to cytotoxicity through a mechanism other than O6-alkylation in CAL77 cells. We next determined the efficacy of nitrososulfamide analogs of fotemustine toward A375 melanoma cells that constitutively express MGMT. Cytotoxicity curves revealed IC50 values of 250 μM for fotemustine as opposed to 75 μM for nitrososulfamide analogs, demonstrating that these new compounds are 3 times more potent on this Mer+ cell line than the parent compound fotemustine. The antiproliferative activity of fotemustine and compound 8 was significantly increased by the MGMT inhibitor O6-benzylguanine which, however, had little effect on the cytotoxicity of compound 4.

These results lead to the hypothesis that the nitrososulfamide analogs of fotemustine are capable of inducing cytotoxicity through a MGMT-independent pathway. Thus, nitrososulfamide analogs could preferentially lead to DNA damage not recognized by MGMT, such as the formation of N7- or N3-alkylguanines. The hard and soft acid/base theory could
offer an explanation as to why it is O-alkylation or N-alkylation of DNA that occurs (Gnewuch and Sosnovsky, 1997). Thus, it is possible, as previously described for busulfan, that nitrosoufamide analogs, especially compound 4, do not produce enough O\(^6\) lesions to have a significant impact on the sensitivity of melanoma cells (Westerhof et al., 2001). Moreover, the activity, despite MGMT expression, that compound 4 retains on both melanoma cell lines, could also be attributed to the ability of bifunctional agents to form lethal cross-links that are not repaired by MGMT, because this enzyme removes DNA adducts before they can be converted into DNA cross-links. However, this difference between both nitrosoufamide analogs was not observed in the MGMT-transfected CAL77 model, suggesting that other biochemical determinants are involved in the genotoxic stress response. Thus, the response of nitrosoufamide analogs could occur through several DNA damage signals that may or not involve the repair of DNA by MGMT. In addition, it is possible that the relative importance of each pathway could vary from one tumor cell type to another. Moreover, it is conceivable that the mismatch repair system could influence the sensitivity of cells to fotemustine analogs, although the precise role of mismatch repair in the response to chloroethynitrosoureas remains to be clarified given that previous publications have reported conflicting results (Aquilla et al., 2000; Pepponi et al., 2003).

When we examined DNA fragmentation using the comet assay on A375 cells, we observed that the nitrosoufamide analogs cause more DNA fragmentation compared with fotemustine. However, we observed that the mean tail length induced by compound 4 is greater, and thus DNA fragments smaller, than for compound 8, thereby suggesting that the pattern of lesions produced by the two analogs could be different. Treatment of A375 cells with O\(^6\)-benzylguanine significantly increased DNA fragmentation caused by fotemustine, whereas BG did not have a significant effect on compound 4-induced damage. We observed that for compound 8, BG significantly intensified DNA damage at low concentrations (10 \(\mu\)M) but not at high concentrations (100 \(\mu\)M), supporting the hypothesis that compounds 4 and 8 can induce distinct DNA lesions in A375 cells. The high percentage of apoptotic comets observed with compound 4 both with and without BG suggests that this analog could cause apoptosis independently of O\(^6\)-alkyl lesions; this was confirmed with a specific apoptosis assay using an ssDNA monoclonal antibody. Previous studies demonstrated that nitrosoureas that cause isocyanate-mediated carbamoylation may selectively activate the human heat shock gene HSP70 and inhibit apoptosis by reducing the activation of caspases (Kroes et al., 1991; Petak et al., 1998). In this way, HSP70 may inhibit apoptosis formation by preventing the binding of Apaf-1 (an activator of the caspases cascade) to cytochrome c (Beere et al., 2000; Saleh et al., 2000). In this context, the absence of carbamoylating activity would be of great interest because DNA damage-induced apoptosis would not be inhibited by isocyanate. Thus, among the two newly synthesized nitrosoufamide analogs of fotemustine, compound 4 seems to be the more promising insofar as it is able to induce DNA damage, apoptosis, and cell death through an MGMT-independent pathway. Further experiments are ongoing to evaluate the potential antitumor activity of these new drugs in vivo.

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