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ANTI-NEOPLASTIC ACTIVITY OF SEQUENCED ADMINISTRATION OF O^6-BENZYLGUANINE, STREPTOZOTOCIN, AND 1,3-BIS(2-CHLOROETHYL)-1-NITROSOUREA *IN VITRO* AND *IN VIVO*

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Abstract—The purpose of this study was to evaluate the anti-tumor activity of sequenced administration of O^6-benzylguanine (BG), streptozotocin (STZ), and 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) *in vitro* and *in vivo*. We measured the recovery of O^6-methylguanine DNA methyltransferase (MGMT) and BCNU cytotoxicity in the human glioma SF767 cell line, and anti-tumor activity against xenografts following exposure to BG, STZ or the combination of BG + STZ combined with BCNU. In SF767 cells, the combination of BG (10 μM) + STZ (0.05 mM) produced sustained inhibition of MGMT activity for at least 24 hr, and a greater potentiation of BCNU cytotoxicity than either agent alone. The combined treatment of BG + STZ increased BCNU-induced cell kill by 0.5 to 1.0 log over BG or STZ alone. The maximally tolerated doses of the combination of BG + STZ + BCNU administered to nude mice i.p. were the following: BG (80 mg/kg), STZ (100 mg/kg), and BCNU (15 mg/kg). Utilizing these doses of BG and STZ, the depletion and repletion profile of MGMT activity in SF767 xenografts was measured. STZ at 100 mg/kg did not affect xenograft MGMT activity. Subsequent to BG treatment, xenograft MGMT activity was inactivated completely for 12 hr, and the tumors gradually recovered approximately 40% of control activity by 24 hr. The combination of BG + STZ produced sustained inhibition of MGMT activity for 24 hr in the xenografts with complete recovery of MGMT activity by 48 hr. Administration of the combination of BG + BCNU to nude mice bearing SF767 tumor resulted in significant inhibition of tumor growth for 23 days. However, the addition of STZ to this combination provided no greater anti-tumor activity than that observed with BG + BCNU. The three-drug combination of BG, STZ, and BCNU produced no more than 2.4 to 13.0% weight loss with occasional lethal toxicity. Collectively, these data suggest that prolonged depletion of MGMT might be required for optimal reversal of BCNU resistance both *in vitro* and *in vivo*.

Key words: O^6-methylguanine DNA methyltransferase; streptozotocin; O^6-benzylguanine; BCNU

The CENUs‖ produce antitumor responses in fewer than 20% of patients with non-hematological malignancies, suggesting that the majority of human malignancies are inherently resistant to the cytotoxic effects of these agents [1]. Substantial evidence suggests that the cytotoxic lesion induced by CENUs is the DNA interstrand cross-link [2]. The formation of this cross-link correlates with the inhibition of DNA replication, reduced RNA transcription, the induction of G_2 arrest, and the subsequent cytotoxicity produced by CENUs [3–5].

There is a correlation between the cellular complement of MGMT, the lack of CENU DNA interstrand cross-link formation, and CENU resistance [6–9]. MGMT is thought to remove the O^6-alkylguanine DNA monoadduct produced by chloroethylating agents prior to cross-link formation [10, 11]. Upon catalytic transfer of the monoadduct to the Cys-145 residue, MGMT is inactivated; therefore, lesion repair occurs in a stoichiometric fashion [12, 13]. In addition, it has been suggested that MGMT may interact with the cyclized intermediate O^6, N^1-ethanoguanine in DNA and result in a bulky protein adduct [14]. Repair of either the O^6-chloroethyl monoadduct or interaction with the cyclized intermediate by MGMT is thought to prevent the formation of CENU-induced DNA interstrand cross-links. Because the expression of MGMT correlates with resistance to CENUs, and the vast majority of human tumor cell lines and biopsies tested are MGMT positive [15, 16], the depletion of MGMT and the reversal of CENU resistance could have potential clinical ramifications.

