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ORIGINAL ARTICLE

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# Potentiation of BCNU antitumor efficacy by 9-substituted $O^6$-benzylguanines. Effect of metabolism

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**Abstract** *Purpose*: $O^6$-Benzylguanine (BG), an $O^6$-methylguanine-DNA methyltransferase (MGMT) inactivator, potentiates the efficacy of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and of other DNA chloroethylating and methylating anticancer drugs and is currently undergoing clinical trials. $O^6$-Benzyl-2′-deoxyguanosine (dBG), a less effective MGMT inactivator than BG in vitro, is at least as effective as BG in combination with BCNU against tumor xenografts in athymic mice. In order to identify the mechanism of dBG activation in in vivo systems we tested the metabolism, ability to inactivate MGMT, and efficacy to potentiate BCNU in vivo of two additional 9-substituted derivatives of BG, namely $O^6$-benzyl-9-cyanomethylguanine (CMBG) and $O^6$-benzylguanosine (BGS). *Methods*: Metabolism and disposition of these drugs was examined in athymic mice and Sprague-Dawley rats. MGMT suppression was determined in human medulloblastoma (Daoy) tumor xenografts in athymic mice following treatment with BGS, dBG, and CMBG and was compared with the loss of resistance to BCNU as determined by tumor growth delays. *Results*: Growth delays at 25 mg/m$^2$ BCNU and 133 mg/m$^2$ BG or equimolar doses of CMBG, BGS or dBG were 23.0, 2.5, 21.3 days, and 30.4 days, respectively. The above differences did not correlate with the $ED_{50}$s of 0.2, 13, 11 μ*M*, and 2 μ*M* determined for the above compounds, respectively, in cell free extracts. Differences in the efficacies of the 9-substituted compounds did correlate, however, with the extent of their metabolic conversion to BG. The maximum concentrations of BG in blood achieved after the administration of equimolar (250 μmol/kg) doses of CMBG, BGS and dBG were 10, 30 μ*M*, and 55 μ*M*, respectively. Although such levels were lower than those achieved in circulation by administration of an equimolar amount of BG, BG levels persisted longer following treatments with BGS or dBG than after treatment with BG itself. Formation of BG was required for continuous and prolonged (> 16 h) suppression of MGMT activity to non-detectable levels (< 5 fmol/mg protein). *Conclusion:* Metabolism of BGS and dBG to BG explains the unexpected high efficacy of these compounds in potentiating the antitumor activity of BCNU in the athymic mouse model. The faster and more effective suppression of tumor MGMT by dBG and its greater efficacy, as compared with BGS, also correlates with a more rapid accumulation of BG in blood after dBG than after BGS administration, which results in faster and complete suppression of MGMT in Daoy xenografts. Thus, metabolism of dBG and BGS to BG appears to be the determining factor for continuous and prolonged suppression of MGMT activity, and that near complete suppression of such activity during and following BCNU administration is required for the higher efficacy of treatments. Similarly, the failure of CMBG to suppress tumor MGMT to the same extent as BGS, in spite of their similar $ED_{50}$ values, could be attributed to the metabolism of this compound mainly by pathways other than conversion to BG.

**Key words** Carmustine · $O^6$-Benzyl-2′-deoxyguanosine · $O^6$-Benzylguanosine · $O^6$-Benzyl-9-cyanomethylguanine · $O^6$-Methylguanine-DNA methyltransferase

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## Introduction

We have previously shown that $O^6$-benzyl-2′-deoxyguanosine (dBG) is an effective inactivator of MGMT in Daoy (medulloblastoma) tumors xenografted in athymic mice, and that such effectiveness results in a remarkable potentiation of the efficacy of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) against this tumor which is comparable with that shown for $O^6$-benzylguanine [1]. Since dBG is ten times less active against $O^6$-methylguanine-DNA methyltransferase (MGMT) than $O^6$-benzylguanine (BG) in cell-free systems [2], it has been proposed that metabolic activation of dBG to a more potent MGMT inactivator might account for its unexpected potency in the athymic mouse model. dBG is metabolized to yield BG, and although the latter is not apparent in urine or bile, it is the prominent metabolite in the circulation after dBG administration [3, 4]. Further studies demonstrated that in rats, a major fraction of dBG was converted to BG over a period of 6 h after its administration [4]. BG itself is an effective inactivator of MGMT in vivo, but it is rapidly metabolized by a variety of pathways, and cleared rapidly in rats with a half-life of only 1.6 h [5]. Prolonged inhibition of MGMT by BG in humans is attributed to $O^6$-benzyl-8-oxoguanine (BOG) a product of direct oxidation of BG, which has an $ED_{50}$ comparable with BG in inactivating MGMT, but slower metabolic and renal clearance [6]. BOG has also been identified in tissues and circulation of animals treated with dBG, at concentrations increasing with time and reaching maximum levels between 8 h and 12 h after dBG injection [4]. The sequential appearance of dBG, BG, and then BOG in blood over a period spanning more than 12 h from the injection of dBG results in nearly complete suppression of MGMT activity in many normal tissues and in Daoy xenografts for at least that time period [1, 4]. Here, we examine the metabolism of two additional 9-substituted derivatives of BG, namely $O^6$-benzylguanosine (BGS) and 2-amino-6-benzyloxy-9-cyanomethylpurine (CMBG), which are five times less potent than dBG in inactivating MGMT in vitro [2]. These compounds, like dBG, are expected to be converted to BG, according to the scheme shown in Fig. 1, but the extent of such metabolic conversion should vary depending on the 9-substituent. The effect of metabolism of these compounds on the suppression of tumor MGMT in vivo and the potentiation of the BCNU antitumor activity against Daoy tumor xenografts is also examined. As expected, extensive metabolic conversion of dBG and BGS to BG according to the scheme shown in Fig. 1 results in enhanced suppression of MGMT and in the potentiation of BCNU antitumor efficacy by the above compounds. On the other hand, lack of significant metabolic conversion of CMBG to BG detracts from the potential of this compound to decisively lower MGMT

