Thresholds of O^6-Alkylguanine-DNA Alkyltransferase which Confer Significant Resistance of Human Glial Tumor Xenografts to Treatment with 1,3-Bis(2-chloroethyl)-1-nitrosourea or Temozolomide[1]

Demetrius M. Kokkinakis,[2] Dora B. Bocangel, S. Clifford Schold, Robert C. Moschel, and Anthony E. Pegg

Department of Neurosurgery, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas 75235-8855 [D. M. K.]; University of Texas Medical Branch, Sealy Center for Molecular Science, Galveston, Texas 77555 [D. B. B.]; National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, Maryland 21702-1201 [R. C. M.]; Departments of Cellular and Molecular Physiology and Pharmacology, Pennsylvania State University, College of Medicine, The Milton Hershey Medical Center, Hershey, Pennsylvania 17033 [A. E. P.]; and University of Pittsburgh Cancer Institute, Pittsburgh, Pennsylvania 15213 [S. C. S.]

ABSTRACT

Bis-2-chloroethylnitrosourea (BCNU) or temozolomide (TMZ) were tested alone or in combination with the AGT inhibitors O^6-benzyl-2′-deoxyguanosine (dBG) or O^6-benzylguanine (BG) against human glial tumor xenografts growing s.c. in athymic mice. Four glioblastoma (SWB77, SWB40, SWB39, and D-54) and one anaplastic oligodendroglioma (SWB61) xenografts having O^6-alkylguanine-DNA alkyltransferase (AGT) activities of 75, 45, 10, <10, and 16 fmol/mg protein, respectively, were used. BCNU at 35 mg/m^2 was ineffective against these tumors, although 70 mg/m^2 (LD_{10}, 75 mg/m^2) produced a marked tumor growth delay (T-C) in D54 but had no effect against SWB40 or SWB77. Coadministration of BG or dBG and BCNU necessitated reduction of the BCNU dose to a maximum of 30 and 35 mg/m^2, respectively, because of increased toxicity. Optimized treatment with dBG (250 mg/m^2) and BCNU (35 mg/m^2) resulted in T-Cs of 30, 29, 11, 16, and 14 days for SWB77, SWB40, SWB39, D-54 and SWB61, respectively. These delays were more pronounced than those induced with optimized, isotoxic treatments with BG (180 mg/m^2) and BCNU (30 mg/m^2). In comparison to BCNU, TMZ was less toxic, with an LD_{10} of 400 mg/m^2. TMZ (300 mg/m^2) was more effective than BCNU against SWB77, SWB40, and SWB61, inducing T-Cs of 23, 53, and 56 days, respectively. BG and dBG enhanced the toxicity of TMZ in athymic mice by decreasing the LD_{10} from 400 to 200 mg/m^2. TMZ (180 mg/m^2) with either BG (180 mg/m^2) or dBG (250 mg/m^2) resulted in T-Cs of 31 and 49 days in SWB77, respectively, as compared with 16 days for TMZ (180 mg/m^2) alone. In SWB40, the combination of TMZ with dBG, but not with BG, was significantly more effective than the maximum tolerated dose of TMZ (300 mg/m^2) alone. The combination of TMZ with AGT inactivators had no benefit, as compared with TMZ alone, against xenografts with marginal AGT activity. In conclusion, at equimolar doses dBG was less toxic than BG in athymic mice when combined with either BCNU or TMZ. In this regard, BCNU or TMZ can be used at higher doses in combination with dBG than with BG. This study further demonstrates that there is a significant benefit of depleting AGT with nonspecific AGT inhibitors prior to treatment with either BCNU or TMZ in tumors having AGT activity >45 fmol/mg protein.

INTRODUCTION

Chemotherapeutic agents that alkylate the O^6 position of guanine in DNA such as BCNU,[3] 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea, fotemustine, dacarbazine, streptozotocin, procarbazine, and TMZ are used primarily to treat brain cancer, melanoma, lymphoma, and gastrointestinal cancers. The effectiveness of these agents, however, is limited by AGT (1), a protein that repairs O^6-alkylguanine adducts and is up-regulated in several tumors during progression (2–5). Furthermore, selection of resistant AGT phenotypic populations after treatment with alkylating agents seems to be the reason for the recurrence of tumors of even a more resistant phenotype (6). Tumor resistance to DNA alkylation could be theoretically reversed with AGT pseudosubstrates that react with and inactivate the protein. The principle that depletion of AGT enhances the antitumor chemotherapeutic potency of DNA alkylating drugs has been tested and well documented experimentally using several O^6-benzyl (7–9) and 4-bromothenyl (10) derivatives of guanine, and clinical trials are under way to test such compounds against

Received 7/21/00; revised 11/9/00; accepted 11/9/00.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] Supported by National Cancer Institute Grants CA 57725 and CA 78561 and the National Cancer Institute, Department of Health and Human Services, under contract with ABL and The Children's Brain Tumor Foundation of the Southwest.
[2] To whom requests for reprints should be addressed, at Department of Neurological Surgery, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, TX 75235-8855. Phone: (214) 648-6314; Fax: (214) 648-2265; E-mail: dkokki@mednet.swmed.edu.

[3] The abbreviations used are: BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; AGT, O^6-alkylguanine-DNA alkyltransferase; BG, O^6-benzylguanine; dBG, O^6-benzyl-2′-deoxyguanosine; MMR, mismatch repair; TMZ, temozolomide; PEG, polyethyleneglycol.

brain tumors. Despite considerable advances in this field, methodologies to sensitize tumors by depleting AGT and the selection of the appropriate chemotherapeutic agent to be combined with AGT depleting drugs are still under evaluation. BG, the prototype AGT inhibitor, is nonspecific, and it also sensitizes normal tissue to alkylating agents, thus increasing their toxicity, mutagenicity, and carcinogenicity, which are limits to its utility to achieve the desirable therapeutic effect (11–13). To overcome the nonspecificity of BG, strategies for the protection of sensitive normal tissue have been considered. The transduction of AGT mutant forms that are resistant to BG-mediated depletion of AGT in hematopoietic bone marrow cells or other cell populations that might be poisoned by the combination of BG and alkylating agents is an interesting approach to avoid unacceptable myelosuppression in patients (14–18), but complications often associated with gene therapy could be avoided with the discovery of more tumor-selective AGT inactivators. Although progress in this direction has been slow, there are numerous reports indicating that the efficacy of chemotherapy could be improved by either modifying the route of administration of BG (19) or by using alternative AGT inhibitors (20, 21). An AGT inhibitor that has not only resulted in impressive tumor growth delays in combination with BCNU but also in eradicating the AGT-efficient, nitrosourea-resistant Daoy tumor xenografts is the deoxyribonucleoside analogue of BG, dBG (9). The effectiveness of this compound, which may be attributed to its water solubility, its systemic availability and persistence in circulation, along with its metabolic conversion to more potent AGT inhibitors (21), warrants further comparisons of BG with additional 9-substituted, water-soluble BG derivatives.