Two agents that inactivate MGMT and sensitize human tumor cell lines to CENUs are the DNA methylating agent STZ and the free base guanine analog BG [17–19]. Repair of STZ-induced O^6-methylguanine lesions in DNA results in the depletion of the protein [20]. STZ readily sensitizes the CENU-resistant cell line HT-29 to the cytotoxic

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‖ Abbreviations: CENU, chloroethylnitrosoureas; STZ, streptozotocin; BG, O^6-benzylguanine; BCNU, 1.3-bis(2-chloroethyl)-1-nitrosourea; and MGMT, O^6-methylguanine DNA methyltransferase.

effects of BCNU [17]. In a phase I clinical trial testing this regimen, the dose-limiting, clinical toxicities of sequenced administration of STZ and BCNU are thrombocytopenia, transient hypophosphatemia, and proteinuria [21]. Additionally, the maximally tolerated dose of BCNU when combined with STZ is 50% lower than when BCNU is used as a single agent [21]. Although STZ readily reverses CENU resistance in cells and potentially in humans, it is highly mutagenic and potentially carcinogenic [22]. Conversely, BG is a direct inhibitor of MGMT which does not introduce promutagenic lesions in DNA [18]. Administration of BG prior to clomesone, BCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU), or chlorozotocin treatment results in the potentiation of cytotoxicity induced by these agents [19]. Furthermore, the combination of BG + BCNU produces significant inhibition of tumor growth rate in human tumor xenografts [23–26].

Using the highly resistant colon carcinoma cell line HT-29, we have observed recently that the combined use of BG and STZ provides prolonged inhibition of MGMT activity and potentiates BCNU cytotoxicity to a greater extent than either agent alone [27]. In the current study, we further examined the effectiveness of the combination of BG and STZ in the inactivation of MGMT and the enhancement of BCNU cytotoxicity in the resistant human glioblastoma cell line and xenograft SF767.

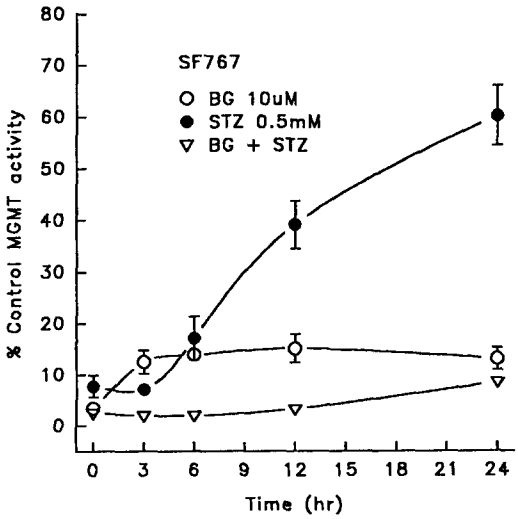

Fig. 1. Inactivation and recovery of MGMT activity in SF767 cells *in vitro*. SF767 cells were treated with BG (10 μM) for 3 hr, STZ (0.5 mM) for 2 hr, or the combination. After 3 hr the drug-containing medium was removed and replaced with fresh medium. The illustrated times refer to the time of cell harvest following drug removal. MGMT activity is expressed as a percentage of activity in untreated SF767 cells. Points represent the means ± SD of three independent experiments.

MATERIALS AND METHODS

Colony formation assay. The human glioma cell line SF767 was provided by The Brain Tumor Research Center, University of California at San Francisco. SF767 cells were cultured in Eagle's minimum essential medium, supplemented with 10% bovine calf serum, HEPES buffer, glutamine, sodium pyruvate, vitamin B_{12}, non-essential amino acids, and gentamicin. Cells were maintained in log phase at 37° in 95% air, 5% CO_2 atmosphere.

SF767 cells were seeded at 150, 300, 1,000, 3,000, and 10,000 cells per flask and incubated for 11–14 hr to allow for complete attachment. When testing the cytotoxicity of the various combinations of BG, STZ, and BCNU, triplicate flasks were individually treated with BG, STZ, BCNU, BG + STZ, BG + BCNU, STZ + BCNU, or BG + STZ + BCNU. BG, STZ, and BCNU were dissolved in dimethyl sulfoxide, 0.9% NaCl, or 95% ethanol, respectively. Cells were cumulatively exposed to BG for 3 hr, STZ for 2 hr, and BCNU for 1 hr. Following treatment, drug-containing medium was aspirated and an equal volume of fresh medium was added. After 9–10 days, cells were fixed in methanol and stained with methylene blue in phosphate buffer. Survival curves show the mean and the standard deviation of three independent experiments.

