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ABSTRACT
Previous studies have demonstrated that optimal reversal of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) resistance requires complete inactivation of the DNA repair protein O6-methylguanine DNA methyltransferase (MGMT) for at least 24 h following BCNU administration. In preparation for clinical trials at this institution, this study was undertaken to compare the efficacy of a conventional single-bolus dose versus double-bolus dose treatments with O6-benzylguanine (BG) in depleting MGMT activity in vivo. In xenograft human glioma SF767 tumors, a single 30-mg/kg bolus dose of BG completely inhibited MGMT activity for at least 8 h, but approximately 50% of the basal MGMT activity recovered within 24 h. To sustain the MGMT depletion for 24 h, a second bolus injection of BG at escalating doses was administered 8 h after the first dose. Second bolus doses of 5, 10, and 15 mg/kg BG attenuated the MGMT recovery in a dose-dependent manner compared with the single 30-mg/kg BG dose alone. When the 15-mg/kg BG dose was administered 8 h after the 30-mg/kg initial dose, MGMT activity was completely inactivated in the tumor xenografts for 24 h. This double-bolus BG treatment also depleted MGMT activity in normal murine tissues, including the liver, kidney, lung, brain, spleen, and bone marrow; and the kinetics of MGMT recovery varied among these tissues. When combined with BCNU treatment, the double-bolus BG treatment would be expected to produce greater antitumor activity in future trials than the conventional single-bolus BG treatment.

The DNA repair protein MGMT is widely expressed in human tumor cell lines and biopsy specimens (Myrnes et al., 1984; Chen et al., 1992), and is a significant source of resistance to chloroethylnitrosoureas such as carmustine (BCNU), lomustine, and fotemustine (methyl-lomustine). These agents are used to treat a variety of neoplastic diseases (Berger, 1993). In particular, BCNU is a frontline chemotherapy for brain malignancies. The cytotoxic action of chloroethylnitrosoureas involves chloroethylation of guanine at the O6 position and formation of a lethal interstrand cross-link (Kohn, 1977). The latter reaction occurs over several hours, providing an extended temporal window for the lesion to be repaired. Removal of the chloroethyl adduct by MGMT produces a covalent bond, thereby inactivating the protein. Hence, cellular MGMT can be depleted temporarily, necessitating de novo protein synthesis for further repair activity. O6-Benzylguanine (BG) is a direct substrate of MGMT that rapidly depletes MGMT in mammalian cells. BG has been used to sensitize a variety of cancer cells and tumor xenografts to BCNU (Dolan et al., 1990, 1991, 1993; Mitchell et al., 1992; Baer et al., 1993; Felker et al., 1993; Gerson et al., 1993; Magull-Seltenreich and Zeller, 1995; Wedge and Newlands, 1996; Kurpad et al., 1997; Phillips et al., 1997). In tumor xenograft studies, a single-bolus dose of BG has been used to deplete MGMT activity. However, between 16 and 33% of basal MGMT levels were regenerated within 24 h of the BG treatments (Gerson et al., 1993; Wedge and Newlands, 1996; Phillips et al., 1997; Wedge et al., 1997). Recent clinical trials of BG also used single-bolus BG treatments (Dolan et al., 1998; Friedman et al., 1998; Spiro et al., 1999). Previous studies by Marathi et al. (1993) and others indi-
cate that optimal reversal of BCNU resistance is achieved by depleting MGMT activity in tumor cells for 24 h post-BCNU treatment. Due to the protracted duration of cross-link formation following BCNU treatment, even partial recovery of MGMT activity greatly attenuates the BG-induced sensitization. For example, in HT-29 human colon carcinoma cells, inactivation of MGMT for 24 h potentiated BCNU toxicity by approximately 3 logs of cell killing more than a BG treatment that depleted MGMT for only a few hours.