An additional important issue in combining DNA alkylating agents with AGT inhibitors is whether to include such inhibitors in the treatment of tumors with no or low AGT content, especially because such a combination limits the dose of the alkylating agent. Dose is important for several reasons, including the fact that the alkylating agent itself might quench low levels of AGT. A case in point is TMZ, which at a dose of 100 mg/kg eliminates all of the AGT activity in tumors having moderate AGT levels for a prolonged time period (11). In addition, the inverse correlation between AGT levels and effectiveness of BCNU against central nervous system tumors (22, 23) suggest that there may be no benefit in treating AGT-deficient tumors with AGT inhibitors. Determining the threshold of AGT activity that could be overcome by alkylating agents without the use of AGT inactivators may be complicated by tumor heterogeneity in AGT levels in some human tumors. However, such heterogeneity has not been demonstrated conclusively in gliomas, and therefore, conclusions derived from xenograft experiments may also apply in patients with such malignancies.

In this report, we address the question of whether AGT depletion prior to the administration of BCNU or TMZ enhances the response of typical glial tumors having low (<50 fmol/mg protein) to moderate (75 fmol/mg protein) levels of AGT activity. It is expected that AGT inhibitors will not enhance the efficacy of TMZ against tumors with modest AGT levels when this agent is administered at its maximum effective dose. On the other hand, the markedly higher toxicity of BCNU as compared with TMZ is expected to render the combination of AGT inhibitors and BCNU more effective than BCNU itself in the treatment of tumors having modest levels of AGT activity, such as those usually found in the central nervous system.

MATERIALS AND METHODS

Chemicals. BG and dBG were synthesized and purified according to methods published previously (24, 25). BCNU was purchased from Bristol-Myers Squibb (Princeton, NJ), and TMZ was donated by Schering-Plough, Inc. (Madison, NJ).

Animals. BALB/c *nu/nu* athymic mice, 4 weeks of age, were purchased from Harlan Labs (San Diego, CA). Mice were maintained under barrier conditions and given sterilized food (Harlan Teklad laboratory diet) and water.

Tumor Lines. The tumor lines D-54, SWB39, SWB40, SWB77 (human glioblastomas), and SWB61 (human anaplastic oligodendroglioma) were chosen for their lack of genomic instability and effective MMR repair.[4] They were grown in 5% fetal bovine serum (Life Technologies, Inc., Gaithersburg, MD) in Eagle's MEM (Life Technologies, Inc.) supplemented with lysine, valine, methionine, and leucine (100 μM each), nonessential amino acids (1:100 dilution of stock from Life Technologies, Inc.), 1 mM sodium pyruvate, 1 μM α- hydroxocobalamin, 10 μM folic acid, and 0.2 mg/ml gentamicin. s.c. tumors grew upon injection of 3–4 million cells/animal. Tumor xenografts D-54, SWB39, SWB40, SWB77, and SWB61 had AGT activities of <10, 10, 45, 75, and 16 fmol/mg protein, respectively, as determined by the biochemical assay (26). Respective mitotic indices in xenografts were 5.5, 8.2, 12.1, 5.7, and 12.9 m/high-powered field. Extensive necrosis was observed in growing xenografts SWB40 and SWB61, which is in agreement with their rapid growth patterns.

Drug Treatment. All treatments were administered i.p. BG and dBG 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 from a stock solution of 20 mg/ml of anhydrous ethanol. Depending on the dose of BCNU, the injected ethanol carrier varied from 10 to 15% ethanol. TMZ was dissolved in 100% DMSO. AGT inhibitors were administered at volumes of 30 ml/m^2 and alkylating drugs at 20 ml/m^2. Drug doses were calculated as mg/m^2 using the formula: meters (m) = weight (g)$^{2/3} \times K \times 10^{-4}$, where K is 10.5 for mice (27). In animals of 20 ± 2 g used in this study, the weight (kg) of the animal is ~2.6 times the area surface (m^2).

Tumor Implantation and Treatment. Two hundred μl containing 3×10^6 cells of D-54, SWB39, SWB40, SWB77, and SWB61 tumor cells in 5% serum media were injected at the left flank of athymic mice, 6 weeks of age, weighing between 18 and 22 g. 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 α is the shorter and β the longer of the two dimensions. Treatment was administered to animals with tumors ranging between 150 and

[4] D. B. Bocangel, S. Mitra, and D. M. Kokkinakis, unpublished observations.

Table 1 Toxicity of carmustine (BCNU) and TMZ: Effect of BG and dBG on potentiating the toxicity of BCNU and TMZ in athymic mice

Alkylating drug[a]	Dose[b] mg/m^2	AGT inhibitor[a]	Dose[b] mg/m^2	Weight loss % of control	Deaths[c]
BCNU	65	none		05 ± 2[d]	0/10[e]
BCNU	70	none		10 ± 3	0/10
BCNU	75	none		15 ± 3	3/10
BCNU	85	none		24 ± 4	6/10
BCNU	35	BG	200	22 ± 6	5/10
BCNU	35	BG	180	23 ± 3	4/10
BCNU	30	BG	200	14 ± 4	1/10
BCNU	30	BG	180	10 ± 2	0/10
BCNU	35	dBG	300	21 ± 4	7/10
BCNU	35	dBG	250	12 ± 3	0/10
BCNU	35	dBG	200	10 ± 2	0/10
TMZ	600	none		21 ± 4	5/10
TMZ	400	none		16 ± 4	1/10
TMZ	200	none		05 ± 2	0/10
TMZ	400	dBG	250	15 ± 3	4/10
TMZ	200	dBG	250	12 ± 3	1/10
TMZ	180	dBG	250	03 ± 1	0/10
TMZ	180	BG	180	03 ± 2	0/10
TMZ	200	BG	180	06 ± 3	2/10

[a] BCNU stock 10 mg/ml in 100% ethanol was diluted to appropriate concentrations with 5% ethanol. TMZ was dissolved in DMSO.