Measurement of MGMT activity. The preparation of methyltransferase extracts and the measurement of MGMT activity from cultured cells have been described previously [27]. In preparing MGMT extracts from SF767 xenografts and organs, the dissected tumors and organs, which had been frozen at −80°, were thawed and homogenized for 30 sec in approximately 3 mL/g wet tissue weight of homogenizing buffer containing 50 mM Tris, 5 mM dithiothreitol, 0.1 mM EDTA, pH 8, with an OMNI International 1000 tissue homogenizer. The homogenate was centrifuged for 5 min at 12,000 *g* at 4°. The cell pellet was reconstituted to approximately 1 mL/g of original wet tissue weight, homogenized for 15 sec, and sonicated for an additional 30 sec. Both supernatants were combined and subjected to 12,000 *g* at 4° for 30 min. Total cellular protein concentration of the supernatant was quantitated utilizing the Bradford micro protein assay (Bio-Rad, Richmond, CA). To measure MGMT activity, a ^{32}P-labeled 18 bp oligomer containing an O^6-methylguanine lesion in the *Pvu* II restriction site was incubated with 10 or 25 μg of whole cell sonicate protein in the homogenizing buffer for 2 hr. After the incubation, the protein was digested with 40 μg of proteinase K in the presence of 1% sodium dodecyl sulfate for 1 hr at 45°. The DNA was purified using a single phenol: chloroform: isoamyl alcohol extraction and ethanol precipitation. This DNA was subsequently digested with 10 units of *Pvu* II, electrophoresed on a 20% denaturing polyacrylamide gel, and autoradiographed. Repair of O^6-methylguanine by MGMT allows *Pvu* II cleavage of the oligomer to an 8 bp ^{32}P-labeled fragment. The percentage of restriction enzyme cleavage is directly proportional to MGMT activity. The radioactivity was quantitated using the Betagen betascope 603 blot analyzer. Percent probe

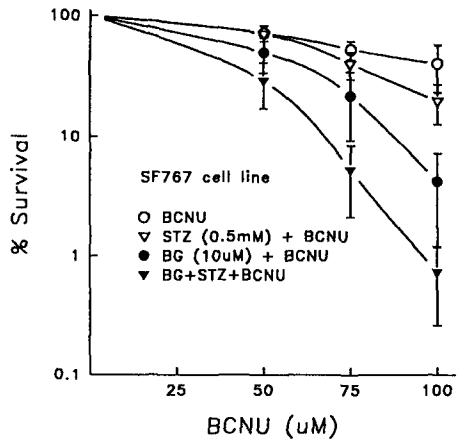

Fig. 2. Comparison of the cytotoxicity induced by BG + BCNU, STZ + BCNU, or BG + STZ + BCNU *in vitro*. SF767 cells were treated with BCNU (50–100 μM) alone, or STZ (0.50 mM) + BCNU (50–100 μM), or BG (10 μM) + BCNU (50–100 μM), or BG (10 μM) + STZ (0.50 mM) + BCNU (50–100 μM). Cells were exposed to BG for 2 hr and STZ for 1 hr prior to a 1-hr exposure to BCNU. Each data point represents the mean ± SD of three independent experiments.

Table 1. Survival following the various pretreatment regimens used to enhance BCNU cytotoxicity in SF767 cells*

	% survival
BG (10.0 μM)†	96.60 ± 0.63
STZ (0.5 mM)†	87.37 ± 5.44
BG (10.0 μM) + STZ (0.5 mM)‡	87.19 ± 6.24

* The survival values reported in this table indicate the mean percentage of cells surviving (± SD) after treatment with the various regimens.

† The cytotoxicity scores for STZ and for BG alone are normalized to untreated control values.

‡ The combination cytotoxicity values are normalized to scores obtained with BG alone, and therefore reflect the cytotoxicity produced by STZ when combined with BG.

Table 2. The IC_{90} values of BCNU when SF767 cells were treated with the various pretreatment regimens

	IC_{90}* (μM)
BCNU alone	>100
BCNU + STZ (0.5 mM)	>100
BCNU + BG 10.0 μM)	87.5
BCNU + BG (10.0 μM) + STZ (0.5 mM)	67.7

* The concentration of BCNU required to inhibit the growth of 90% of the seeded cells. The values depicted in this table were extrapolated from the data illustrated in Fig. 2.

cleaved = cpm of 8 mer/cpm of 18 mer + cpm of 8 mer. Incubation of the radiolabeled oligomer with 10 μg of untreated cultured cell or 25 μg of xenograft extract produced 23.2 ± 2.0 or 62.5 ± 3.2% probe cleavage, respectively. MGMT activity reported was normalized to these untreated control cells.

Animal studies. Female NIH Swiss nude mice and NIH Swiss normal mice were purchased from the Frederick Cancer Research and Development Center (Frederick, MD). Animals were housed in a sterile environment and provided with food and water *ad lib*. The nude mice were injected subcutaneously in the flank region with 7–10 × 10^6 SF767 cells. In tumor growth studies, animals were randomized and treated with various regimens when tumor volumes reached 30–112 mm^3. Animal weights and tumor volumes were measured twice per week, and animals were euthanized when the tumor exceeded 1500 mm^3. Tumor volumes were calculated utilizing the formula: length × $width^2$ × 0.53. For the MGMT repletion studies, tumor volume ranged from 200 to 400 mm^3 at the time of treatment. BG, STZ, and BCNU were dissolved in 10% Cremophor EL, normal saline, and 10% ethanol/90% normal saline, respectively. At various times following drug exposure, mice were euthanized via cervical dislocation. The tumors and organs were dissected, quickly frozen in liquid nitrogen, and stored at −80° for subsequent MGMT analysis.