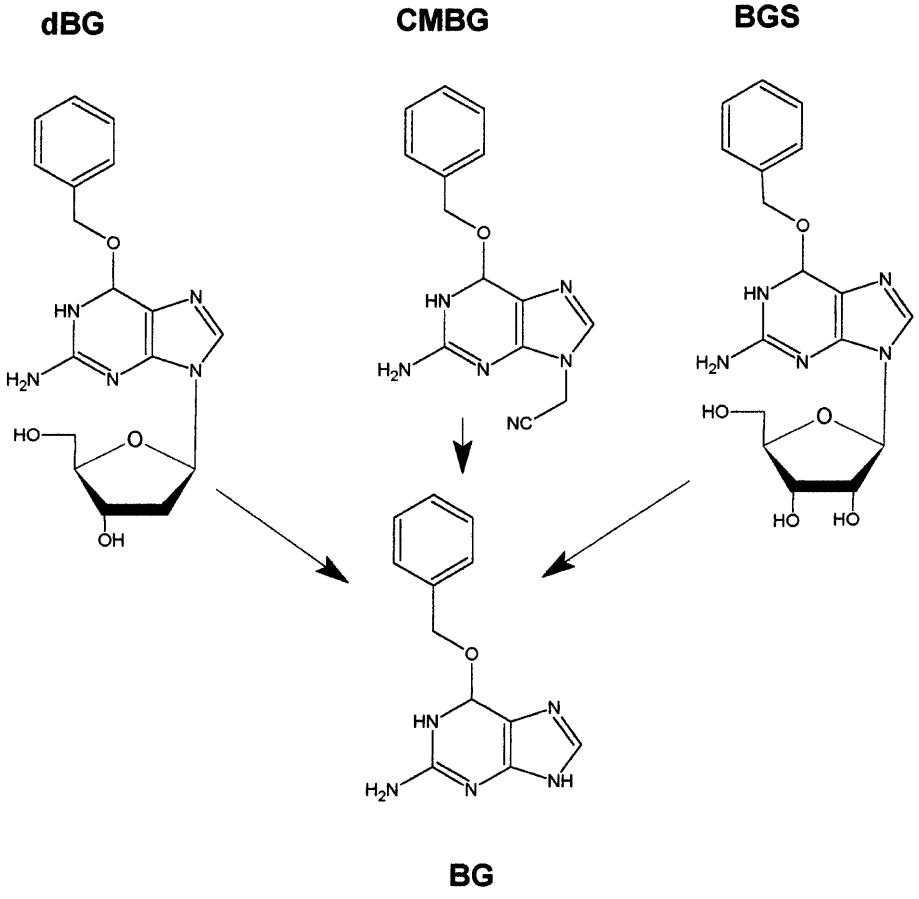


**Fig. 1** Postulated pathway for the metabolism of 9-substituted $O^6$-benzylguanines. The extent of such metabolic conversion should depend on the 9-substituent

activity in tumors and to substantially increase the therapeutic index of BCNU treatments.

## Materials and methods

### Chemicals

dBG, BGS, CMBG, and $O^6$-*p*-hydroxymethylbenzylguanine were synthesized and purified according to methods published previously [2, 7, 8, 9]. [8-$^3$H] $O^6$-Benzyl-2′-deoxyguanosine and [8-$^3$H] $O^6$-benzylguanosine were prepared by Amersham (Arlington Heights, Ill., USA) by tritium exchange, and their specific activities were determined as 880 Ci/mmol and 670 Ci/mmol, respectively. $^3$H-Methylated DNA was prepared as previously described [10] with [$^3$H-$CH_3$]MNU purchased from Amersham (specific activity 17.5 Ci/mmol).

### Animals

For tumor treatment studies, 6-week-old Balb/c nu/nu athymic mice were purchased from Simonson (Gilroy, Calif., USA). Mice were maintained under barrier conditions and given sterilized food (Harlan Teklad laboratory diet) and water. For metabolic studies, both Sprague-Dawley rats weighing 200 g (Charles River, Wilmington, Del., USA) and athymic mice weighing 20–25 g were used. In all experiments involving animals the NIH and Institutional guidelines for the welfare of animals were observed.

### Tumor lines

Daoy, a hyperdiploid human medulloblastoma line, grows s.c. in athymic mice with a doubling time of 4.9 days. Tumors grow upon injection of shredded tumor fragments. Its untreated MGMT activity when growing as xenograft in athymic mice is 375 ± 25 fmol/mg protein.

### Drug treatment

All treatments were administered i.p. at a volume of 30 ml/m$^2$ (approximately 0.2 ml per mouse). 9-Substituted BGs were dissolved in 40% PEG 400/60% PBS. The pH of the solvent was corrected to 7.0 with sodium bicarbonate before the addition of the drug. BCNU was administered in 5% ethanol in water.