[b] dBG and BG were dissolved in 40% PEG in PBS. BCNU and TMZ were injected i.p. (injection volume, 0.15–0.2 ml/animal) 2 h after treatment with i.p. dBG, BG, or vehicle (injection volume, 0.2–0.3 ml/animal).

[c] Number of deaths per number of animals treated.

[d] Mean ± SD.

[e] Animals were monitored for 20 days from initiation of treatment.

200 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 individual animals 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. The number of tumor regressions (number of tumors measuring smaller than the volume on the treatment day) occurring in each group was also determined. Groups were compared with the two-tailed Fisher's exact test. Two control groups received 40% PEG:60% PBS, followed by 10% ethanol:water in 2 h or 40% PEG:60% PBS and BCNU in 10% ethanol. Unless otherwise indicated, 10–12 animals were used in each experimental group.

RESULTS

Toxicity of BCNU and Temozolomide. The toxicity of BCNU, TMZ, and their combinations with BG and dBG were determined in the Balb/c athymic mouse (Table 1). The LD_{10} for BCNU is between 70 and 75 mg/m^2 when the drug was delivered i.p. Treatments with doses of 70 mg/m^2 induced a temporary loss of weight of ~10% and no deaths. All deaths at higher doses occurred within 72 h from treatment. The AGT inhibitors BG and dBG sensitized animals to BCNU, and their use necessitated a decrease of the dose of the chemotherapeutic drug to 30 and 35 mg/m^2, respectively. The combination of dBG and BCNU at 250 and 35 mg/m^2, respectively, was the maximum tolerated exposure to this drug combination, inducing a 12% weight loss but no deaths. Increasing the dBG dose to 300 mg/m^2 depleted the intestinal AGT in mice and sensitized the animals to the toxicity of BCNU, even at doses <35 mg/m^2. At 180 mg/m^2 BG, a dose that is equimolar to 250 mg/m^2 dBG, 35 mg/m^2 BCNU induced a 40% incidence of death and a weight loss 2-fold greater than that induced by an equimolar doses of dBG and 35 mg/m^2 BCNU. Animal deaths were prevented by reducing the dose of BCNU to 30 mg/m^2. Therefore, administration of equimolar doses of BG and dBG required treatment with 30 and 35 mg/m^2 BCNU, respectively, to achieve the same extent of weight loss and no deaths (equitoxic treatments). Comparisons between BG and dBG at equimolar doses with slight variation in the BCNU dose to achieve comparable toxicity is justified because such treatments achieve the best possible tumor response for the combination of these two AGT inactivators with BCNU without causing any animal deaths.

TMZ was tolerated well by athymic mice at a single dose of 400 mg/m^2 with only 1 of 10 animals dying 5 days after treatment. At 600 mg/m^2, 5 of 10 animals died acutely (in <24 h), whereas an additional 2 died within 27 days from treatment. No deaths were seen in animals treated with doses <400 mg/m^2, and toxicity was manifested only as mild weight loss. Acute toxicities of TMZ and BCNU, based on weight loss, were comparable at maximum tolerated doses of 300 and 70 mg/m^2, respectively. As it was the case with BCNU, the toxicity of TMZ was also potentiated by administration of AGT inhibitors 2 h prior to treatment. Equimolar doses of BG or dBG in 40% PEG of 180 and 250 mg/m^2, respectively, caused acute animal deaths when combined with TMZ at doses >200 mg/m^2. Sporadic deaths observed between 5 and 12 days after treatment with dBG (250 mg/m^2) or BG (180 mg/m^2) and TMZ at 200 mg/m^2 necessitated further reduction of the TMZ dose to 180 mg/m^2.

Treatment Efficacy of BCNU against Glial Tumors. The efficacy of BCNU against glial tumors was tested using the D-54, SWB39, SWB61, SWB40, and SWB77 xenografts growing s.c. in nude mice. The median time and range for these tumors to quintuple in size was 8 (7–11), 11 (9–12), 12 (7–16), 11 (9–15), and 11 (9–13) days, respectively. The effect of BCNU at doses ranging between 35 and 75 mg/m^2 on growth delay of these xenografts and the toxicity of BCNU at these doses are shown on Table 2. A dose of 35 mg/m^2 BCNU was ineffective against all tumor xenografts tested, including those with negligible levels of AGT activity (D- 54, SWB61, and SWB39). However, SWB61 SWB39, and D-54 tumor xenografts, with AGT activity <20 fmol/mg protein, responded to a single BCNU treatment at doses of 65–70 mg/m^2, whereas those with higher AGT activities did not respond (SWB77) or responded weakly (SWB40). Further escalation of the dose induced animal deaths, as expected from the toxicity data without further delaying tumor growth in the survivors.

Treatment Efficacy of BCNU and dBG. Table 3 shows the effect of depletion of AGT prior to BCNU treatment. Depletion of AGT was accomplished with dBG, which has been shown previously to suppress AGT activity to <2% of its baseline for a period lasting for at least 16 h after its administration (21). BCNU was administered at 2 h after 250 mg/m^2 dBG, which is approximately the molar equivalent to a dose of 180 mg/m^2 BG. Escalation of the dBG dose above this level did

Table 2 Response of human glial tumor xenografts in athymic mice to a single i.p. treatment with BCNU: Effect of dose

Tumor	BCNU[a] mg/m^2	No. of animals	No. of deaths	Weight loss Mean %	No. of regressions	Median to 5× treatment volume	T-C[b] Days
D-54	35	6	0	2	0	10	2
SWB61	35	6	0	1	0	12	0
SWB39	35	6	0	0	0	10	−1
SWB40	35	6	0	1	0	11	0
SWB77	35	6	0	0	0	10	−1
D-54	65						
SWB61	65	10	0	3	6	21	9[c]
SWB39	65	10	0	3	6	22	11[c]
SWB40	65	13	0	5	3	16	4[c]
SWB77	65	10	0	3	3	12	1
D-54	70	12	1	10	6	26	18[c]
SWB61	70	6	0	8	3	20	8[c]
SWB39	70	6	0	9	3	21	10[c]
SWB40	70	6	0	9	2	19	8[c]
SWB77	70	6	0	9	0	14	3
D-54	75	12	4	23	4	27	19[c]

[a] BCNU stock 10 mg/ml in 100% ethanol was diluted to appropriate concentrations with 5% ethanol. BCNU was injected i.p. (injection volume, 0.15–0.2 ml/animal).
[b] Tumor growth delay as compared with untreated controls. Animals were killed when tumor exceeded 1200 mm^3.
[c] Statistically significant ($P < 0.05$) as compared with untreated controls or to those treated with 35 mg/m^2 BCNU.

