**O6-Benzylguanine Potentiates the In Vivo Toxicity and Clastogenicity of Temozolomide and BCNU in Mouse Bone Marrow**

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The effects of treatment of mice with O6-benzylguanine (O6-BeG) on the levels of O6-alkylguanine-DNA alkyltransferase (ATase) in the hematopoietic progenitor cells and on the in vivo sensitivity of hematopoietic progenitor cells to the toxic and clastogenic effects of the antitumor agents 1,3-bis(2-chloroethyl)-nitrosourea (BCNU) and temozolomide were studied. When the overall effects of BCNU alone or with O6-BeG pretreatment were compared, dose potentiating factors of 4.17 for marrow cellularity, 4.57 for granulocyte macrophage-colony forming cells (GM-CFC) and 8.25 for colony forming unit-spleen (CFU-S) in O6-BeG pretreated versus nonpretreated animals were observed. A similar trend of dose potentiation was observed for temozolomide, although it was of lower magnitude: 1.20 for marrow cellularity, 1.63 for GM-CFC, and 1.68 for CFU-S. When the clastogenic effects of BCNU and temozolomide were examined in the mouse bone marrow micronucleus assay, a significantly (P < .05 to .001) higher frequency of micronuclei formation was observed in mice that received O6-BeG pretreatment compared with mice that received no pretreatment. These data suggest that the use of O6-BeG as a tumor-sensitizing agent before treatment of patients with O6-alkylating agents may lead to more severe hematological toxicity and possibly to an increased incidence of secondary leukemias as a result of elevated mutation frequencies in these patients.

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**Materials and Methods**

Chemicals

Temozolomide was obtained from the Cancer Research Campaign Formulation Unit, Department of Pharmaceutical Sciences, University of Strathclyde, Glasgow, Scotland. BCNU (carmustine) was purchased from Bristol-Myers Pharmaceuticals, Hounslow, Middlesex, England. O6-BeG was a generous gift from Dr R.S. McElhinney and Prof B. McMurry, Trinity College, Dublin, Ireland. Temozolomide and BCNU were dissolved in DMSO (30 mg/mL) and ethanol (25 mg/mL), respectively and both were diluted appropriately in phosphate-buffered saline (PBS). O6-BeG was homogeneously suspended in corn oil. All the solutions were freshly prepared immediately before use.

Mice

Male and female B6D2F1 mice aged 9 to 11 weeks and weighing between 24 g and 32 g were obtained from the Paterson Institute’s breeding colony. The mice were housed three to a cage with wooden chip bedding and given commercial food pellets and water ad libitum. All experimental procedures were performed under United Kingdom Home Office Licence in accordance with the Animals (Scientific Procedures) Act (1986).

Cytotoxicity to Bone Marrow Cells

Groups of three male mice were treated with a single dose of either corn oil (vehicle control) or O6-BeG (30 mg/kg body weight)
in corn oil intraperitoneally (IP) 2 hours before the administration of temozolomide or BCNU. Animals were killed by cervical dislocation 48 hours after the last injection and femur cell suspensions were prepared in Isove’s modified Dulbecco’s (IMDM) medium. Nucleated cells in the marrow were counted using a Sysmex Microcellcounter (TOA Medical Electronics Co, Ltd, Kobe, Japan).

**Granulocyte-Macrophage Colony-Forming Cell (GM-CFC) Assay**

The method used was as described by Heyworth and Spooner. Briefly, an aliquot of the femoral bone marrow suspension was added to IMDM supplemented with 20% fetal calf serum, 10% bovine serum albumin (BSA) (100 mg/mL), penicillin (at a final concentration of 0.2 mg/mL), streptomycin (at a final concentration of 0.033 mg/mL) and 5% murine lung conditioned medium as a source of colony-stimulating factor. Agar Noble (Difco, Detroit, MI) was added to 0.33% and aliquots of 1 mL were plated in 35 mm dishes and incubated at 37°C in humidified air plus 5% CO₂ for 7 days. Colonies of at least 50 cells were then counted, and the number of GM-CFC per femur was calculated.