RESULTS

Inactivation of MGMT activity and potentiation of BCNU cytotoxicity by the following pretreatment regimens in SF767 cells: BG, STZ, and the combination of BG + STZ. The human astrocytoma cell line SF767 contains appreciable levels of MGMT and consequently is highly resistant to the cytotoxic effects of CENUs [19]. In Fig. 1, the repletion kinetics of MGMT activity following treatment with BG (10 μM), STZ (0.5 mM), and the combination of BG + STZ are compared. Immediately following BG exposure, MGMT activity was inactivated completely with a recovery of approximately 15% of control MGMT activity by 3 hr, which remained at that level for 24 hr. Following STZ treatment, MGMT activity was inactivated by greater than 90% of control activity at 0 hr, and recovered to approximately 60% of control activity by 24 hr. However, sequenced administration of BG and STZ produced near complete inactivation of MGMT activity through 24 hr. The combination of BG + STZ produced a more prolonged inhibition of MGMT activity than either agent alone.

Figure 2 illustrates the potentiation of BCNU cytotoxicity by the various pretreatment regimens. BG, STZ, or the combination of BG + STZ were essentially non-toxic at the doses used in these studies (Table 1). The survival values measured after BG + BCNU, STZ + BCNU, or BG + STZ + BCNU treatment were normalized to BG, STZ, or BG + STZ survival scores, respectively. Therefore, the enhancement of BCNU cytotoxicity illus-

Table 3. Toxicities of BG + BCNU and BG + STZ + BCNU combinations in normal NIH Swiss female mice

Combination (mg/kg)	Mean maximum weight loss (%)	No. of animal deaths*
BG (80) + BCNU (15)	7.50	0/4
BG (80) + BCNU (15) + STZ (25)	13.0	1/4
BG (80) + BCNU (15) + STZ (50)	11.0	0/4
BG (80) + BCNU (15) + STZ (100)	5.90	0/4
BG (80) + BCNU (15) + STZ (200)	10.5	4/12†
BG (80) + BCNU (20)	15.6	2/4
BG (80) + BCNU (20) + STZ (25)	13.4	1/4
BG (80) + BCNU (20) + STZ (50)	18.1	2/4
BG (80) + BCNU (20) + STZ (100)	22.8	3/4

Mice were sequentially administered BG, STZ, and/or BCNU i.p. at 1-hr intervals. Animal weights were measured twice weekly for 28 days.

* Number of animal deaths/number of animals treated. These data reflected the number of spontaneous deaths and the number of animals killed following 20–25% reduction in total body weight.

† Survival through 14 days.

trated in Fig. 2 by the different pretreatment regimens is synergistic. The combinations of STZ + BCNU, BG + BCNU, and BG + STZ + BCNU at 100 μM BCNU potentiated cell killing by approximately 0.5, 1.0 and 2.0 logs, respectively. The enhancement of BCNU cytotoxicity, as reflected in the decrease in the IC_{90} of BCNU, is illustrated in Table 2. The addition of STZ at a concentration of 0.5 mM to the BG + BCNU regimen resulted in a further 22.9% reduction in the IC_{90} of BCNU. The combination of BG + STZ, which produced the most prolonged MGMT depletion, produced the greatest sensitization of SF767 cells to BCNU cytotoxicity.

Effects of the combination of BG + STZ on the inactivation of MGMT activity and the enhancement of BCNU anti-tumor activity in vivo. Mitchell *et al.* [24] have observed that the growth rate of SF767 xenografts can be inhibited significantly by the i.p. administration of BG (80 mg/kg) followed by BCNU (20 mg/kg) without appreciable systemic animal toxicity (i.e. in terms of animal death and nadir in weight loss). We used this optimized BG + BCNU regimen and asked whether the addition of STZ would induce any greater inhibition of tumor growth, or produce tumor regressions when compared with the combination of BG + BCNU alone. Preliminary acute toxicity studies using female NIH Swiss regular mice were performed to determine the maximally tolerated doses of the three drug combination of BG + STZ + BCNU. Animals were sequentially administered i.p. BG, STZ and BCNU at 1-hr intervals. The maximally tolerated doses of the three drug combination (BG 80 mg/kg, STZ 100 mg/kg, and BCNU 15 mg/kg) (Table 3) were used to examine the inactivation and recovery of MGMT activity and BCNU anti-tumor activity in female nude mice bearing SF767 xenografts. Animals were injected with BG, STZ, or the combination at 1-hr intervals, and animals were killed at the times illustrated in Fig. 3. Figure 3 shows the effectiveness of the combination of BG and STZ on the inactivation and recovery of MGMT activity in SF767 xenografts.