Table 3 Enhancement of the efficacy of BCNU with dBG against human glial tumor xenografts in athymic mice

Tumor	BCNU[a] mg/m^2	dBG mg/m^2	No. of animal	No. of deaths	Weight loss Mean %	No. of regressions	Median to 5× treatment volume	T-C[b] Days
D-54	35	300	12	7	29	3	17	9
	35	250	12	0	12	9	24	16
	30	250	12	0	10	6	21	13
SWB61	35	250	12	0	10	8	26	14
	35	200	12	0	7	7	25	13
SWB39	35	250	12	0	9	7	22	11
SWB40	35	250	13	0	10	11	40	29
SWB77	35	250	12	0	9	12	49	38
SWB77	35	250	14	0	12	14	41	30

[a] BCNU stock 10 mg/ml in 100% ethanol was diluted to appropriate concentrations with 5% ethanol. dBG was dissolved in 40% PEG in PBS. BCNU was injected i.p. (injection volume, 0.15–0.2 ml/animal) 2 h after treatment with i.p. dBG (injection volume, 0.2–0.3 ml/animal).
[b] T-C tumor growth delay. All values were statistically significant as compared with untreated controls.

not cause toxicity by itself but resulted in unacceptable toxicity by BCNU with no increase in tumor growth delay. The increased toxicity of BCNU with higher doses of dBG is likely attributable to depletion of intestinal AGT (9) and the exposing of the brush epithelium to cross-linking DNA damage. The toxicity of the optimal dBG/BCNU combination, as determined from acute loss of weight, was similar to that achieved for the maximum tolerated dose of BCNU alone. However, the tumor growth delay was markedly enhanced in glioblastomas expressing AGT activity at levels >45 fmol/mg protein. Although the increase in tumor growth delay induced by dBG (250 mg/m^2) and BCNU (35 mg/m^2) observed in tumors with low AGT activity (<20 fmol/mg protein) was not statistically significant as compared with an equitoxic dose of BCNU alone (70 mg/m^2), there was a definite benefit ($P < 0.025$) as compared with 35 mg/m^2 BCNU alone (Tables 2 and 3).

Comparison between BG and dBG in Enhancing the Efficacy of BCNU. A direct comparison between BG and dBG in enhancing the efficacy of BCNU against the AGT-efficient SWB77 tumor xenografts is shown in Fig. 1. Treatments with either dBG-BCNU (at 250 and 35 mg/m^2) or BG-BCNU (at 180 and 30 mg/m^2) were isotoxic to a treatment with 70 mg/m^2 BCNU alone (Tables 2 and 4). Tumor delays with either drug combination were markedly greater than those observed when the same tumors were treated with the full dose (70 mg/m^2 of BCNU alone, but dBG was more effective than BG ($P < 0.001$) in enhancing the efficacy of BCNU. A statistically significant difference ($P < 0.001$) between BG and dBG was also determined when these two compounds were used in combination with BCNU against SWB40 tumor xenografts (Table 4). It was noted that dBG-BCNU caused similar delays in SWB77 and SWB40, but BG increased the efficacy of BCNU, more notably in SWB40 than in SWB77. There was a small but statistically significant ($P < 0.05$) advantage in using the reduced dose of BCNU and AGT inhibitors rather than the maximum tolerated dose of BCNU without AGT inhibition (70 mg/m^2) against the SWB61 tumor xenografts, which have marginal AGT activity, but not against D-54, which has negligible AGT levels.

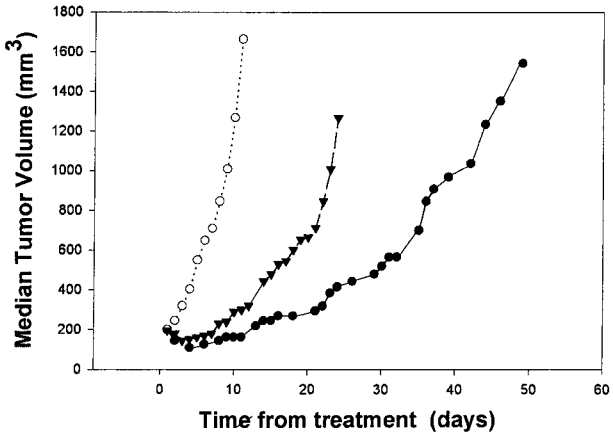

Fig. 1 Treatment of s.c. SWB77 human glioblastoma xenografts in athymic mice with BG or dBG and BCNU. The treatments were administered i.p. when tumors had reached volumes of 150–200 mm^3. ○, vehicle plus BCNU 35 mg/m^2 at 2-h intervals; ▼, BG 180 mg/m^2 plus 30 mg/m^2 BCNU; ●, dBG 250 mg/m^2 plus 35 mg/m^2 BCNU. Vehicle for dBG and BG was 40% PEG 400 in PBS. BCNU was administered as a solution in 10% ethanol.

Treatment Efficacy of TMZ. Three tumor xenografts, SWB61, SWB40 and SWB77, were treated with TMZ at single i.p. doses of 300 mg/m^2 and at 200 mg/m^2 (Table 5). Although none of the implanted tumors were eradicated by TMZ treatment alone, the growth of SWB61 and SWB40 was retarded for >50 days. TMZ induced a moderate response in SWB77, where tumor growth delay was ~20 days. There were no statistical differences in the tumor response between the doses of 200 and 300 mg/m^2 TMZ in any of the tumors treated, despite evident greater toxicity of the latter dose. Further escalation of the dose to 600 mg/m^2 against SWB61 did not delay growth but had a rather profound effect in toxicity. It was concluded that the maximum therapeutic effect of TMZ (single-dose i.p.) was achieved at 200–300 mg/m^2.