**Colony-Forming Unit-Spleen (CFU-S) Assay**

CFU-S were assayed in potentially lethally irradiated (15.25 Gy ⁶Co γ-rays; dose rate of 0.95 Gy/h) mice as previously described by Lord. Groups of 10 irradiated recipient female mice were intravenously injected with 0.2 mL of appropriately diluted femoral bone marrow suspension. Twelve days later mice were killed, spleens were removed and fixed, and colonies were counted and the number of CFU-S per femur was calculated. Irradiation-only control groups of mice that did not receive any injected cells showed zero endogenous colony growth in all experiments.

**Micronucleus Assay**

Groups of four male B2D6F1 mice were treated with a single dose of either corn oil or O⁶-BeG (30 mg/kg) in corn oil IP. Two hours after this treatment, mice received a single dose of 0, 0.25, or 0.50 mg/kg BCNU or 0, 5, 10, or 15 mg/kg temozolomide IP and were killed 24 hours later. Femurs were removed and cells flushed out with fetal calf serum. The slides were prepared from the pellet, allowed to air dry, and fixed in methanol for 15 minutes. Slides were then stained with acridine orange as described by Tinwell and Ashby and scored blindly by two separate investigators for micronucleated polychromatic erythrocytes (MPE) among 2,000 polychromatic erythrocytes.

**ATase Assay**

Groups of four male B2D6F1 mice were treated with a single dose of O⁶-BeG (30 mg/kg IP) and killed at 2, 4, 8, 12, 16, 20, 24, 30, 36, 48, or 72 hours posttreatment. Bone marrow from both the femurs were flushed with 0.55 mL of Buffer I (50 mmol/L Tris-HCl, pH 8.3, 1 mmol/L EDTA, 3 mmol/L diithiothreitol containing 5 μg/mL leupeptin) and a piece of liver (≈0.1 g) was removed and immediately frozen in liquid nitrogen and stored at -70°C until further analysis. The ATase activity was measured in individual paired Student’s t-test was used to compare the bone marrow colony-stimulating factor. Agar Noble (Difco, Detroit, MI) was added to 0.33% and aliquots of 1 mL were plated in 35 mm dishes and incubated at 37°C in humidified air plus 5% CO₂ for 7 days. Colonies of at least 50 cells were then counted, and the number of GM-CFC per femur was calculated.

**Statistics**

The dose response curves of bone marrow cellularity, GM-CFC, and CFU-S for temozolomide and BCNU in the absence and presence of O⁶-BeG were compared by analysis of variance using MINITAB statistical software (MINITAB Inc, Pennsylvania). The baseline levels of ATase in liver and bone marrow mice were compared by analysis of variance using MINITAB statistical software (MINITAB Inc, Pennsylvania). The baseline levels of ATase in liver and bone marrow mice were compared by analysis of variance using MINITAB statistical software (MINITAB Inc, Pennsylvania). The baseline levels of ATase in liver and bone marrow mice were compared by analysis of variance using MINITAB statistical software (MINITAB Inc, Pennsylvania). The baseline levels of ATase in liver and bone marrow mice were compared by analysis of variance using MINITAB statistical software (MINITAB Inc, Pennsylvania). The baseline levels of ATase in liver and bone marrow mice were compared by analysis of variance using MINITAB statistical software (MINITAB Inc, Pennsylvania). The baseline levels of ATase in liver and bone marrow mice were compared by analysis of variance using MINITAB statistical software (MINITAB Inc, Pennsylvania). The baseline levels of ATase in liver and bone marrow mice were compared by analysis of variance using MINITAB statistical software (MINITAB Inc, Pennsylvania).