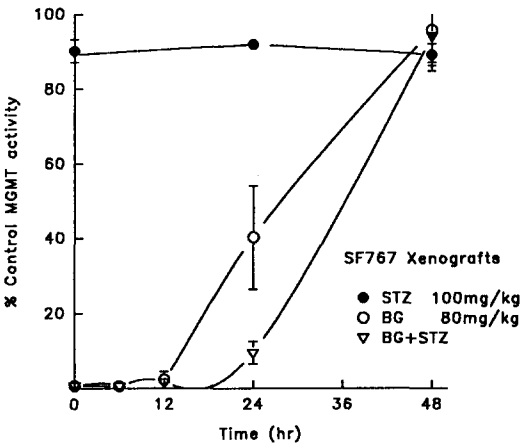

Fig. 3. Repletion of MGMT activity in SF767 xenografts following treatment with BG, STZ, or BG + STZ. Mice carrying SF767 xenografts were administered 80 mg/kg BG 1 hr prior to the administration of 100 mg/kg STZ, i.p. Animals were killed at the indicated times. The 0-hr time point refers to 3 and 2 hr following BG and STZ treatment, respectively. MGMT activity is expressed as a percentage of activity measured in untreated xenografts. Measurements of STZ alone at 0, 24, and 48 hr, and BG and BG + STZ at the 0 and 6 hr time points represent the mean MGMT activity of two animals. The remaining data points represent the mean ± SD of three animals.

As a single agent, STZ (100 mg/kg) did not affect MGMT activity appreciably in the xenografts, whereas complete inhibition of MGMT activity for 12 hr was observed following BG (80 mg/kg) administration with recovery to approximately 40% of control activity by 24 hr. Sequenced administration of BG (80 mg/kg) and STZ (100 mg/kg) slows the recovery rate of MGMT activity relative to BG alone. The combination produced near complete