Treatment Efficacy of TMZ and dBG or BG. AGT inhibitors enhanced the efficacy of TMZ alone against the AGT-positive SWB77 xenografts compared with the efficacy of TMZ alone at its maximum tolerated dose of 300 mg/m^2. At equimolar doses, BG was less effective than dBG in enhancing TMZ antitumor activity against SWB77, although the two treatments produced equal toxicity in the animals (Table 6). A significant advantage in using lower levels of TMZ in combination with BG or dBG rather than greater doses of TMZ alone was also seen in SWB40 tumor xenografts. However, with SWB40, the difference between BG and dBG in enhancing the efficacy of TMZ (Fig. 2; Table 6) was not statistically significant. There was no enhancement of TMZ efficacy against SWB61 by BG or dBG (data not shown).

DISCUSSION

The goals of this study were to test the importance of low to modest (10–75 fmol/mg protein) levels of AGT activity in the resistance of gliomas to both methylating and chloroethylating agents and to determine the threshold of AGT activity required to completely block the chemotherapeutic effectiveness of BCNU and of TMZ. Furthermore, this study aimed to determine the benefit, if any, of using AGT inactivators in combination with either TMZ or BCNU against brain neoplasms with low or nondetectable AGT activity and whether the selectivity of the combination of AGT inhibitors and alkylating drugs against tumors outweighs the imposed toxicity of such combinations on normal tissue in the mouse model.

DNA repair of the damage induced by alkylating agents occurs with the aid of several systems (28), such as direct reversal of O^6-alkylguanine, nucleotide excision repair, MMR, base excision repair, error-free and error-prone tolerance during DNA replication, and recombinational repair. Although BCNU is toxic via the formation of cross-links, DNA excision repair seems to be of minor importance as compared with O^6-alkylguanine reversal (29). However, BCNU ultimately bridges the N^1 of G and N^3 of C residues via the intermediate formation of O^6-N^1 cycloethyl derivative of guanine yielding cross-links that are not subject to repair by either excision or by AGT (30). Inhibition of the O^6-2-chloroethyl reversal for a period of at least 18 h, which is required for the cross-links to be formed, is therefore necessary to preserve the cytotoxic effect of BCNU. TMZ, on the other hand, exerts its toxicity by invoking the response of the MMR system via the recognition of O^6-methylguanine containing odd bp by the MSH2, MLH1, and PMS2 complexed to MSH3 or MSH6. As a result of the different mechanisms by which BCNU and TMZ exert their toxicities, an inverse correlation between BCNU toxicity and AGT levels is expected with all tumors, whereas such a correlation is expected with TMZ only with tumors that have at least a functional MMR and a rapid rate of turnover. The tumors used in this communication express similar levels of MLH1, MSH2, and PMS2, which migrate to the nucleus after exposure to TMZ and show no apparent instability after TMZ treatment, as determined by microsatellite assays.[4] The above indicate the presence of functional MMR systems (31). Therefore, the resistance of SWB77 and to a lesser extent that of SWB40 to TMZ was attributable, at least partially, to AGT. The poorer response of SWB77 to TMZ, as compared with that of SWB40 and SWB61, even after the depletion of the AGT activity, indicates that SWB77 may resist alkylating damage by mechanisms not yet identified. Different responses of SWB77 on one hand and SWB40 or SWB61 on the other, in cell cycle responses to DNA damage that could determine the efficiency of death via the action of MMR-related mechanisms, could also account for the resistance of SWB77 to TMZ (32).

Gliomas with low levels of AGT (<20 fmol/mg protein), such as SWB61 and SWB39, were minimally responsive to BCNU, and their resistance to this agent was not improved by pretreatment with AGT inhibitors, indicating the presence of yet unknown mechanisms of resistance. In this regard, SWB39 and SWB61 differ from SWB40 and SWB77, which are resistant to BCNU mainly because of their moderate levels of AGT. SWB39, SWB61, D-54, and even the AGT-effective tumor SWB40 were responsive to TMZ alone in the absence of AGT inhibition, which indicates that AGT activity <45 fmol/mg protein did not significantly contribute to the resistance of these tumors to TMZ. Furthermore, the high efficacy of TMZ against SWB61 suggests that mechanisms of resistance other than AGT, which contribute to the weak response of these tumors to

Table 4 Comparison of the enhancement of efficacy of BCNU by BG and dBG against SWB77, SWB40, SWB61, and D-54 human glial tumor xenografts in athymic mice

Tumor	BCNU[a] mg/m^2	dBG or BG mg/m^2	No. of animals	No. of deaths	Weight loss Mean %	No. of regressions	Median to 5× treatment volume	T-C[b] Days
SWB77	35	250 (dBG)	14	0	12	14	41	30
	30	180 (BG)	12	0	11	8	22	11
SWB40	35	250 (dBG)	13	0	10	11	40	29
	30	180 (BG)	12	0	12	11	31	20
SWB61	35	250 (dBG)	12	0	10	9	26	14
	30	180 (BG)	12	0	11	10	24	12
D-54	35	250 (dBG)	12	0	12	9	24	16
	30	180 (BG)	12	0	10	3	18	10

[a] BCNU stock 10 mg/ml in 100% ethanol was diluted to appropriate concentrations with 5% ethanol. dBG was dissolved in 40% PEG in PBS. BCNU was injected i.p. (injection volume, 0.15–0.2 ml/animal) 2 h after treatment with i.p. dBG (injection volume, 0.2–0.3 ml/animal).
[b] T-C tumor growth delay as compared with untreated controls. All values were statistically significant as compared with untreated controls.

Table 5 Response of human glial tumor xenografts in athymic mice to a single i.p. treatment with TMZ

Tumor	TMZ[a] mg/m^2	No. of animals	No. of deaths	Weight loss Mean %	No. of regressions	Median to 5× treatment volume	T-C[b] Days
SWB77	300	10	0	9	10	34	23
SWB77	200	10	0	6	10	30	19
SWB40	300	10	0	10	10	66	53
SWB40	200	10	0	5	10	59	46
SWB61	600	12	6	10	10	61	54
SWB61	300	10	0	10	9	63	56
SWB61	200	10	0	6	10	61	54

[a] TMZ was dissolved in DMSO and injected i.p. (injection volume, 0.15–0.2 ml/animal).
[b] T-C tumor growth delay as compared with untreated controls. All values were statistically significant as compared with untreated controls.