In preparation for clinical trials of BG at this institution, we have investigated BG treatments that deplete MGMT activity in xenograft tumors for 24 h. Based on in vitro studies demonstrating the efficacy of residual BG at maintaining MGMT inactivation following single or multiple washes (Gerson et al., 1993; Marathi et al., 1993), we initially developed a BG treatment that depleted MGMT activity for 24 h in human glioma xenografts by combining a single-bolus dose with a continuous, low-dose infusion administered by mini-osmotic pumps (Krekau et al., 1999). For human trials, continuous infusion of BG is feasible but requires hospitalization or the use of portable infusion devices. A practical and economic alternative is a bolus infusion schedule that can be administered in an outpatient clinic setting. A double-bolus BG treatment used by Gerson et al. (1993) depleted MGMT activity in xenograft tumors for 24 h. In combination with BCNU, the double-bolus BG treatment significantly delayed the growth of xenograft tumors compared with BCNU alone. In that study, the vehicle for BG was a Cremophor-EL formulation, which has been associated with hypersensitivity reactions in clinical trials (Weiss et al., 1990). Since then, a polyethylene glycol (PEG-400) formulation has been found to be a less toxic and more efficacious vehicle for BG treatment in vivo (Dolan et al., 1994), and the PEG-400 formulation has been employed in BG clinical trials. Thus, in preparation for future clinical trials, we compared MGMT depletion in human glioma xenografts following conventional single-bolus BG treatment to double-bolus treatment using the PEG-400 vehicle.

The efficacy of BG as a chemomodulator also depends on the extent of MGMT depletion in normal tissues, and the optimal therapeutic index for combination BG and BCNU therapy should be achieved by depleting MGMT in the target tumor for 24 h with minimal depletion in normal tissues. Hence, we also investigated differences in MGMT depletion and regeneration between the target tumor and normal tissues following BG treatment. Finally, we have developed a novel fluorometric oligonucleotide assay to measure MGMT activity in cellular and tissue extracts, which is reported for the first time in the current study.

**Experimental Procedures**

**Materials.** BG was purchased from Sigma Chemical Co. (St. Louis, MO). For in vivo administration, BG was dissolved in PEG-400 and subsequently diluted to 40% PEG-400 (v/v) in phosphate-buffered saline. The final solution was filter-sterilized through a 0.2-µm syringe filter immediately before administration. Unless otherwise stated, other chemical reagents were obtained from Sigma Chemical Co., Fisher Scientific (Chicago, IL), or USB (Cleveland, OH).

**Cell Culture.** The SF767 human glioma cell line was provided by The Brain Tumor Research Center, University of California at San Francisco. SF767 and HeLa cells were cultured in Dulbecco's modified essential medium supplemented with 10% bovine calf serum (Hyclone Laboratories, Logan, UT), 1% l-glutamine, 1% HEPES buffer, and 2% penicillin-streptomycin (Life Technologies, Grand Island, NY). Murine leukemia L1210 cells were maintained in RPMI 1640 medium supplemented with 15% bovine calf serum, 1% l-glutamine, 1% HEPES buffer, and 2% penicillin-streptomycin. Cells were maintained in logarithmic growth phase at 37°C in 5% CO₂ atmosphere.

**Xenograft Tumor Studies.** Animal protocols were approved by the Animal Use Committee of Indiana University School of Medicine. NOD/SCID mice were maintained in microisolator cages with sterile bedding, food, and water under 12-h light/dark cycle. Male and female NOD/SCID mice at 9 to 12 weeks of age were sublethally irradiated with 300 radiation-absorbed dose (RAD), and then subcutaneously inoculated in the flank three weeks later with 10^6 SF767 cells suspended in 0.1 ml of sterile Hank's buffered saline solution containing 1 mM HEPES. When tumors were palpable (about 3 weeks later, tumor volume 200–300 mm³), the mice received i.p. bolus injections of BG or vehicle. The BG-treated mice were first administered a bolus BG dose (3.75 mg/ml BG) of 5 mg/kg, followed 8 h later by a second bolus injection of vehicle or 5, 10, or 15 mg/kg BG. Mice were euthanized 24 h after the first injection. The tumor, liver, kidneys, lung, brain, spleen, and bone marrow (obtained from femurs and tibia) of each mouse were excised, snap-frozen in liquid nitrogen, and stored at −80°C for analysis.