**RESULTS**

**General Toxicity**

Administration of a single IP dose of O⁶-BeG in corn oil, alone or in combination with either temozolomide or BCNU at any of the doses used did not produce any mortality or obvious morbidity in treated mice during the 48-hour test period.

**Effect of O⁶-BeG on ATase Levels in Liver and Bone Marrow**

The base line levels of ATase in liver and bone marrow before treatment with O⁶-BeG were 131 ± 5 and 102 ± 0.85 fmol/mg total protein, respectively. The ATase inactivation and the kinetics of recovery in mouse liver and bone marrow cells were studied after administration of 30 mg/kg O⁶-BeG (Fig 1). Exposure to O⁶-BeG reduced ATase activity in liver to undetectable levels (<2 fmol/mg) at the earliest time point studied and the level remained undetectable over the first 8 hours posttreatment. However, detectable levels of ATase (16% of untreated control; 16.6 ± 0.49 fmol/mg) were found in the bone marrow cells over this same time period. Thus, depletion of ATase in liver was greater in absolute terms than in bone marrow. There was a subsequent rise in ATase activity both in bone marrow and in liver from 12 hours onward with bone marrow ATase levels reaching 91% of untreated control levels at 48 hours and liver ATase levels reaching 79% of control values at 72 hours post-O⁶-BeG treatment.

**Potentiation of Cytotoxicity in Bone Marrow**

Treatment of mice with 30 mg/kg O⁶-BeG alone did not alter the bone marrow cellularity (corn oil control = 2.13 ± 0.152 × 10⁷, O⁶-BeG = 2.10 ± 0.210 × 10⁷ cells per femur; mean ± standard deviation (SD) of eight independent observations). Treatment with temozolomide alone decreased marrow cellularity in a dose-dependent manner and pretreatment of mice with O⁶-BeG potentiated this cytotoxicity as reflected by a significant shift in the dose response curve (Figs 2 and 3). The dose required to reduce marrow cellularity to 50% of control levels (toxic dose 50%, TD₅₀, Table
Fig 1. Inactivation and regeneration kinetics of ATase after O6-BeG treatment in liver (●) and bone marrow (■). Male B6D2F1 mice received 30 mg/kg O6-BeG and were killed at different time intervals. Data points represent mean ± SE of four animals.

1) was calculated to be 89 mg/kg and 74 mg/kg for temozolomide in the absence and presence of O6-BeG, respectively, which is equivalent to a 1.20-fold potentiation of toxicity. The effects of O6-BeG pretreatment on BCNU toxicity were even more dramatic, with the TD50 for BCNU being 20 mg/kg and 4.8 mg/kg in the absence and presence of O6-BeG, respectively (P < .001), equivalent to a 4.17-fold potentiation of toxicity.

Potentiation of Cytotoxicity to Hematopoietic Progenitor Cells

GM-CFC. Exposure of mice to O6-BeG had no significant effect on the number of femoral GM-CFC (corn oil control = 40,200 ± 2,860; O6-BeG = 48,200 ± 6,880 per femur, mean ± SD of six independent observations). Treatment with temozolomide or BCNU alone reduced the number of GM-CFU in a dose-dependent manner and this was potentiated by O6-BeG pretreatment (Figs 2 and 3). TD50 was calculated as 68.9 mg/kg for temozolomide alone and 42.2 mg/kg for O6-BeG plus temozolomide, equivalent to a...
1.63-fold potentiation of toxicity. As with marrow cellularity, the effects on BCNU-induced toxicity were more pronounced than those with temozolomide, with TD50 values of 20 mg/kg for BCNU alone and 4.38 mg/kg with pretreatment with O6-BeG, a 4.57-fold potentiation of toxicity.