Table 6 Response of human glial tumor xenografts in athymic mice to a single treatment of BG or dBG, followed by a single i.p. treatment with TMZ

Tumor	TMZ[a] mg/m^2	AGT inhibitor mg/m^2	No. of animals	No. of deaths	Weight loss Mean %	No. of regressions	Median to 5× treatment volume	T-C[b] Days
SWB77	180	180 (BG)	12	0	9	12	43	31
SWB77	180	250 (dBG)	12	0	10	12	61	49
SWB77	200		12	0	6	10	28	16
SWB40	180	180 (BG)	12	0	10	12	68	56
SWB40	180	250 (dBG)	12	0	11	12	75	63
SWB40	200		12	0	4	12	53	41
SWB40	300		12	0	6	12	67	55

[a] TMZ was dissolved in DMSO. dBG and BG were dissolved in 40% PEG in PBS. TMZ was injected i.p. (injection volume, 0.15–0.2 ml/animal) 2 h after treatment with i.p. dBG (injection volume, 0.2–0.3 ml/animal).
[b] T-C tumor growth delay as compared with untreated controls. All values were statistically significant as compared with untreated controls.

BCNU, can be overcome by TMZ. These data indicate that the threshold of AGT activity required for resistance to TMZ is markedly higher than that determined for BCNU. These experiments also demonstrate that the maximum benefit of the combination of AGT inhibitors and BCNU requires fine adjustments of the doses of these two agents.

In mice, the doses of BG and dBG required for maximum efficacy were 180 and 250 mg/m^2, respectively. Doses of BG and dBG can be lowered to 150 and 200 mg/m^2 with only a mild decline in the enhancement of antitumor efficacy of the alkylating agents. Doses of 300 dBG induced unacceptable depletion of AGT from normal mouse tissues (21) without improving the suppression of AGT from tumors, and therefore, such doses rendered these tissues extremely sensitive to the alkylating drug, causing severe weight loss and deaths to the treated animals. Overdosing with AGT inhibitors did not improve the tumor response in the survivors, indicating that BG and dBG cause maximal AGT depletion in the tumor when administered at 150–180 and 200–250 mg/m^2, respectively.

One of the important findings of this study is that the potential of other AGT inhibitors to enhance the efficacy of chemotherapy with DNA alkylating drugs and theoretically to surpass BG, the prototype AGT inhibitor currently undergoing clinical trials. It has been shown previously that dBG, which has a small but significant advantage to suppress tumor AGT as compared with O^6-benzyl-9-cyanomethylguanine and O^6-benzylguanosine, is also more effective in enhancing the efficacy of BCNU against human medulloblastoma (Daoy) tumors

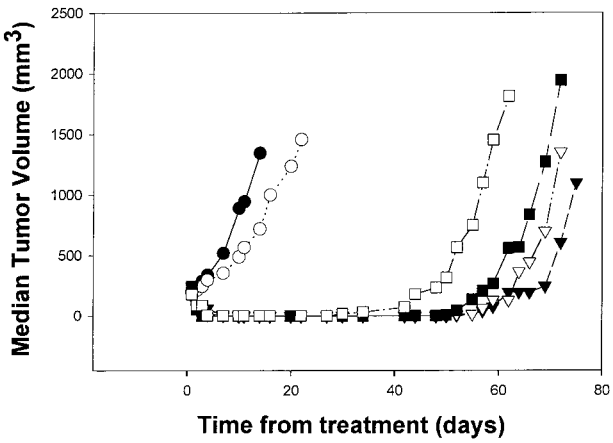

Fig. 2 Treatment of s.c. SWB40 human glioblastoma xenografts in athymic mice with BG or dBG and TMZ. The treatments were administered i.p. when tumors had reached volumes of 150–200 mm^3. ●, 40% PEG in PBS followed with 20 ml/m^2 DMSO 2 h later; ○, dBG 250 mg/m^2 followed by 20 ml/m^2 DMSO 2 h later; □, 40% PEG 400 in PBS vehicle followed in 2 h with 200 mg/m^2 temozolomide in DMSO; ■, 40% PEG 400 in PBS vehicle followed in 2 h with 300 mg/m^2 temozolomide in DMSO; ▽ BG 180 mg/m^2 plus 200 mg/m^2 temozolomide; ▼, dBG 250 mg/m^2 plus 200 mg/m^2 TMZ in DMSO. Vehicle for dBG and BG was 40% PEG 400 in PBS.

(21). Comparisons between these 9-substituted derivatives of BG have shown that small variations in both the extent and also the duration of suppression could translate into marked differences in the response of tumor to BCNU chemotherapy. Careful screening of additional compounds should therefore allow the discovery of AGT inactivators that are more effective than those presently at hand. In this report, we demonstrate that dBG is significantly more effective than BG in enhancing the efficacy of BCNU against two AGT-efficient glioblastomas. This could be attributed to the more prolonged exposure of tumors to AGT inhibitors derived from dBG than from BG metabolism (21), causing a more protracted AGT suppression. Comparison of BG and 8-oxo-BG levels in plasma and cerebrospinal fluid of primates given systemic BG and dBG, respectively, may suggest a greater potency of BG over dBG (33). However, the comparisons were made at equal doses and not at equimolar or equitoxic doses that would have allowed direct comparisons between that report and the data presented here. Our results with TMZ contrast with those reported earlier showing marginal benefit on TMZ efficacy by BG in single-administration regimens (7, 34). This is probably because of the optimization of the chemotherapeutic regimens and fine adjustment of the TMZ and BG or dBG doses in this study.