**Measurement of MGMT Activity.** Tumor and tissue extracts were prepared by resuspending each sample in approximately 3 ml of assay buffer (50 mM Tris, pH 8.0; 1 mM dithiothreitol; 1 mM EDTA; 5% glycerol) per milligram of tissue weight. Tissues were then homogenized on ice three times for 30 to 60 s each, pulse-sonicated on ice five times for 5 s each, and then centrifuged at 14,000g at 4°C for 30 min. Protein content was quantitated using the Bradford protein assay. The MGMT assay was performed as previously described (Futschek et al., 1989) with some modification. To measure MGMT activity, this laboratory has used a double-stranded 18-bp oligonucleotide containing a single O6-methylated guanine residue nested within a PvuII restriction site (Genosys Biotechnologies, Inc., The Woodlands, TX). Previously, the oligonucleotide was radiolabeled by filling in the 3’ recessed end of the complementary 16-bp strand with [α-32P]TPP. We have also designed a modified oligo that incorporates a fluorometric 5’-hexachloro-fluorescein phosphoramidite (HEX) molecule in the synthesis. The HEX molecule was incorporated into the 5’ end of the complementary strand to minimize steric hindrance of either the MGMT- or PvuII-oligonucleotide interaction. This modification resulted in a 10-bp HEX-labeled digestion cleavage product instead of the 8-bp 32P-labeled product. The radiolabeled oligo was detected and quantitated using a Storm 860 PhosphorImager (Molecular Dynamics, Sunnyvale, CA), and the HEX-labeled oligo was detected and quantitated using a Hitachi FMBIO II Fluorescence Imaging System (Hitachi Genetic Systems, South San Francisco, CA). The HEX fluorophore is excited by a solid-state laser at 532 nm (Perkin-Elmer, Norwalk, CT) and emits a fluorescent light signal at 560 nm, which is then isolated using a 585-nm filter. Fluorescence intensity units were quantitated using FMBIO software. MGMT activity was measured by incubating 0.2 pmol of the 32P- or HEX-labeled oligo with 5 to 200 μg of total cellular protein at 37°C for 2 h, followed by phenol-chloroform extraction to remove cellular protein and ethanol precipitation of the oligonucleotide. The purified oligo was then digested with PvuII (Boehringer-Mannheim, Indianapolis, IN) and electrophoresed on a 20% denaturing polyacrylamide gel. MGMT specific activity (fmol of O6-methylguanine removed/mg of protein) was calculated according to the following equation:

\[
\text{cpm of 8-bp (or fluorescence units of 10-bp) fragment} \times \left( \frac{\text{cpm (or fluorescence units) of 18-bp fragment}}{200 \text{ fmol}} \right)
\]
Statistical Analysis. MGMT activity, as measured with either the $^{32}$P- or HEX-labeled oligonucleotide, was analyzed as a function of cellular protein by linear regression. The difference in MGMT activity between the $^{32}$P- or HEX-labeled oligonucleotides was compared by ANOVA. BG treatments were compared with control treatment by Student’s $t$ test to determine the significance of differences. Single- and double-bolus BG treatments were also compared by Student’s $t$ test. Differences between treatments were considered significant at $p < 0.01$ unless otherwise stated. All data are presented as the mean ± S.E. from at least six independent measurements.