### Treatment of s.c. tumors

Fifty microliters of Daoy tumor was implanted at the left flank of animals. Visible tumors appeared in most of the animals within 3 weeks after implantation. The tumors were subsequently measured in two perpendicular dimensions, and their volumes were estimated using the formula $(\alpha^2 \times \beta)/2$, where $\alpha$ is the shorter and $\beta$ the longer of the two dimensions. Treatment was administered to animals with tumors ranging between 200 and 300 mm$^3$. Tumors were measured every other day until their volumes exceeded five times the volume of the tumor at treatment. The data were analyzed using the Wilcoxon rank sum test, comparing the time from treatment to five times treatment volume in each of the groups. Growth delay was the difference between the median time to five times treatment volume in the treatment group, minus the median time to five times treatment volume in the control group. *P* values were corrected for the number of comparisons made between data sets using the Bonferroni correction test. The number of tumor regressions (number of tumors measuring smaller than the volume of the treatment day) occurring in each group was also measured. Groups were compared with the two-tailed Fisher exact test. Two control groups received 40% PEG/60% PBS followed in 1 h by either 5% ethanol in water or BCNU in 5% ethanol. All treatments were administered at volumes of 30 ml/m$^2$. Between 10 and 12 animals were used in each experimental group.

### Determination of toxicity

The toxic effect of BCNU was determined in athymic mice by observing weight loss following treatment with the MGMT inhibitor and BCNU. The percent loss of weight for each of the treated animals was determined and the mean value ± standard deviation (SD) of such percentages was reported.

### Tissue MGMT assay

Animals bearing Daoy xenografts were injected i.p. with various doses of BG, dBG, BGS, or CMBG in 40% PEG/60% PBS. Animals were killed at 1-, 2-, and subsequently 2-h intervals after injection, and tumors were removed. Three animals were used for each of the doses and time points selected. Tissue preparation for MGMT determinations and the MGMT assay were performed as previously described [10]. Using a highly specific activity substrate (> 15 Ci/mmol), the assay can accurately determine MGMT activities as low as 5 fmol/mg protein.

### Metabolism

Four groups of nine mice each were injected i.p. with 750 μmol/m$^2$ of [8-$^3$H]dBG (sp. act. 2.5 Ci/mol) and, [8-$^3$H]BG (sp. act. 3.1 Ci/mol) or CMBG, respectively, in 30% polyethylene glycol 400 (PEG 400). The animals were placed individually in Nalgene metabolic cases (Fisher Scientific, Pittsburgh, Pa., USA) to collect urine. At 2, 4 h and 6 h three animals from each group were placed under anesthesia, and 1 ml blood was collected from the heart before they were killed. Urine was also collected from the bladder and combined with that already excreted. Blood samples (1 ml) were homogenized in 9 ml of 75% ethanol water with an electric biohomogenizer (Bartesville, Okla., USA) set at high speed; 10 nmol $O^6$-p-hydroxymethylbenzylguanine was added to each sample as internal standard. Extracts were centrifuged at 5,000 *g* to remove precipitated material which was re-extracted with 5 ml 75% ethanol water. Extracts were combined and the organic solvent was removed by rotatory evaporation. Acetonitrile was added to a final concentration of 25% and the samples were centrifuged to remove debris. Volumes were then adjusted to 10 ml with 25% acetonitrile in water and samples were passed through Supelclean LC-18 cartridges (Supelco, Bellefonte, Pa., USA), which then were washed with 5 ml 25% acetonitrile in water. The sample volume was again reduced to 1 ml by rotatory evaporation. Samples were analyzed for metabolites retaining their label by reverse phase chromatography according to published procedures [3]. BG and the parent metabolite were determined by their UV absorption (254 nm) and by fluorescence (ex: 305, em: 370). BG and other radioactive metabolites were also quantitated when applicable by collecting fractions at 0.5-min intervals and counting for tritium with a scintillation counter. The same experiment was repeated for [8-$^3$H] BGS using Sprague-Dawley rats in which the bile duct was cannulated to collect bile according to the method previously described [3].

## Results

### Metabolism of 9-substituted $O^6$-benzylguanines

The metabolism and disposition of dBG in Spargue-Dawley rats has been previously described [3]. Following i.p. administration of dBG, 38% of the dose was excreted unchanged in the urine, and 2% in the bile, while

the rest was metabolized. The major metabolite in the urine that maintained the label at the 8-position was BG, totaling ~2% of the injected dose. In the bile, the glucuronic acid conjugate of the parent compound predominated, reaching 10% of the total dose, while BG did not exceed 1% of the total dose. A similar pattern of renal and biliary clearance was observed with [8-$^3$H] BGS. Thirty-three percent of the BGS injected in Sprague-Dawley rats was excreted in the urine within 24 h and only 6% of the dose was found unchanged in the bile. There were no appreciable levels of metabolites retaining the tritium in the urine or bile. Disposition of BGS was not substantially different in mice. Table 1 shows that BGS was excreted during the first 8 h after injection, while accumulation of additional label in the urine after that time was primarily due to excretion of volatile tritium, probably tritiated water, which was released from catabolism of the purine ring. As is the case with dBG, BGS also yielded high concentrations of BG and BOG in the circulation. Levels of BGS in blood reached a maximum between 0 h and 2 h from its administration and declined rapidly after that, while BG and BOG reached maximum levels in 6 h and remained detectable up to 16 h after BGS administration (Fig. 2). Lack of availability of a tritiated CMBG did not allow exact measurements on routes of disposition and metabolism of this compound other than recording the kinetics of BG and BOG formation in the circulation (Fig. 3). Since only 5% of the total dose was excreted unchanged in the urine and 11% was secreted in the bile of rats over a period of 24 h, the bulk of the CMBG must have been rapidly metabolized. Low levels of CMBG and BG and only traces of BOG in the circulation, as well as the absence of any additional hydrophobic metabolites in blood (Fig. 3) suggests that conversion of CMBG to BG is a minor metabolic pathway. These experiments indicate that 9-substituted derivatives of BG are subject to the enzymatic removal of the adduct at the 9-position, and that the extent of such a reaction in overall metabolism depends on the type of substituent.