Overall, we have shown that for glial tumors with AGT activity <45 fmol/mg protein, there is no significant benefit in using an AGT inhibitor and a reduced dose of BCNU as compared with an isotoxic full dose of BCNU. However, tumors with AGT of 45 fmol/mg protein or greater activity are unresponsive to BCNU alone and require pretreatment with an AGT inhibitor to respond to BCNU treatment. It is interesting that tumors, which are resistant to BCNU because of their AGT content, are more responsive to treatment combining BCNU and AGT inhibitors than tumors with low AGT activity and responsive to BCNU alone. This indicates that the latter may rely on mechanisms of resistance other than the AGT. Unlike BCNU, the performance of TMZ is not as dependent on tumor AGT at the AGT levels tested. Consistent improvement of tumor delay because of AGT inhibition prior to TMZ treatment is observed with SWB77. The similar efficacy of TMZ against SWB61, SWB39, and SWB40 suggests that the (different) mechanisms of resistance these tumors may relay on are easily overcome by a single dose of TMZ. On the other hand, the failure to obtain the same response with SWB77 as with SWB61 and SWB40, even under AGT inhibition conditions, indicates that the efficacy of TMZ may also be limited by yet uncharacterized mechanisms of resistance in some tumors. The tumors tested here are neither rich in AGT nor have MMR defects, which may confer resistance to TMZ.

ACKNOWLEDGMENTS

We acknowledge Jacquelynn Wick for assistance in the completion of xenograft experiments.

REFERENCES

1. Dolan, M. E., and Pegg, A. E. O^6-Benzylguanine and its role in chemotherapy. Clin. Cancer Res., *3:* 837–847, 1997.

2. Ishibashi, T., Nakabeppu, Y., Kawate, H., Sakumi, K., Hayakawa, H., and Sekiguchi, M. Intracellular localization and function of DNA repair methyltransferase in human cells. Mutat. Res., *315:* 199–212, 1994.

3. Ishibashi, T., Nakabeppu, H., and Sekiguchi, M. Artificial control of nuclear translocation of DNA repair methyltransferase. J. Biol. Chem., *269:* 7645–7650, 1994.

4. Citron, M., Schoenhaus, M., Rothenberg, H., Kostroff, K., Wasserman, P., Kahn, L., White, A., Burns, G., Held, D., and Yarosh, D. O^6-Methylguanine-DNA methyltransferase in normal and malignant tissue of the breast. Cancer Investig., *12:* 605–610, 1994.

5. Kokkinakis, D. M., Ahmed, M., Delgado, R., Fruitwala, M., Mohiuddin, M., and Albores-Saavedra, J. Role of O^6-methylguanine-DNA methyltransferase in the resistance of pancreatic tumors to alkylating agents. Cancer Res., *57:* 5360–5368, 1997.

6. Lage, H., and Dietel, M. Involvement of the DNA mismatch repair system in antineoplastic drug resistance. J. Cancer Res. Clin. Oncol., *125:* 156–165, 1999.

7. Wedge, S. R., Porteous, J. K., and Newlands, E. S. Effect of single and multiple administration of an O^6-benzylguanine/temozolomide combination: an evaluation in a human melanoma xenograft model. Cancer Chemother. Pharmacol., *40:* 266–272, 1997.

8. Phillips, W. P., Jr., Willson, J. K., Markowitz, S. D., Zborowska, E., Zaidi, N. H., Liu, L., Gordon, N. H., and Gerson, S. L. O^6-Methylguanine-DNA methyltransferase (MGMT) transfectants of a 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU)-sensitive colon cancer cell line selectively repopulate heterogeneous MGMT+/MGMT− xenografts after BCNU and O^6-benzylguanine plus BCNU. Cancer Res., *57:* 4817–4823, 1997.

9. Kokkinakis, D. M., Moschel, R. C., Pegg, A. E., and Schold, S. C. Eradication of human medulloblastoma tumor xenografts with a combination of O^6-benzyl-2′-deoxyguanosine and 1,3-bis(2-chloroethyl)-1-nitrosourea. Clin. Cancer Res., *5:* 3676–3681, 1999.

10. Middleton, M. R., Kelly, J., Thatcher, N., Donnelly, D. J., McElhinney, R. S., McMurry, T. B., McCormick, J. E., and Margison, G. P. O^6-(4-Bromothenyl)guanine improves the therapeutic index of temozolomide against A375M melanoma xenografts. Int. J. Cancer, *85:* 248–252, 2000.

11. Chinnasamy, N., Rafferty, J. A., Hickson, I., Ashby, J., Tinwell, H., Margison, G. P., Dexter, T. M., and Fairbairn, L. J. O^6-benzylguanine potentiates the *in vivo* toxicity and clastogenicity of temozolomide and BCNU in mouse bone marrow. Blood, *89:* 1566–1573, 1997.

12. Wali, R. K., Skarosi, S., Hart, J., Zhang, Y., Dolan, M. E., Moschel, R. C., Nguyen, L., Mustafi, R., Brasitus, T. A., and Bissonnette, M. Inhibition of O^6-methylguanine-DNA methyltransferase increases azoxymethane-induced colonic tumors in rats. Carcinogenesis (Lond.), *20:* 2355–2360, 1999.

13. Kurpad, S. N., Dolan, M. E., McLendon, R. E., Archer, G. E., Moschel, R. C., Pegg, A. E., Bigner, D. D., and Friedman, H. S. Intraarterial O^6-benzylguanine enables the specific therapy of nitrosourea-resistant intracranial human glioma xenografts in athymic rats with 1,3-bis(2-chloroethyl)-1-nitrosourea. Cancer Chemother. Pharmacol., *39:* 307–316, 1997.

14. Kleibl, K., and Margison, G. P. Increasing DNA repair capacity in bone marrow by gene transfer as a prospective tool in cancer therapy. Neoplasma, *45:* 181–186, 1998.

15. Loktionova, N. A., Xu-Welliver, M., Crone, T. M., Kanugula, S., and Pegg, A. E. Protection of CHO cells by mutant forms of O^6-alkylguanine-DNA alkyltransferase from killing by 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU) plus O^6-benzylguanine or O^6-benzyl-8-oxoguanine. Biochem. Pharmacol., *58:* 237–244, 1999.

16. Marathi, U. K., Harris, L. C., Venable, C. C., and Brent, T. P. Retroviral transfer of a bacterial alkyltransferase gene (*ada*) into human bone marrow cells protects against O^6-benzylguanine plus 1,3-bis(2-chloroethyl)-1-nitrosourea cytotoxicity. Clin. Cancer Res., *3:* 301–307, 1997.