Results

$^{32}$P- versus HEX-Labeled Oligonucleotide. To compare the reactivity of the HEX-labeled oligonucleotide to the $^{32}$P-labeled oligonucleotide, varying amounts of SF767 cellular extract were incubated separately with each oligonucleotide. A representative experiment is shown in Fig. 1, and the quantitated results are summarized in Table 1. When 0.2 pmol of HEX-labeled oligonucleotide was incubated with 5, 10, 25, and 50 µg of SF767 cellular extract in separate reactions, 5.3, 15.1, 38.6, and 68.5%, respectively, of labeled cleavage product were formed, with a linear regression coefficient ($r^2$) of 0.992. When the same reactions were performed using 0.2 pmol of $^{32}$P-labeled oligonucleotide, 6.8, 14.9, 34.3, and 72.9% of labeled cleavage product were formed, respectively, with an $r^2$ of 0.999. Hence, the HEX-labeled oligonucleotide exhibits similar MGMT reactivity and sensitivity to the $^{32}$P-labeled oligonucleotide, and no difference was detected between the oligonucleotides by ANOVA ($p = 0.941$). Similar results were also obtained in HeLa cells and MGMT-transfected L1210 cells (data not shown). The HEX-labeled oligonucleotide was used in the remaining experiments. In addition to eliminating $^{32}$P from the assay, the HEX-labeled oligonucleotide assures reproducibility of the substrate because thousands of reactions can be performed from a single synthesis product. The HEX fluorochrome is considered stable for more than 1 year when stored in Tris-EDTA (pH 8.0) buffer at −20°C. Furthermore, the cost of using the HEX-labeled substrate is substantially lower than that incurred by continual $^{32}$P-labeling of the substrate, handling, and disposal.

Depletion of MGMT Activity by BG in SF767 Xenograft Tumors. Mice bearing SF767 xenograft tumors were initially administered an i.p. bolus injection of BG at 30 mg/kg (time 0). Basal tumor MGMT activity was determined in control animals at time 0. Tumor MGMT activity was also measured 8 h after administering the 30-mg/kg BG dose to determine MGMT depletion before administration of the second BG dose. In mice that received a second i.p. bolus injection of vehicle or BG at 5, 10, or 15 mg/kg, MGMT activity in the tumors was measured at 24 h post the first dose. The basal mean MGMT activity of the SF767 xenograft tumors was 1234 fmol of $O^6$-methylguanine ($O^6$-MeG) removed per milligram protein (Table 2). As shown in Fig. 2, the 30 mg/kg BG dose completely inactivated the tumor MGMT, reducing its activity to an undetectable level at 8 h postadministration. However, within 24 h of this dose alone, the MGMT activity had recovered to 48% (617 ± 298 fmol of $O^6$-MeG/mg of protein) of the basal level. The second bolus BG dose of 5 mg/kg administered 8 h after the 30-mg/kg dose did not attenuate the MGMT recovery (47%; 585 ± 546 fmol of $O^6$-MeG/mg of protein). When a second bolus BG dose of 10 mg/kg was administered 8 h after the first dose, the MGMT activity recovered to 18% (226 ± 128 fmol of $O^6$-MeG/mg of protein) of the basal level at 24 h. Hence, the second bolus dose of 10 mg/kg BG attenuated the MGMT recovery by 63% compared with the 30 mg/kg BG bolus dose alone. In animals that received a second bolus BG dose of 15 mg/kg at 8 h after the initial 30-mg/kg dose, the MGMT activity in the tumors at 24 h was only 1.6% (20 ± 24 fmol of $O^6$-MeG/mg of protein) of the basal level. Thus, the tumor MGMT activity was completely depleted for 24 h by the double-bolus 30 mg/kg + 15 mg/kg BG treatment.

Depletion of MGMT by BG in Normal Tissues of NOD/SCID Mice. Normal tissues, including the liver, kidney, lungs, brain, spleen, and bone marrow, were also harvested from the control and BG-treated mice for MGMT measurement. The basal MGMT activities are summarized in Table 2. Basal MGMT activity in these tissues ranged from 143 ± 28 fmol of $O^6$-MeG/mg of protein in the brain to 863 ±