**CFU-S.** In the same experiments, O6-BeG treatment alone did not alter the number of CFU-S in mice (corn oil control = 2,330 ± 200; O6-BeG = 2,310 ± 270 per femur, mean ± SD of six independent observations). Treatment of mice with increasing doses of temozolomide or BCNU significantly depleted the number of CFU-S in a dose-dependent manner (Figs 2 and 3). Potentiation of toxicity by O6-BeG was observed with temozolomide as well as with BCNU. However, the effect with BCNU was more pronounced than with that of temozolomide as evidenced by the TD50 values. TD50 values for temozolomide in the absence and presence of O6-BeG were 88.4 mg/kg and 52.6 mg/kg, respectively, whereas corresponding figures for BCNU were 13.2 mg/kg and 1.60 mg/kg. These data indicate 1.68-fold potentiation of temozolomide toxicity and a dramatic 8.25-fold potentiation of BCNU toxicity to CFU-S following O6-BeG pretreatment (Table 1).

**Potentiation of Micronucleus Formation in the Polychromatic Erythrocytes of the Bone Marrow**

Micronucleus formation in bone marrow was used as a monitor of clastogenicity after treating mice with low doses of temozolomide or BCNU. A dose-dependent increase in the frequency of micronucleus formation was observed when animals were treated with temozolomide or BCNU alone, while O6-BeG on its own did not alter the frequency of micronucleus formation when compared with vehicle controls. However, O6-BeG pretreatment significantly (P < .05 to .001) increased the frequency of micronuclei observed in the bone marrow of animals that subsequently received temozolomide or BCNU (Fig 4).

**DISCUSSION**

There is compelling evidence that alkylation at the O6-position of guanine in DNA is one of the major lesions responsible for the cytotoxicity, mutagenicity, clastogenicity, and carcinogenicity of alkylating agents1 and that the DNA repair protein, ATase, plays a pivotal role in protection against these effects.2 Indeed, the pronounced sensitivity of the hematopoietic compartment to the cytotoxic effects of the O6-alkylating agents is almost certainly related to the finding that rat, mouse, and human bone marrow exhibit low...
levels of ATase compared with many other organs. The levels of ATase detected in bone marrow do presumably confer some protection of the hematopoietic tissue from the effects of O\textsuperscript{6}-alkylating agents. Thus, pretreatment of animals or patients systemically with ATase inhibitors (to sensitize the tumor cells) might be predicted to deplete repair activity in the normal cells and as a consequence increase the toxicity to the normal tissues. Given that treatment with O\textsuperscript{6}-alkylating agents (like most chemotherapeutic agents) results in significant myelosuppression, the use of O\textsuperscript{6}-BeG may greatly exacerbate this effect. In this study, we have examined the effects of O\textsuperscript{6}-BeG pretreatment on the cytotoxic and clastogenic effects of O\textsuperscript{6}-alkylating agents in vivo.

We first examined the extent of ATase depletion and the kinetics of recovery of activity in mice following O\textsuperscript{6}-BeG treatment. As expected, ATase activity in liver decreased to undetectable levels within 2 hours of treatment of animals with O\textsuperscript{6}-BeG. ATase activity in bone marrow also decreased over the same time period, but only to 16% of levels in untreated mice at the dose of O\textsuperscript{6}-BeG tested. The residual ATase activity seen in bone marrow following O\textsuperscript{6}-BeG treatment of mice may reflect the presence of an inactivator resistant population of cells in this tissue. In addition to hematopoietic cells, bone marrow contains a number of other cell types, including fibroblasts and adipocytes, which collectively make up the bone marrow stroma. An O\textsuperscript{6}-BeG resistant cell population might be derived from either the hematopoietic or stromal elements of the bone marrow. Alternatively, differences in the partitioning of O\textsuperscript{6}-BeG between liver and bone marrow, or in the rate of recovery of ATase levels in these tissues might account for this preliminary observation.

Of perhaps more importance than absolute depletion levels is the rate of recovery of ATase in both liver and bone marrow of O\textsuperscript{6}-BeG–treated mice. In such animals, ATase began to rise by 12 hours posttreatment. The recovery of activity, and particularly the timing of the recovery, has important implications for the effects of O\textsuperscript{6}-BeG pretreatment on the cytotoxicity of the agents used in this study and this will be discussed in greater detail below.

