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O^6-Benzylguanine-mediated enhancement of nitrosourea activity in Mer^- central nervous system tumor xenografts – implications for clinical trials

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Abstract *Purpose*: To evaluate the role of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) plus O^6-benzylguanine (O^6-BG) in the treatment of both Mer^+ and Mer^- tumors. *Methods*: The effect of pretreatment with O^6-BG on the activity of BCNU against Mer^- human central nervous tumor xenografts D-54 MG and D-245 MG was evaluated in athymic nude mice. *Results*: BCNU (1.0 LD_{10}; dose lethal to 10% of treated animals) produced growth delays of 8.9 days and 7.5 days and tumor regressions in six of ten and one of nine animals against D-54 MG, which was derived from a human malignant glioma xenograft. Dose reduction of BCNU to 0.38 LD_{10} eliminated antitumor activity. The combination of BCNU (0.38 LD_{10}) plus O^6-BG produced growth delays of 8.8 days and 7.9 days, with tumor regressions in four of ten and two of nine animals, respectively. BCNU (1.0 LD_{10}) produced a growth delay of 49.8 days and ten of ten tumor regressions against D-245 MG, which was derived from a glioblastoma multiforme. BCNU (0.38 LD_{10}) produced a growth delay of 19.4 days, with nine of ten tumor regressions. The combination of BCNU (0.38 LD_{10}) plus O^6-BG produced a growth delay of 65.7 days and seven of eight tumor regressions. *Conclusion*: These results suggest that the combination of BCNU plus O^6-BG may be a rational intervention for both Mer^+ as well as Mer^- tumors.

Key words Nitrosourea · Glioma · Brain tumors

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Introduction

Alkylating agents, particularly the chloroethylnitrosoureas such as 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), combined with surgery and external beam radiotherapy, are the community standard of care for the treatment of malignant gliomas [16]. Unfortunately, BCNU produces only a modest benefit, with de novo or rapidly acquired evidence of tumor resistance to this agent. The major mechanism of resistance to BCNU identified to date is O^6-alkylguanine-DNA alkyltransferase (AGT), which removes BCNU-induced chloroethyl adducts through a stoichiometric transfer of the adduct to the cysteine moiety within the active site of the protein, thereby preventing formation of DNA interstrand crosslinks [17].

The observation that O^6-benzylguanine (O^6-BG) can deplete central nervous system tumor AGT levels with subsequent enhancement of nitrosourea antineoplastic activity has been shown with several human xenografts [7, 9, 11, 19]. These results, with one exception, were seen in Mer^+ xenografts with measurable AGT activity. Felker et al. [9] reported O^6-BG-mediated enhancement of BCNU activity in the Mer^- xenograft D-245 MG, with an increase in growth delay from 14.0 days to 22.2 days. More recently, Wedge and Newlands [21] reported O^6-BG-mediated enhancement of BCNU activity against the Mer^- human glioblastoma xenograft U87 MG. These results led us to further define the interaction between O^6-BG and BCNU in additional Mer^- xenografts, and to explore the implications of these results for the soon-to-open phase 22 trials of O^6-BG + BCNU.

Materials and methods

Measurement of O^6-alkylguanine-DNA alkyltransferase activity

Extracts from tissue for alkyltransferase assay were prepared as described previously [8, 18]. Alkyltransferase activity was measured as removal of [^{3}H]methyl from O^6-[^{3}H]methylguanine, which was prepared by allowing [^{3}H]methylnitrosourea to react with calf thymus DNA. The extracts were incubated with a [^{3}H]methylated DNA at 37 °C for 30 min. The DNA was precipitated by adding ice-cold perchloric acid at a final concentration of 0.25 *M* and hydrolyzed in 0.1 *M* HC1 at 70 °C for 30 min [8]. The modified bases were separated by reverse-phase HPLC [5]. Protein was determined using Bradford's [2] method and the results are expressed as fmol of O^6-methylguanine released for the DNA per mg of protein.

Animals/human xenografts

Male and female athymic BALB/c mice (*nu*/*nu* genotype, 6 weeks or older) were used for all studies and were maintained as described previously [3]. Human xenografts D-54 MG and D-245 MG were derived from malignant gliomas. The xenografts were maintained and used for in vivo studies as previously described [11, 12].