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<td>Basal MGMT activity in SF767 tumor xenografts and normal murine tissues</td>
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Fig. 1. MGMT activity in SF767 cells using $^{32}$P-labeled versus HEX-labeled oligonucleotide. Varying amounts of SF767 cells were incubated with either $^{32}$P-labeled or HEX-labeled oligonucleotide to measure MGMT activity. Lanes 1 to 4: HEX-labeled oligonucleotide incubated with 5, 10, 25, and 50 µg of SF767 cellular extract, respectively. Lanes 5 to 8: $^{32}$P-labeled oligonucleotide incubated with 5, 10, 25, and 50 µg of SF767 cellular extract, respectively. Data shown are from a representative experiment.
66 fmol of $O^6$-MeG/mg of protein in the liver. At 8 h after the 30 mg/kg BG dose, MGMT activity was undetectable in all of the tissues. However, MGMT activity recovered significantly within 24 h of the single-dose treatment. The liver, kidneys, and bone marrow showed the greatest recoveries relative to their respective basal levels (81%, 83%, and 79%, respectively), whereas the spleen, brain, and lungs recovered only moderately (35, 27, and 25%, respectively), as shown in Fig. 3A. In animals that received the double-bolus 30 mg/kg + 15 mg/kg BG treatment (Fig. 3B), only the liver exhibited substantial recovery of MGMT activity (83% of the basal level) at 24 h. The kidneys and bone marrow recoveries were reduced to 14 and 21%, respectively, whereas MGMT activity in the spleen, brain, and lungs recovered to only 4, 6, and 6%, respectively, of basal levels. Hence, the double-bolus BG treatment dramatically reduced the MGMT regeneration in all normal tissues except the liver compared with the single-bolus treatment. Furthermore, the kinetics of MGMT repletion following either the single- or double-bolus BG treatment among the normal tissues exhibited four distinct trends, or rates, of MGMT recovery. Using a normalized standard unit of enzymatic activity (1 fmol of $O^6$-MeG removed/mg of cellular protein = 1 enzymatic unit), the liver regenerated MGMT at the rate of 43.4 enzymatic units/h following the single-bolus treatment. The respective rates of MGMT recovery in the kidneys and bone marrow under these conditions were 25.4 and 17.5 enzymatic units/h following the single-bolus treatment. The respective rates of MGMT recovery in the kidneys and bone marrow under these conditions were 25.4 and 17.5 enzymatic units/h following the single-bolus treatment. The respective rates of MGMT recovery in the kidneys and bone marrow under these conditions were 25.4 and 17.5 enzymatic units/h following the single-bolus treatment. The respective rates of MGMT recovery in the kidneys and bone marrow under these conditions were 25.4 and 17.5 enzymatic units/h following the single-bolus treatment. The respective rates of MGMT recovery in the kidneys and bone marrow under these conditions were 25.4 and 17.5 enzymatic units/h following the single-bolus treatment. The respective rates of MGMT recovery in the kidneys and bone marrow under these conditions were 25.4 and 17.5 enzymatic units/h following the single-bolus treatment. The respective rates of MGMT recovery in the kidneys and bone marrow under these conditions were 25.4 and 17.5 enzymatic units/h following the single-bolus treatment. The respective rates of MGMT recovery in the kidneys and bone marrow under these conditions were 25.4 and 17.5 enzymatic units/h following the single-bolus treatment. The respective rates of MGMT recovery in the kidneys and bone marrow under these conditions were 25.4 and 17.5 enzymatic units/h following the single-bolus treatment.

Discussion

An important goal of future clinical trials of BG at this institution will be to inactivate MGMT activity in the target tumor for 24 h. Studies in vitro have shown that maximal sensitization of chloroethylnitrosourea-resistant cells requires complete depletion of cellular MGMT for 24 h post-BCNU treatment (Marathi et al., 1993; Kreklau et al., 1999). The reason for this characteristic is likely due to the delayed...
Figure 4. Residual MGMT activity in xenograft tumor and murine tissues following single- and double-bolus BG treatments. NOD/SCID mice bearing SF767 tumor xenografts were administered a bolus dose of 30 mg/kg BG at time 0 and a second bolus dose of vehicle or 15 mg/kg BG 8 h later. Mice were euthanized to measure MGMT activity at 24 h post the first bolus dose. MGMT activity (fmol of O6-methylguanine removed/mg of protein) in the tumor, liver, lung, kidney, brain, spleen, and bone marrow are shown following the single-bolus BG treatment (A) and the double-bolus BG treatment (B). Data are summarized as the mean ± S.E. of at least six samples per treatment from three independent experiments.