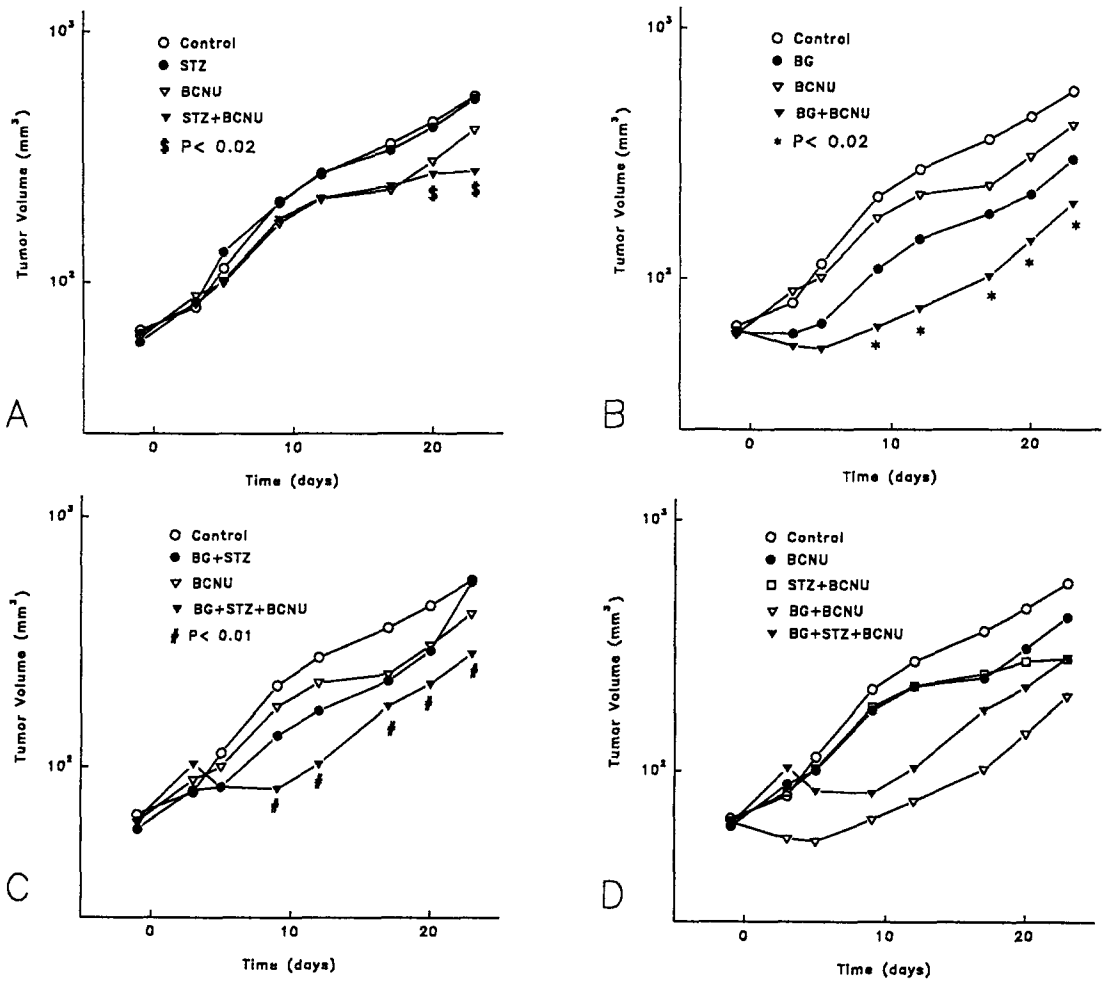

Fig. 4. Tumor growth inhibition assay utilizing subcutaneously implanted SF767 xenografts. Nude mice bearing the xenografts were injected i.p. with BG (80 mg/kg), STZ (100 mg/kg), and/or BCNU (15 mg/kg) at 1-hr intervals. Each data point represents the mean tumor volumes of 5–9 animals. Panels A, B, and C depict the anti-tumor activity in animals receiving the combination of STZ + BCNU, BG + BCNU, and BG + STZ + BCNU, respectively. The identical tumor growth curves from the untreated and BCNU-treated animals are reproduced in panels A–D. Furthermore, within panel D, the tumor growth curves of untreated, BCNU, BG + BCNU, STZ + BCNU, and BG + STZ + BCNU treated animals are reproduced for comparison. *Statistical analysis*: the data were analyzed with a multivariate ANOVA using repeated measures with a post-hoc test of single degree of freedom contrast. The analysis was performed with the SYSTAT™ statistical package.

inhibition of MGMT activity for 24 hr. Subsequently, the tumors recovered control levels of MGMT activity by 48 hr. These data are consistent with our *in vitro* observations. The sequential administration of BG and STZ probably allowed a greater number of STZ-induced O^6-methylguanine lesions in DNA, which subsequently allowed the repair-mediated inactivation of nascent MGMT for 24 hr.

Because the O^6-methylguanine is a cytotoxic and clastogenic lesion [28], we reasoned that a greater number of STZ-induced lesions would form when MGMT was initially depleted with BG. These additional STZ lesions could act in concert with the genotoxic effects of BCNU and provide greater anti-tumor activity than BG + BCNU. To test this hypothesis, nude mice bearing SF767 xenografts

Table 4. Anti-tumor activity (T/C%) of various BCNU based regimens against SF767 xenografts subcutaneously implanted in nude mice

Combination	T/C%*
BCNU alone	73.21
STZ + BCNU	54.89
BG + BCNU	6.30
BG + STZ + BCNU	19.44

* T/C% = DT/DC × 100. DT represents the mean tumor volume of the treated group on day 12 minus the pretreatment tumor volume. DC is the change in mean tumor volume in the untreated control group over the same period.

Table 5. Toxicities of STZ, BCNU, or the combinations of BG + STZ, BG + BCNU, STZ + BCNU, and BG + STZ + BCNU

Combination (mg/kg)	Mean maximum weight loss (%)	No. of animal deaths*
STZ	<1	0/7
BCNU	<1	0/6
BG + STZ	<1	0/7
BG + BCNU	4.8	0/8
STZ + BCNU	<1	0/7
BG + STZ + BCNU	2.4	0/7

Female NIH Swiss nude mice bearing SF767 xenografts were sequentially administered BG, STZ, and/or BCNU i.p. at 1-hr intervals. Animal weights were measured twice weekly for 23 days.