17. Reese, J. S., Davis, B. M., Liu, L., and Gerson, S. L. Simultaneous protection of G156A methylguanine DNA methyltransferase gene-transduced hematopoietic progenitors and sensitization of tumor cells using O^6-benzylguanine and temozolomide. Clin. Cancer Res., *5:* 163–169, 1999.

18. Chinnasamy, N., Rafferty, J. A., Hickson, I., Lashford, L. S., Longhurst, S. J., Thatcher, N., Margison, G. P., Dexter, T. M., and Fairbairn, L. J. Chemoprotective gene transfer II: multilineage *in vivo* protection of haemopoiesis against the effects of an antitumour agent by expression of a mutant human O^6-alkylguanine-DNA alkyltransferase. Gene Ther., *5:* 842–847, 1998.

19. Kreklau, E. L., Kurpad, C., Williams, D. A., and Erickson, L. C. Prolonged inhibition of O^6-methylguanine DNA methyltransferase in human tumor cells by O^6-benzylguanine *in vitro* and *in vivo*. J. Pharmacol. Exp. Ther., *291:* 1269–1275, 1999.

20. Dolan, M. E., Roy, S. K., Garbiras, B. J., Helft, P., Paras, P., Chae, M. Y., Moschel, R. C., and Pegg, A. E. O^6-Alkylguanine-DNA alkyltransferase inactivation by ester prodrugs of O^6-benzylguanine derivatives and their rate of hydrolysis by cellular esterases. Biochem. Pharmacol., *55:* 1701–1709, 1998.

21. Kokkinakis, D. M., Moschel, R. C., Pegg, A. E., and Schold, S. C. Potentiation of BCNU antitumor efficacy by 9-substituted O^6-benzylguanines. Effect of metabolism. Cancer Chemother. Pharmacol., *45:* 69–77, 2000.

22. Belanisch, M., Pastor, M., Randall, T., Guerra, D., Kibitel, J., Alas, L., Li, B., Citron, M., Wasserman, P., White, A., Eyre, H., Jaeckle, K., Schulman, S., Rector, P., Prados, M., Coons, S., Shapiro, W., and Yarosh, D. Retrospective study of the correlation between DNA repair protein alkyltransferase and survival of brain tumor patients treated with carmustine. Cancer Res., *56:* 783–788, 1996.

23. Jaeckle, K. A., Eyre, H. J., Townsend, J. J., Schulman, S., Knudson, H. M., Belanich, M., Yarosh, D. B., Bearman, S. I., Giroux, D. J., and Schold, S. C. Correlation of tumor O^6-methylguanine-DNA methyltransferase levels with survival of malignant astrocytoma patients treated with bis-chloroethylnitrosourea: a Southwest Oncology Group study. J. Clin. Oncol., *16:* 3310–3315, 1998.

24. Robins, M. J., Khwaja, T. A., and Robins, R. K. Purine nucleosides: XXIX. The synthesis of 2′-deoxy-L-adenosine and 2′-deoxy-L-guanosine and their α-anomers. J. Org. Chem., *35:* 636–639, 1970.

25. Pauly, G. T., Powers, M., Pei, G. K., and Moschel, R. C. Synthesis and properties of H-ras DNA sequences containing O^6-substituted-2′-deoxyguanosine residues at first, second, or both positions of codon 12. Chem. Res. Toxicol., *1:* 391–397, 1988.

26. Kokkinakis, D. M., von Wronski, M. A., Vuong, T. H., Brent, T. P., and Schold, S. C., Jr. Downregulation of O^6-methylguanine-DNA alkyltransferase and sensitization of human tumor cell lines to nitrosoureas by methionine deprivation. Br. J. Cancer, *75:* 779–788, 1997.

27. Hawk, C. T., and Leary, S. L., Formulatory for Laboratory Animals, p. 78. Ames, IA: Iowa State University Press, 1995.

28. Lengauer, C., Kinzler, K. W., and Vogelstein, B. Genetic instabilities in human cancers. Nature (Lond.), *396:* 643–649, 1998.

29. Friedberg, E. C., Walker, G. C., and Siede, W. DNA Repair and Mutagenesis. Washington, DC: American Society of Microbiology, 1995.

30. Fischhaber, P. L., Gall, A. S., Duncan, J. A., and Hopkins, P. B. Direct demonstration in synthetic oligonucleotides that *N*,*N*′-bis(2-chloroethyl)-nitrosourea cross-links N^1 of deoxyguanosine to N^3 of deoxycytidine on opposite strands of duplex DNA. Cancer Res., *59:* 4363–4368, 1999.

31. Christmann, M., and Kaina, B. Nuclear translocation of repair protein MSH2-MSH6 as response of cells to alkylating agents. J. Biol. Chem., *275:* 36256–36262, 2000.

32. Davis, T. W., Wilson-Van Patten, C., Meyers, M., Kunugi, K. A., Cuthill, S., Reznikoff, C. G., Boland, C. R., Kinsella, T. J., Fishel, R., and Boothman, D. A. Defective expression of the DNA mismatch repair protein, MLH1, alters G_2-M cell cycle checkpoint arrest following ionizing radiation. Cancer Res., *58:* 767–778, 1998.

33. Long, L., Berg, S. L., Roy, S. K., McCully, C. L., Song-Yoo, H., Moschel, R. C., Balis, F. M., and Dolan, M. E. Plasma and cerebrospinal fluid pharmacokinetics of O^6-benzylguanine and analogues in nonhuman primates. Clin. Cancer Res., *6:* 3662–3669, 2000.

34. Friedman, H. S., Dolan, M. E., Pegg, A. E., Marcelli, S., Keir, S., Catino, J. J., Bigner, D. D., and Schold, S. C., Jr. Activity of temozolomide in the treatment of central nervous system tumor xenografts. Cancer Res., *55:* 2853–2857, 1995.

Clinical Cancer Research

Thresholds of O^6-Alkylguanine-DNA Alkyltransferase which Confer Significant Resistance of Human Glial Tumor Xenografts to Treatment with 1,3-Bis(2-chloroethyl)-1-nitrosourea or Temozolomide

Demetrius M. Kokkinakis, Dora B. Bocangel, S. Clifford Schold, et al.

Clin Cancer Res 2001;7:421-428.