A comparison of levels of the parent 9-substituted compounds tested and their major benzylated metabolites in blood and tissues of athymic mice bearing Daoy tumors following treatment with dBG, BGS, and CMBG is shown in Table 2. It is evident that all three of these compounds yield BG as a common metabolite, since it was found in the circulation and all tissues examined. BG levels varied among types of tissue and

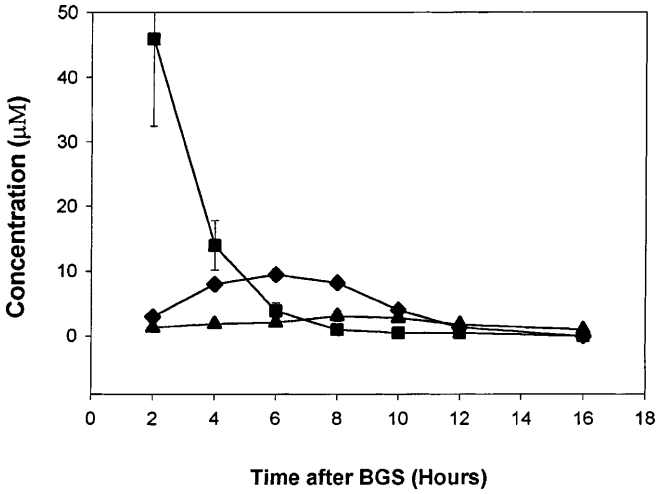


**Fig. 2** Levels of BGS (*square*), BG (*diamond*) and BOG (*triangle*), in blood of Sprague-Dawley rats treated with a single i.p. injection of 100 mg/kg BGS at time zero. Data represent the mean of at least 3 independent determinations and bars the standard deviation. *BGS* $O^6$-benzylguanosine

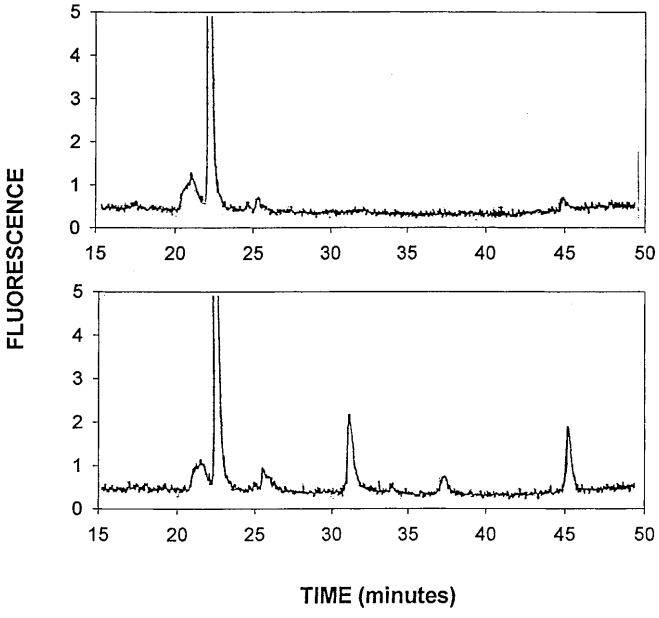


**Fig. 3** HPLC detection of CMBG and metabolites in the blood of Sprague-Dawley rats following i.p. injection of 100 mg/kg CMBG. Peaks at 22.5, 31.0, 37.3 min, and 45.3 min represent the internal standard $O^6$-(*p*-hydroxybenzyl)guanine, BG, BOG, and CMBG, respectively. A run from control animals is shown in the *upper panel*. *BG* $O^6$-benzylguanine, *BOG* $O^6$-benzyl-8-oxoguanine, *CMBG* $O^6$-benzyl-9-cyanomethylguanine

**Table 1** Excretion of tritium label in the urine of Sprague–Dawley rats treated with 100 mg/kg (8-$^3$H)$O^6$-benzylguanosine (BGS; sp. act. 2.2 Ci/mol)

| Time[a] (h) | Excreted (μCi) | Non-volatile[b] (μCi) | BGS (%)[c] |
|---|---|---|---|
| 2 | 1.4 ± 0.4[d] | 1.4 ± 0.6 | 12 |
| 4 | 2.7 ± 0.7 | 2.7 ± 0.8 | 22 |
| 6 | 3.4 ± 0.9 | 3.2 ± 1.1 | 25 |
| 8 | 4.5 ± 1.1 | 4.1 ± 1.4 | 33 |
| 12 | 6.2 ± 0.8 | 4.3 ± 1.1 | 33 |
| 16 | 6.9 ± 1.3 | 4.3 ± 1.4 | 33 |
| 24 | 7.7 ± 2.8 | 4.3 ± 1.9 | 33 |

[a] Time from injection of labeled BGS
[b] Label remaining after complete removal of solvent by vaccum
[c] Percentage of BGS in urine compared with the total dose
[d] Mean ± SD