* Number of animal deaths/number of animals treated.

were sequentially administered the maximally tolerated doses of BG, STZ, and BCNU. Figure 4 illustrates the anti-tumor activity of all combinations of BG, STZ, and BCNU. Panels A, B, and C depict the results of growth inhibition assays in animals receiving STZ + BCNU, BG + BCNU, and BG + STZ + BCNU, respectively. The combination of STZ + BCNU produced significantly greater inhibition of tumor growth on days 20 and 23 ($P < 0.02$) than untreated controls. However, BG, which depleted xenograft MGMT for 12 hr, produced significant inhibition of tumor growth when combined with BCNU for 9–23 days relative to the untreated controls ($P < 0.02$). These results are consistent with the observations made by Mitchell *et al.* [24] in that the combination of BG + BCNU produced significant inhibition of SF767 xenograft growth for greater than 20 days. Similarly, the three-drug combination of BG + STZ + BCNU also significantly inhibited tumor growth for 23 days as compared with the untreated controls ($P < 0.01$). However, tumor regressions, defined as reductions in tumor volume over two consecutive measurements, were not observed in any of the combinations. In panel D, the tumor growth profiles from untreated animals and those treated with BCNU, STZ + BCNU, BG + BCNU and BG + STZ + BCNU are reproduced for comparative analysis. The combination of BG + BCNU produced significantly greater anti-tumor activity on days 9, 12, 17, and 20 ($P < 0.03$) than STZ + BCNU. Similarly, the combination of BG + STZ + BCNU produced greater anti-tumor activity than STZ + BCNU on days 9 and 12 ($P < 0.05$). However, the three-drug combination of BG + STZ + BCNU provided no greater anti-tumor activity than BG + BCNU. The two combinations did not differ statistically at any time after drug treatment. The effective anti-tumor activity of the various combinations is also illustrated as T/C% in Table 4. A 58% inhibition of tumor growth, or a T/C% of 42% by a given regimen is considered to be significant anti-tumor activity in murine models [29]. According to this criterion, the combinations of BG + BCNU and BG + STZ + BCNU produced significant anti-tumor activity. Additionally, the administration of all combinations of BG, STZ, and BCNU did not appreciably increase nude mouse toxicities (Table 5). These data demonstrate the feasibility of using the combination of BG and STZ to provide sustained inhibition of MGMT activity without exacerbating BCNU toxicities. However, the potential therapeutic benefit by the addition of STZ to the BG + BCNU combination could not be demonstrated in the nude mouse subcutaneous anti-tumor model. The doses of STZ in this model were limited by mouse toxicities, probably due to low levels of MGMT activity in host tissues. Consistent with this interpretation was that a single dose of 100 mg/kg STZ, which was ineffective against the xenograft, however, produced complete ablation of renal and hepatic MGMT activity (data not shown). Any potential therapeutic advantage of the combination of BG + STZ + BCNU over BG + BCNU may be examined in humans, who may better tolerate the toxicities of the combination due to a greater constitutive level of MGMT.

DISCUSSION

The primary goal of this study was to evaluate the effectiveness of the combined use of two MGMT depleting agents, BG and STZ, in reversing BCNU resistance in cultured cells and tumor xenografts. We observed that the co-administration of BG and STZ sensitized SF767 cells to BCNU cytotoxicity and produced a more prolonged inhibition of MGMT activity than either agent alone. The combined use of BG + STZ depleted MGMT activity for 24 hr and produced approximately 2 logs of synergistic BCNU cell kill. Consistent with these observations, the combination of BG + STZ also produced a more prolonged inhibition of MGMT activity in SF767 xenografts than either agent alone. The combination produced sustained inactivation of MGMT activity for 24 hr in the xenografts. Both combinations, BG + BCNU and BG + STZ + BCNU, produced significant inhibition of tumor growth for 9–23 days after treatment and demonstrated a greater anti-tumor activity than STZ + BCNU.

The single use of STZ as a CENU resistance modulator *in vivo* appears to be limited. We observed that STZ at 300 mg/kg administered i.p. produced a modest 35% reduction in SF767 xenograft MGMT activity, and a 3-fold lower dose effectively ablated renal and hepatic MGMT activity (data not shown). Because this particular dose for STZ is close to the acute LD_{50} dose (360 mg/kg) [30], the doses required to inhibit neoplastic MGMT activity may produce unacceptable host toxicity and thereby provide no beneficial change in the therapeutic index of BCNU. Similarly, Friedman *et al.* [26] administered 300 mg/kg of STZ to BALB/c mice bearing medulloblastoma and glioblastoma tumors and found that this dose of STZ did not deplete MGMT activity appreciably, nor did it enhance BCNU sensitivity. Moreover, the DNA methylating agents temozolomide and decarbazine were also ineffective in sensitizing SF767 xenografts to BCNU cytotoxicity [31]. These data suggest that methylating agents should not be used as single agents for CENU resistant modulation. However, STZ might be useful