We then went on to determine the effects of O\textsuperscript{6}-BeG pretreatment on the sensitivity of the hematopoietic compartment to alkylating agents in vivo. The overall effect on the hematopoietic system was studied by monitoring marrow cellularity, while the effects on particular progenitor populations within the bone marrow were studied using in vitro and in vivo colony-forming assays. These colony-forming

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**Table 1. Potentiation of Temozolomide and BCNU Toxicity to Mouse Bone Marrow by O\textsuperscript{6}-Benzylguanine**

<table>
<thead>
<tr>
<th>Toxic Dose&lt;sub&gt;50&lt;/sub&gt; (mg/kg)</th>
<th>Temozolomide</th>
<th>BCNU</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BG</td>
<td>+BG</td>
</tr>
<tr>
<td>Cellularity</td>
<td>89.0</td>
<td>74.0</td>
</tr>
<tr>
<td>GM-CFU</td>
<td>68.9</td>
<td>42.2</td>
</tr>
<tr>
<td>CFU-S</td>
<td>88.4</td>
<td>52.6</td>
</tr>
</tbody>
</table>

* DPF = TD<sub>50</sub> (drug)/TD<sub>50</sub> (O\textsuperscript{6}-BeG + drug).
O\textsuperscript{-}BENZYLGUANINE POTENTIATES MYELOTOXICITY

indicating that O\textsuperscript{-}BeG dose of agent. It will be of great interest and importance for temozolomide was between 1.20 and 1.68 AID Blood 0032 / 5H2F$$$621 01-31-97 09:57:13 blda WBS: Blood TD 50 (temozolomide or BCNU alone)/TD 50 (GM-CFC and CFU-S) assays measure the levels of particular (experimentally defined) subpopulations of the hematopoietic tissue at the time of bone marrow harvest. Temozolomide or BCNU treatment of mice led to a dose-dependent depletion of marrow cellularity, GM-CFC, and CFU-S. However, pretreatment with O\textsuperscript{-}BeG substantially increased the sensitivity of these populations to the cytotoxic effects of temozolomide and BCNU. Quantitatively this enhanced toxicity was expressed as the dose potentiating factor (DPF) = TD\textsubscript{50} (temozolomide or BCNU alone)/TD\textsubscript{50} (O\textsuperscript{-}BeG + temozolomide or BCNU) as summarized in Table 1. The O\textsuperscript{-}BeG DPF for temozolomide was between 1.20 and 1.68 indicating that O\textsuperscript{-}BeG pretreatment marginally increased the temozolomide sensitivity of all hematopoietic cells. The general trend was that sensitization varied with the cell population assayed and the more primitive the cell population examined, the more marked was the sensitization effect of O\textsuperscript{-}BeG: thus sensitization followed the order of CFU-S > GM-CFC > marrow cellularity. A similar trend in the potentiation of toxicity was seen with BCNU, although in this case, the extent of sensitization, as reflected in the DPF, was considerably greater than that seen with temozolomide (eg, 8.25 v 1.68 for sensitization of CFU-S to BCNU and temozolomide, respectively, see Table 1). The greatest drop in progenitor concentration occurred in the day 12 CFU-S compartment, suggesting a greater sensitization of these cells (as compared with GM-CFC) to alkylating agents by O\textsuperscript{-}BeG. It should be stressed, however, that the apparent differences in sensitization of individual populations of progenitors noted here may reflect modest temporal differences in the initial kill and subsequent short-term recovery of these cell populations. Further work will be necessary to address this point. The observed trend is consistent, however, with the proliferative activity of bone marrow progenitor cells: the effect on cellularity is least pronounced because of the large number of postmitotic cells (both hematopoietic and stromal) present in the bone marrow. More pronounced potentiation of temozolomide toxicity by O\textsuperscript{-}BeG is seen in the proliferating cell compartment (GM-CFC) and the cells that will be recruited into proliferative activity (CFU-S) following cytotoxicity.