**Table 2** Levels of parent compound and metabolites in blood and tissues of athymic mice 2 h after the i.p. injection of 100 mg/kg dBG, BGS and CMBG. *dBG* $O^6$-benzyl-2′-deoxyguanosine, *BGS* $O^6$-benzylguanosine, *CMBG* $O^6$-benzyl-9-cyanomethylguanine, *ND* non-detectable

| | *dBG* | | | *BGS* | | | *CMBG* | | |
|---|---|---|---|---|---|---|---|---|---|
| | Parent | BG | BOG | Parent | BG | BOG | Parent | BG | BOG |
| Blood | 85 ± 5[a] | 22 ± 4 | 4 ± 2 | 60 ± 7 | 8 ± 2 | 2 ± 1 | 55 ± 3 | 10 ± 2 | ND |
| Liver | 60 ± 8 | 60 ± 5 | 8 ± 3 | 156 ± 13 | 22 ± 4 | 16 ± 3 | 209 ± 29 | 12 ± 1 | 7 ± 2 |
| Kidney | 100 ± 14 | 60 ± 15 | 20 ± 7 | 165 ± 18 | 21 ± 3 | 7 ± 3 | 120 ± 7 | 10 ± 2 | 4 ± 2 |
| Lungs | 25 ± 3 | 40 ± 6 | 3 ± 1 | 148 ± 14 | 15 ± 2 | 11 ± 3 | 147 ± 18 | 4 ± 1 | 3 ± 1 |
| Tumor | 49 ± 6 | 12 ± 3 | ND | 38 ± 3 | 6 ± 2 | 4 ± 1 | 83 ± 14 | 3 ± 1 | ND |

[a] Concentration (μ*M*), mean of at least three determinations ± standard deviation

were dependent on the compound injected. In general, BG was present in higher concentrations in all tissues following administration of dBG than BGS or CMBG. Higher levels of BG in the liver than in circulation with all three compounds suggest that BG was formed primarily in this organ and that at 2 h it was still produced at rates greater than its catabolism or removal by the circulation. Differences between hepatic and blood concentrations of BG were more pronounced with dBG than BGS or CMBG, which also confirmed a more effective removal of the 2′-deoxyribose compared with the ribose or the cyanomethyl group from 9-substituted BG derivatives. Table 3 shows the concentration of the parent compounds and their common metabolite BG in circulation as a function of time after treatment with the above three derivatives of BG. BGS was more resistant to metabolism than either dBG or CMBG. Since dBG and BGS yielded higher levels of BG in the circulation than CMBG, in spite of their significantly greater elimination by excretion in the urine compared with the latter, it is unlikely that conversion of CMBG to BG is a major pathway in its metabolism. This is further supported by the barely detectable levels of BOG in the circulation of animals treated with CMBG.

**Table 3** Concentrations of MGMT inhibitors and their metabolites in blood at various time intervals after administration of equimolar (250 μmol/kg) doses of BG, dBG, BGS, and CMBG. *BG* $O^6$-benzyl-guanine, *dBG* $O^6$-benzyl-2′-deoxyguanosine, *BGS* $O^6$-benzyl-guanosine, *CMBG* $O^6$-benzyl-9-cyanomethylguanine, *ND* non-detectable

| Compound injected | Time (h) | BG (μ*M*) | dBG | BGS | CMBG |
|---|---|---|---|---|---|
| BG | 2 | 88 ± 12[a] | – | – | – |
| | 4 | 57 ± 6 | – | – | – |
| | 6 | 5 ± 2 | – | – | – |
| dBG | 2 | 22 ± 3 | 85 ± 11 | – | – |
| | 4 | 55 ± 7 | 13 ± 2 | – | – |
| | 6 | 26 ± 4 | < 1 | – | – |
| BGS | 2 | 8 ± 2 | – | 60 ± 7 | – |
| | 4 | 30 ± 5 | – | 26 ± 3 | – |
| | 6 | 21 ± 4 | – | 11 ± 3 | – |
| CMBG | 2 | 10 ± 3 | – | – | 55 ± 10 |
| | 4 | 7 ± 2 | – | – | 19 ± 4 |
| | 6 | 2 ± 1 | – | – | ND |

[a] Mean of at least three independent determinations ± SD

## MGMT suppression in tumors and tissues by 9-substituted BG derivatives

The effect of dBG, CMBG, and BGS on suppression of MGMT activity in Daoy xenografts implanted in athymic mice is shown in Fig. 4. All three compounds lowered the MGMT activity to levels below 5% of its base value. However, substantial differences were observed among them with respect to the overall suppression of the activity and also the kinetics of suppression. The marginal suppression of MGMT activity by BGS 1 h after its administration suggests that this compound was not effective by itself, and that it probably needed metabolic activation to BG in order to suppress the MGMT activity in the tumor. However, CMBG reduced MGMT activity to 5% of its base value within 1 h of its administration, but failed to further suppress activity to levels achieved with BGS and dBG (1% of the base value). Furthermore, CMBG did not keep tumor MGMT suppressed as long as dBG or BGS, and activity nearly doubled in the tumor between 4 h and 16 h from administration of CMBG. On the other hand, dBG and BGS suppressed tumor MGMT activity to about 1% of

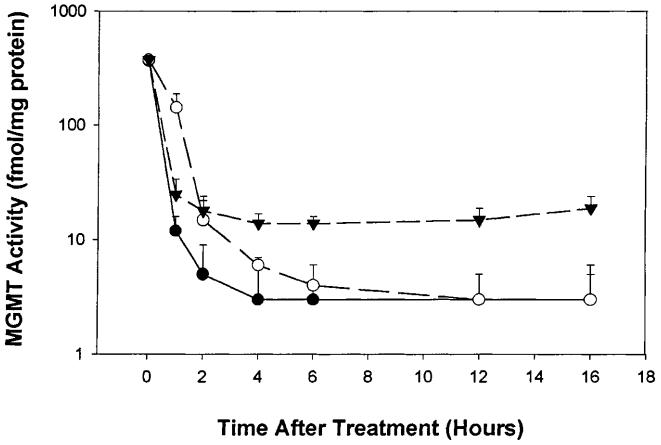


**Fig. 4** MGMT activity in Daoy tumors harvested from athymic mice following the i.p. injection of equimolar doses of 133 mg/m$^2$ dBG (*closed circles*), 138 mg/m$^2$ BGS (*open circles*) or 104 mg/m$^2$ CMBG (*triangles*). Data represent the mean of 3 independent determinations and bars the standard deviation. *MGMT* $O^6$-methylguanine-DNA methyltransferase, *dBG* $O^6$-benzyl-2′-deoxyguanosine, *BGS* $O^6$-benzylguanosine, *CMBG* $O^6$-benzyl-9-cyanomethylguanine