formation of lethal cross-links following BCNU exposure, which has been shown in cell-free systems to take at least 8 to 14 h to occur (Brent et al., 1987). In previous preclinical xenograft tumor studies, the antitumor modulatory effect of BG was examined following a single-bolus dose of BG treatment 1 h before BCNU treatment. When MGMT activity was measured at 24 h post-BG treatment in these studies, up to one-third of the basal MGMT activity had recovered (Phillips et al., 1997). In the current study, nearly half of the basal MGMT activity had recovered (Phillips et al., 1998) and enhanced myelosuppression (Chinnasamy et al., 1997). Drug-related mortality, loss of body weight, and clinical signs of toxicity have primarily been observed in mice receiving both drugs. In humans, no major toxicities have
been associated with BG treatment alone (Dolan et al., 1998; Friedman et al., 1998; Spiro et al., 1999). Nonetheless, it has been suggested that the maximally tolerated dose of BCNU will also be reduced by BG in humans, and the expected dose-limiting toxicity for combination BG/BCNU therapy is myelosuppression (Friedman et al., 1998; Spiro et al., 1999). Although neither myelosuppression nor dose-limiting toxicity was observed among patients who received combined treatment with 120 mg/m² BG and 13 mg/m² BCNU (Spiro et al., 1999), the proposed double-bolus BG treatment in combination with BCNU may produce enhanced myelotoxicity, neurotoxicity, or pulmonary toxicity.

Basal MGMT activity among normal tissues varies significantly in both mice and humans. In the current study, MGMT activity was found to be highest in the liver and lowest in the brain of NOD/SCID mice, and the relative tissue levels from highest to lowest was liver > kidney > bone marrow > spleen > brain. In CD-1 mice, the relative MGMT levels among normal tissues exhibited a different pattern with the bone marrow and liver containing the highest levels, and the brain and kidney containing the lowest (Gerson et al., 1986). In the same study, the pattern of relative MGMT activity found in normal human tissues was similar to the pattern found in NOD/SCID mice, with highest activity found in the liver and lowest in the brain. However, the specific activities for MGMT normalized to cellular protein in the CD-1 murine and human tissues were markedly lower than those reported here for NOD/SCID mice. These differences in MGMT specific activity and in relative MGMT activity among normal tissues may be due to interspecies variations or differences in the assays used.

This report is the first to investigate the differences in MGMT depletion and regeneration among a variety of normal tissues following BG treatment. The single-bolus dose of 30 mg/kg BG depleted MGMT activity to undetectable levels in all tissues of the mice within the first 8 h. Interestingly, the rates of MGMT recovery following BG treatment did not correlate with basal MGMT levels. For example, although the bone marrow and spleen have similar basal MGMT activities, the bone marrow regenerated 2-fold greater MGMT activity than the spleen by 24 h after the single 30-mg/kg BG dose. The mechanism(s) responsible for the variable rates of MGMT regeneration among normal tissues is unknown. Activated MGMT protein has been shown to be ubiquitinated and degraded by proteolysis in human and murine tumor cells (Pegg et al., 1991; Srivengupol et al., 1996), providing further evidence that regeneration of MGMT activity requires de novo protein synthesis. The half-life of MGMT mRNA is at least 10 to 12 h, and MGMT transcription is nearly undetectable under normal conditions in HT-29 human colon carcinoma cells (Kroes and Erickson, 1995). Surprisingly, no changes in MGMT mRNA levels have been observed following BG treatment in vitro (Marathi et al., 1993), although MGMT transcription has not been measured following BG treatment. MGMT regulation, like that of many ubiquitously expressed cellular proteins, may vary due to tissue-specific transcription and/or translation factors. Furthermore, it is not known whether human tissues exhibit a similarly wide variation or pattern of MGMT regeneration following BG treatment.

Understanding differences in MGMT regeneration between the target tumor and normal tissues may potentially be important to optimize the therapeutic index of BG and BCNU combination treatment. The optimal BG treatment would maintain MGMT depletion for 24 h in the tumor with minimal loss of MGMT activity in normal tissues, particularly in BCNU target tissues such as bone marrow and lungs. In this study, the double-bolus BG treatment decreased the MGMT recovery in all of the tissues except for the liver compared with the single-bolus treatment, underscoring the importance of using the lowest, effective BG dosing treatment in future trials to minimize systemic BCNU toxicity.

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