The difference in the levels of toxicity seen with temozolomide and BCNU is likely to be due to the nature of the DNA lesion produced. Temozolomide undergoes rapid chemical degradation at physiological pH to 5-(3-methyltriazeno) imidazole-4-carboxamide (MTIC), which reacts with DNA bases leading to methylation at several sites including O\textsuperscript{6}-guanine, N3-adenine, and N7-guanine. BCNU is known to form O\textsuperscript{6}-chloroethylguanine, which gives rise to the cyclic intermediate N1, O\textsuperscript{6}-ethanoguanine and this subsequently forms a DNA interstrand cross-link with the cytosine of the opposite strand, an event that may be fatal for the cell. ATase prevents the formation of cross-links by removing O\textsuperscript{6}-chloroethylguanine groups from the DNA, but does not remove the cross-link itself. In those animals treated with BCNU alone, ATase would start repairing the O\textsuperscript{6}-chloroethylguanine adducts before cross-links had formed, a process with a half-life of around 7.6 hours. In the ATase recovery experiment shown in Fig 1, recovery of activity was not seen until around 12 hours after O\textsuperscript{-}BeG pretreatment. Thus, in mice treated with BCNU following O\textsuperscript{-}BeG pretreatment, the inactivation of ATase probably prevented cross-link precursor repair during this crucial 7.6-hour period, thereby maximizing cross-link formation and manifesting greater BCNU-induced cytotoxicity.

Comparatively less is known about cell killing by O\textsuperscript{-}methylguanine (O\textsuperscript{6}-meG). However, it seems likely that the abnormal O\textsuperscript{6}-meG: thymine base pair that would be generated after the replication of DNA containing O\textsuperscript{6}-meG is the substrate for a mismatch repair system, which cleaves the newly synthesized DNA strand. Since the excision repair process then restores the mismatch, the cleavage substrate is perpetuated and effectively results in a permanent cytotoxic strand break. Recently, however, it has been observed that O\textsuperscript{6}-meG can cause DNA chain termination in in vitro assays and if this occurs in vivo, it may represent an important mode of cytotoxicity for this class of agent. In either case, it is likely that the time before commitment of cells to death following formation of O\textsuperscript{6}-meG is longer than that following the formation of O\textsuperscript{6}-chloroethylguanine lesions. Therefore, the window of opportunity for the repair of methyl lesions is probably wider, allowing some repair of potentially lethal O\textsuperscript{6}-meG lesions if the regeneration of the ATase protein occurs before DNA replication.

Extrapolation of the in vivo data presented here, taken together with our earlier study reporting a potentiating effect of O\textsuperscript{-}BeG on the in vitro toxicity of temozolomide in the human granulocyte/macrophage lineage, suggests that enhanced toxicity to hematopoietic progenitor cells is likely to occur in patients receiving O\textsuperscript{-}BeG before O\textsuperscript{-}alkylating agent chemotherapy. Given this, at least two further avenues of investigation seem important in further understanding the likely effect of O\textsuperscript{-}BeG/nitrosourea combinations on hematopoietic progenitors. The first of these concerns the rate of recovery of hematopoiesis following treatment. Earlier studies with MNU have indicated that recovery of hematopoiesis (as measured by marrow GM-CFC numbers) occurs over a 7- to 9-day period with an approximate twofold overshoot of total CFU-GM around day 11, following a single dose of agent. It will be of great interest and importance to further our investigations by comparing the kinetics of hematopoietic recovery in mice following treatment with BCNU or temozolomide plus or minus O\textsuperscript{-}BeG pretreatment. None the least of reasons for this would be that several doses of O\textsuperscript{-}BeG might be necessary to keep the tumor ATase levels low for an extended period to inhibit regeneration of ATase and thus reduce the survival of tumor cells after chemotherapy. In this case, multiple treatment of patients with combinations of O\textsuperscript{-}BeG and nitrosoureas might be required, causing further exacerbation of the toxicity in bone marrow. Clearly, careful consideration and monitoring of the effects of such regimens on normal sensitive tissues will be vital.