its base value for at least 16 h from administration. The data suggest that extensive metabolic conversion to BG is needed for the suppression of MGMT to below 10 fmol/mg protein in Daoy tumors, and that the yields of BG may be sufficient for such effective suppression with BGS and dBG, but not with CMBG.

## Levels of 9-substituted $O^6$ benzylguanines in brain

Table 4 shows the levels of BG, BGS, dBG, and CMBG in brain at various intervals after the administration of these compounds to athymic mice at equimolar doses. Such concentrations and those of their metabolite BG were below those found in the circulation. Ratios of the parent compound in brain tissue versus that in circulation were markedly greater with BG and CMBG than dBG and BGS, which is in accordance with the difference in lipophilicity between these two groups of inhibitors. Concentrations of BG in the brain were lower than expected following administration of dBG and especially BGS. However, while concentrations of BG in the brain declined with time (between 2 h and 6 h) following BG administration, such concentrations increased over time when BGS or BdG are injected. Again, the administration of dBG may be more advantageous for lowering the MGMT activity of intracranial tumors than administration of BG itself, since dBG yields BG levels that increase with time. Administration of CMBG results in relatively high concentrations of this compound in brain tissue, but as in the case with BG, such concentrations decline rapidly with time. No detectable BG was found in the brain of CMBG-treated animals between 2 h and 4 h after treatment.

**Table 4** Concentrations of MGMT inhibitors and their active metabolites in brain at various time intervals after administration of equimolar (250 μmol/kg) doses of BG, dBG, BGS, and CMBG. *MGMT* $O^6$-methylguanine-DNA methyltransferase, *BG* $O^6$-benzylguanine, *dBG* $O^6$-benzyl-2′-deoxyguanosine, *BGS* $O^6$-benzylguanosine, *CMBG* $O^6$-benzyl-9-cyanomethylguanine

| Compound injected | Time (h) | BG ($\mu M$) | dBG | BGS | CMBG |
|---|---|---|---|---|---|
| BG | 2 | 12 ± 3[a] | – | – | – |
| | 4 | 8 ± 3 | – | – | – |
| | 6 | 2 ± 1 | – | – | – |
| dBG | 2 | 6 ± 2 | 4 ± 1 | – | – |
| | 4 | 9 ± 2 | <1[c] | – | – |
| | 6 | 13 ± 3 | ND[b] | – | – |
| BGS | 2 | <1 | – | 3 ± 2 | – |
| | 4 | 2 ± 1 | – | <1 | – |
| | 6 | 4 ± 2 | – | ND | – |
| CMBG | 2 | ND[b] | – | – | 12 ± 3 |
| | 4 | ND | – | – | 6 ± 2 |
| | 6 | ND | – | – | 3 ± 2 |

[a] Mean of at least 3 independent determinations ± standard deviation
[b] Non-detectable
[c] Detectable (less than 1 nmol per milliliter of blood)

## Relative efficacies of BG, BGS, CMBG, and dBG against Daoy xenografts implanted s.c. in athymic mice

The median times from treatment to five times the tumor volume at treatment for Daoy medulloblastoma were 8.0 days when tumor-bearing animals were injected with the vehicle (40% PEG/60% PBS and 5% ethanol), and 8.2 days when animals were treated with the same vehicle plus BCNU (25 mg/m$^2$ ). This was not a significant difference. On the other hand, when animals with Daoy tumors were treated with the equimolar (370 μmol/m$^2$) doses of dBG, BGS or CMBG (133, 138 mg/m$^2$, and 104 mg/m$^2$ , respectively) 1 h prior to treatment with BCNU, growth delays were highly dependent on the MGMT inhibitor used (Fig. 4; Table 5). The combination of BCNU and dBG was the most effective, resulting in tumor growth delay of 30.4 days, with no mortalities due to toxicity and a weight loss of 12.1%. BGS in combination with BCNU was nearly as effective as dBG, showing a tumor growth delay of 21.3 days, but there were two mortalities due to the acute toxicity of this combination. CMBG was the least effective of all three, yielding a growth delay of only 2.5 days when combined

**Table 5** Treatment of medulloblastoma Daoy xenografts in athymic mice with 9-substituted $O^6$-benzylguanine analogs and BCNU. *BCNU* 1,3-bis(2-chloroethyl)-1-nitrosourea, *BGS* $O^6$-benzylguanosine, *dBG* $O^6$-benzyl-2′-deoxyguanosine, *CMBG* $O^6$-benzyl-9-cyanomethylguanine, *MGMT* $O^6$-methylguanine-DNA methyltransferase, *NS* not significant

| Agent | Dose[a] (mg/m$^2$) | Median time to 5× treatment volume (days) | Tumor regressions | Mortality | Weight loss (%) | T-C[b] | *P* vs control[c] |
|---|---|---|---|---|---|---|---|
| BGS/BCNU (1 h) | 133/25 | 29.5 | 8/8 | 2/10 | 4.6 | 21.3 | <0.01 |
| dBG/BCNU | 138/25 | 38.6 | 10/10 | 0/10 | 12.1 | 30.4 | <0.01 |
| CMBG/BCNU | 104/25 | 11.1 | 5/9 | 1/10 | 4.2 | 2.9 | NS |
| BGS/BCNU (1 h)[d] | 200/25 | 30.8 | 8/8 | 0/10 | 5.1 | 22.6 | <0.01 |
| BGS/BCNU(2 h) | 200/25 | 47.7 | 8/8 | 2/10 | 7.0 | 39.5 | <0.01 |
| PEG/ethanol | –/– | 8.0 | 0/12 | 0/12 | 0.0 | – | NS |
| PEG/BCNU | –/25 | 8.2 | 0/10 | 0/10 | 0.0 | – | NS |