when added to the BG + BCNU regimen. It may be advantageous to use an MGMT-depleting agent with intrinsic anti-tumor activity, such as STZ, in an adjuvant fashion with BG. In the sequential administration of BG, STZ, and BCNU, BG can deplete MGMT, and thereby allow a greater number of STZ-induced O^6-methylguanine lesions to persist and deplete MGMT for extended periods. These additional STZ lesions could act in concert with BCNU adducts to increase the tumor cell lesion burden and manifest a greater clinical anti-tumor activity. The O^6-methylguanine lesion has been considered to be more mutagenic than cytotoxic. However, recent observations by Kaina *et al.* [28] demonstrated that transfection of MGMT and *ada* complementary DNAs produces significant protection against methylating agent cytotoxicity, as well as mutagenesis. This cytotoxic potential of STZ-induced O^6-methylguanine lesions could be maximized when combined with BG.

Consistent with our data in the SF767 cell line, we demonstrated in a previous study with HT-29 cells that pretreatment regimens that provide sustained inhibition of MGMT for at least 24 hr after BCNU treatment produce the greatest potentiation of BCNU cytotoxicity. This greater temporal inhibition of MGMT activity probably inhibited cross-link precursor repair, maximizing cross-link formation, and thereby manifested the greater cytotoxicity. The cross-link precursor and an MGMT substrate, O^6, N^1-ethanoguanine, have been observed in CENU-treated DNA for greater than 8 hr after drug removal [11]. Additionally, in MGMT-deficient cells and purified DNA treated with CENU, the cytotoxic DNA interstrand cross-links continue to form for greater than 8 hr after drug removal [11, 32]. Collectively, these data suggest that in order to maximize cross-link formation and cytotoxicity in MGMT expressing cells, the protein should be inactivated for extended periods following BCNU exposure. Furthermore, our data suggest that clinical regimens targeting the inactivation of MGMT should be optimized by preventing the regeneration of MGMT for extended periods.

At the maximally tolerated doses, the combination of BG + STZ + BCNU produced significant inhibition of tumor growth. However, this three-drug combination provided no greater anti-tumor activity than BG + BCNU. Both regimens produced significant inhibition of tumor growth for 9–23 days and produced minimal host toxicity. These data suggest that the utility of STZ in an optimized BG + BCNU regimen may be limited. In the mouse, ample tumor concentrations of BG could be reached to inactivate MGMT for 12 hr, and this prolonged inhibition appears to be sufficient in maximizing tumor responses in the xenograft model. These and other data strongly recommend the clinical testing of the combination of BG + BCNU [18, 19, 23–26]. However, due to solubility limitations, one may not achieve high enough plasma concentrations of BG to inactivate tumors with high levels of MGMT for a prolonged period. If extended depletion of MGMT cannot be achieved with BG due to solubility limitations in a clinical setting, then this difficulty may be overcome in two ways: (i) repeated administrations of BG could provide complete depletion for 24 hr and potentiate BCNU cytotoxicity *in vitro* and *in vivo* [25]; and (ii) alternatively, BG could be combined with STZ. The latter regimen could act as a proverbial double-edged sword. Serial administration of BG and STZ could not only provide extended depletion of MGMT, but also the enhanced number of O^6-methylguanine lesions could contribute to anti-tumor activity.

If clinical CENU resistance modulation were to require multiple cycles of chemotherapy, the combined use of BG and STZ could also prevent the possible selection of BG-resistant tumor cells. A single point mutation within MGMT has been demonstrated to impair the MGMT-mediated repair of BG, whereas the repair of O^6-methylguanine in DNA was unaffected [33]. The addition of STZ to a BG + BCNU regimen could provide ample levels of O^6-methylguanine in DNA, which could inactivate BG-resistant MGMT and thereby facilitate BCNU cell killing.

A potential clinical disadvantage to the BG + STZ + BCNU regimen could be an increased risk of secondary malignancies. MGMT has been demonstrated to play a pivotal role in the prevention of methylating agent-induced mutagenesis and carcinogenesis [28, 34]. Presentation of methylating agent-induced pro-carcinogenic lesions when MGMT is depleted with BG could conceivably increase mutation rate, and thereby the possibility of secondary neoplastic transformation.

The combination of BG + BCNU has produced promising results in pre-clinical studies. The clinical testing of this regimen should consider the importance of extended depletion of MGMT in maximizing BCNU cytotoxicity. One possible means of producing extended depletion of MGMT is the combined use of BG and STZ in BCNU resistance modulation.

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