A second consideration may be whether or not a differential effect of O\textsuperscript{-}BeG on the sensitization of tumor and normal tissue might occur, leading to an increased therapeutic index. Were this to be the case, it might be possible to use low levels of chemotherapeutic agent and hence cause less...
collateral damage in bone marrow, while still achieving significant tumor kill. In a number of xenograft studies reported, increased tumor killing without significant mortality in mice has certainly been seen. In terms of collateral toxicity, there are reports of qualitative changes in normal tissue pathology in animals receiving O6-BeG pretreatment. However, most studies of collateral toxicity have concentrated on mortality in mice and clearly, for human studies, this would be an inappropriate expression of dose-limiting toxicity. The studies reported here are the first quantitative analysis of in vivo bone marrow damage and it will be important to extend our studies further with long-term and extensive xenograft experiments to fully assess the clinical potential of a combination of O6-BeG with a range of clinically active alkylating agents. Furthermore, the observations of tissue pathology in mouse models, even in the absence of mortality, raises the issue of the potential for longer term disease as a consequence of treatment.

To assess potential effects of O6-BeG on the clastogenicity of alkylating agents, we exploited the mouse bone marrow micronucleus test. This test is sensitive to all known human carcinogens including leukemia. Although it is not in itself directly predictive of leukemogenicity. In the present study, analysis of polychromatic erythrocytes from the bone marrow of mice exposed to various doses of temozolomide or BCNU clearly showed that the number of clastogenic events detected was significantly higher in mice that had received O6-BeG pretreatment than in those that had not. This is in agreement with our earlier report showing that overexpression of ATase can protect cells against the clastogenic effects of alkylating agents, indicating that O6-alkylguanine is a clastogenic agent. O6-alkylguanine is also a mutagenic lesion, giving rise to G → A point mutations, as is well-established, for example, in the methylyating agent induced activation of the ras oncogene. It is also important to note that the effects seen here were achieved at very low (nontoxic) doses of alkylating agent, far below those normally achieved in patients undergoing chemotherapy. Extrapolation of all of these data to the clinical situation raises the possibility that increased mutations may occur in the hematopoietic cells of patients receiving O6-BeG before chemotherapy, and this could lead to an increased incidence of iatrogenic leukemias.

We and others have recently shown that retroviral transduction of bone marrow with vectors encoding human ATase leads to protection of hematopoietic cells against the toxic effects of nitrosoureas, and a number of investigators are pursuing ATase gene therapy as a means of protecting the hematopoietic compartment from collateral damage during antitumor chemotherapy. However, ATase produced as a result of retroviral transduction would still be sensitive to O6-BeG pretreatment and its activity would be depleted, leading to renewed sensitivity of the hematopoietic system to alkylating agents. One recent report detailed the ability of singly mutated forms of ATase to confer a moderate level of O6-BeG resistance and protect cells from nitrosoureas. We have recently shown that a double mutated form of ATase, which is completely insensitive to inactivation by O6-BeG, is able to fully protect cells expressing this mutated protein from a combination of O6-BeG and nitrosoureas, which proves toxic to cells expressing even large amounts of the wild type protein. Based on such observations, our proposed gene therapy strategy will involve the use of autologous bone marrow transplantation to reconstitute an O6-alkylating agent resistant bone marrow in which the constitutive expression of the transduced mutant ATase gene capable of expressing a protein resistant to O6-BeG should protect hematopoietic cells from chemotherapy-induced toxicity, while allowing sensitization of tumor tissues to chemotherapy.

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