[a] Drugs were administered i.p. at a volume of 20–25 ml/m$^2$
[b] T-C median time to 5 times treatment volume in treatment group − median time to 5 times treatment volume in control (drug vehicle)
[c] Bonferroni-corrected *P* values for comparisons of median time to 5 times treatment volume of each of the groups compared with the PEG/BCNU control
[d] Interval between treatments with MGMT inhibitor and BCNU, as indicated

with BCNU. This last treatment was associated with one mortality, but low toxicity as manifested by loss of weight immediately following treatment (Table 5). None of the animals treated with CMBG/BCNU or BGS/BCNU survived more than 75 days after the treatment and animals were killed at that point due to large tumor burden. On the other hand two of ten animals treated with dBG/BCNU were free of tumors for that time period and were considered cured.

Since MGMT levels were not fully suppressed 1 h after BGS treatment, BCNU was not expected to be fully effective due to repair of its DNA lesions by unsuppressed MGMT. To test this hypothesis, we increased the time interval between BGS and BCNU treatment from 1 h to 2 h. The dose of BGS was also increased from 133 to 200 mg/m$^2$ in order to ensure maximum possible suppression of MGMT levels. Treatment with 200 mg/m2 BGS followed with 25 mg/m$^2$ BCNU 1 h later delayed Daoy xenograft growth by 22.6 days (Fig. 5; Table 5), but when BCNU was administered 2 h after BGS, the growth delay was significantly increased to 39.5 days. Acute toxicity was seen in two out of ten animals, and there was an average weight loss of 5.8% in the survivors. The results demonstrate that an increase in the dose of BGS had no effect on tumor delay or toxicity when BGS was given 1 h prior to BCNU treatment. However, when BGS was allowed to substantially reduce tumor MGMT by delaying the time of BCNU injection, this drug significantly potentiated the therapeutic effect of BCNU without increasing its toxicity.

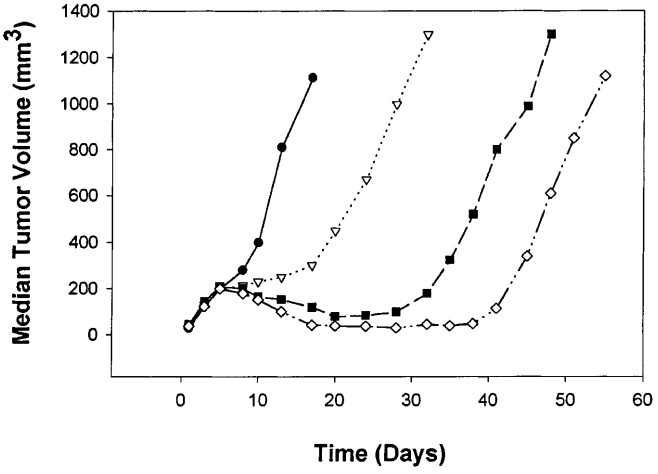


**Fig. 5** Treatment of s.c. Daoy xenografts in athymic mice with 25 mg/m$^2$ BCNU alone (*circles*), or 133 mg/m$^2$ dBG (*diamonds*), 138 mg/m$^2$ BGS (*squares*), or 104 mg/m$^2$ CMBG (*triangles*) followed by 25 mg/m$^2$ BCNU. BCNU was administered 1 h after treatment with the MGMT inhibitor or vehicle 40% PEG in water in a volume of 30 ml/m$^2$ in 5% ethanol. *BCNU* 1,3-bis(2-chloroethyl)-1-nitrosourea, *dBG* $O^6$-benzyl-2′-deoxyguanosine, *BGS* $O^6$-benzylguanosine, *CMBG* $O^6$-benzyl-9-cyanomethylguanine, *MGMT* $O^6$-methylguanine-DNA methyltransferase

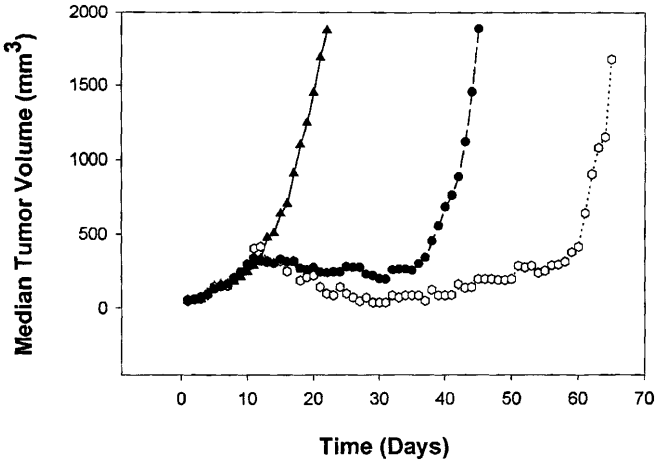


**Fig. 6** Effect of time interval between administration of BGS and BCNU on the growth delay of Daoy xenograft in athymic mice; 25 mg/m$^2$ BCNU was administered i.p. 1 h (*closed circles*) or 2 h (*open circles*) after treatment with 200 mg/m$^2$ BGS. Treatment with 25 mg/m$^2$ BCNU alone is also shown (*triangles*). *BGS* $O^6$-benzylguanosine, *BCNU* 1,3-bis(2-chloroethyl)-1-nitrosourea

## Discussion

Inhibitors of MGMT potentiate the effect of chloroethylating and methylating antitumor drugs that produce $O^6$-substituted guanine adducts. Such potentiation has been shown against a variety of tumors both in culture [11, 12, 13, 14, 15, 16, 17] and in animal models [1, 18, 19] using the prototype MGMT inhibitor BG, which is currently undergoing clinical trials [20, 21]. BG is one of the most potent compounds in suppressing MGMT with an $ED_{50}$ of only 0.2 $\mu M$ [11]. BG and other similar compounds such as those containing electron withdrawing groups at the 8-position or methyl or hydroxyethyl substituted benzyl rings are the most potent inactivators of MGMT, but at the same time they are only marginally soluble in aqueous solvents [22]. Such insolubility could impede adequate systemic distribution to poorly perfused sites, such as tumor cores, from the site of administration of the drug. In addition to insolubility, BG is rapidly cleared from the circulation, primarily by metabolism to yield BOG (oxidation) and guanine (debenzylation), at ratios that are dependent on P-450 and aldehyde-oxidase-catalyzed reactions in the liver [5]. BOG formation could be extensive and since it is as potent as BG in inhibiting MGMT, it could account for prolonged continuous suppression of MGMT following administration of BG even when the parent compound has declined to levels below detection in circulation or tissues [6]. Inactivation of BG involving loss of the benzyl group and of its MGMT inhibitory activity is believed to be a prevailing pathway in the metabolism of BG accounting for as much as 60% of its metabolic clearance in rodents [5]. The insolubility and rapid clearance of BG and BOG have stimulated development of additional inhibitors, with $ED_{50}$s compa-

rable with that of BG, but with greater solubilities in aqueous solvents to facilitate rapid systemic distribution to target sites.

Early tests with dBG, one of the most soluble derivatives of BG, but having about one tenth of its ability to inactivate MGMT in vitro, have shown that this compound is actually more potent than BG in enhancing BCNU efficiency against tumors in the athymic mouse model [1]. This has been explained by its efficient metabolic conversion to BG and BOG [3, 4]. Evidently, systemic administration of more water soluble prodrugs that yield BG either in the tumor or even in the circulation may be a more effective method of sensitizing tumors to chemotherapy than administration of BG itself.

In the case of BGS and dBG, which were considered because of their solubility in aqueous solvents, hepatic metabolism yields BG at systemic concentrations that surpass those of the parent compound after the first 2 h of treatment. Such levels may also be higher than those found in the circulation after injection of an equimolar amount of the BG itself, suggesting that conversion of dBG to BG in the liver results in a better distribution of the latter than if it was administered as a single injection (i.p.). A comparison between dBG and BGS indicates that the two compounds may yield the same levels of BG, although at different rates. BGS is metabolized more slowly than dBG, thus yielding lower concentrations of BG in the circulation, but for a more prolonged period of time. This is also reflected by the markedly slower kinetics of suppression of MGMT in xenografts following BGS administration than after treatment with dBG. The fact that MGMT is suppressed to a very similar extent (less than 10 fmol/mg protein), but at a slower rate with BGS than BdG, indicates that complete ($>98\%$) suppression of MGMT requires adequate systemic levels of BG, and that although such levels are not present one h after treatment with BGS, they can be reached at later times. The dramatic delay in tumor suppression when BGS was given at 2 h instead of 1 h prior to BCNU further supports the idea and demonstrates the importance of having the tumor MGMT fully suppressed when DNA is targeted by the alkylating agent.

The moderate enhancement of BCNU antitumor activity by CMBG, relative to dBG and BGS, is apparently due to insufficient conversion to BG. Although the extent of conversion of CMBG to BG was not determined, it is probably rather low compared with the BG yields obtained from dBG and BGS. The kinetics of disappearance of CMBG from the circulation following i.p. administration indicates that this compound is absorbed rapidly by the gut and is metabolized by pathway(s) that result mainly in loss of activity. This is also supported by its rapid, but incomplete inactivation of MGMT activity in Daoy xenografts. The rather poor performance of CMBG in the athymic mouse model and the observed toxicity does not justify further examination of this compound.

In conclusion, some 9-substituted derivatives of BG enhance the antitumor activity of BCNU if their metabolism yields high levels of BG. The 9-substituted derivatives may inactivate MGMT shortly after their administration, while continuous subsequent formation of BG and BOG ensures that suppression is maintained for extended time periods. Both an effective initial suppression of MGMT and subsequent maintenance of low MGMT activity levels are best achieved with dBG, which is the most effective of all MGMT inhibitors tested so far in potentiating BCNU against a strongly MGMT-positive xenograft (Daoy). Although additional experiments need to be performed in order to fully evaluate the effectiveness of BGS, it seems that this compound may also be of interest, since it is metabolized to BG over a longer time period than dBG. The mechanism of conversion of dBG and BGS to BG is not known. The liver appears to effectively remove the 9-substituent from both BGS and dBG, but the enzyme responsible for this reaction has not been identified. The characterization of this enzymatic activity is of importance for the further development of additional MGMT inhibitors.

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