Subcutaneous xenograft transplantation, drugs, and tumor measurements

Subcutaneous tumor transplantation into the right flank of the animals was performed as described previously, with inoculation volumes of 50 µl s.c. [10]. O^6-BG was prepared as previously described [4]. BCNU was provided by the Drug Synthesis and Chemistry Branch of the National Cancer Center, Bethesda, Md., USA. Tumors were measured twice weekly with hand-held vernier calipers (Scientific Products McGraw, III., USA). Tumor volume was calculated according to the following formula: $[(\text{width})^2 \times (\text{length})]/2$.

Xenograft therapy

BCNU ± O^6-BG was given to mice via intraperitoneal (i.p.) injection. O^6-BG was delivered at a dose of 240 mg/m^2 in 40% polyethyleneglycol (PEG) in saline. BCNU was delivered at a dose of 100 mg/m^2 in 10% ethanol in saline (1.0 LD_{10}). This dose of BCNU is lethal to 10% of treated non-tumor-bearing animals. For combination studies, BCNU was administered at a dose of 38 mg/m^2 (0.38 LD_{10}) 1 h following O^6-BG. Groups of randomly selected mice began receiving treatment when the median tumor volume exceeded 200 mm^3 and were compared with control animals receiving 10% ethanol in saline alone.

Assessment of response

The response of subcutaneous xenografts was assessed by delay in tumor growth and by tumor regressions. Growth delay, expressed as T-C, is defined as the difference in days between the median time required for tumors in treated (T) and control (C) animals to reach a volume five times greater than that measured at the start of treatment. Tumor regression is defined as a decrease in tumor volume over two successive measurements. Statistical analyses were performed using the Wilcoxon rank order test for growth delay and Fisher's exact test for tumor regression as described previously [10].

Results

O^6-alkylguanine-DNA alkyltransferase activity, subcutaneous xenograft therapy, and toxicity

Neither D-54 MG nor D-245 MG exhibited undetectable AGT activity, measured as activity against [^{3}H]methylated DNA (assay sensitivity 10 fmol/mg protein). BCNU (1.0 LD_{10}) produced growth delays of 8.9 days and 7.5 days against D-54 MG in replicate experiments (Table 1). Tumor regressions were noted in six of ten and one of nine animals, respectively. Reduction of the dose of BCNU to 0.38 LD_{10} removed any evidence of antitumor response. O^6-BG alone produced no antitumor activity. However, the combination of BCNU plus O^6-BG produced growth delays of 8.8 days and 7.9 days, with four of ten and two of nine tumor regressions, respectively.

BCNU (1.0 LD_{10}) produced a growth delay of 49.8 days, with ten of ten tumor regressions against D-245 MG. Reduction of the dose of BCNU to 0.38 LD_{10} reduced the growth delay to 19.4 days, with nine of ten tumor regressions. O^6-BG alone produced a growth delay of −0.3 days, with zero of ten tumor regressions. The combination of O^6-BG plus BCNU produced a growth delay of 65.7 days, with seven of eight

Table 1 Antitumor activity of BCNU ± O^6-BG against human central nervous system xenografts in athymic nude mice. *BCNU* 1,3-bis(2-chloroethyl)-1-nitrosourea; LD_{10} dose lethal to 10% of treated animals; *T-C* difference in days between the median time required for tumors in treated (*T*) and control (*C*) animals to reach a volume five times greater than that measured at the start of treatment; *Regression*: decrease in tumor volume over two successive measurements

Xenograft	Experiment	Treatment[a]	T-C	Regressions
D-54 MG	1	BCNU (0.38)	0.1	0/10
		O^6-BG	0.0	0/10
		BCNU + O^6-BG	8.8*	4/10*
		BCNU (1.0)	8.9*	6/10*
	2	BCNU (0.38)	0.8	0/9
		O^6-BG	−0.6	0.8
		BCNU + O^6-BG	7.9*	2/9
		BCNU (1.0)	7.5*	1/9
D-245 MG	3	BCNU (0.38)	19.4*	9/10*
		O^6-BG	−0.3	0/10
		BCNU + O^6-BG	65.73*	7/8*
		BCNU (1.0)	49.8*	10/10[a]

[a] BCNU was given at 0.38 or 1.0 of the LD_{10} (100 mg/m^2)
* *P* value ≤0.05 compared with controls

tumor regressions. One death among 113 treated animals (BCNU + O^6-BG group) was attributable to drug toxicity. The median nadir weight loss was as follows: BCNU (1.0 LD_{10}) 4.3%, BCNU (0.38 LD_{10}) 0.5%, O^6-BG 0.1%, and the combination of BCNU plus O^6-BG 14.6%.

Discussion

Enhancement of BCNU antineoplastic activity against human CNS tumor Mer^+ xenografts has been reported many times [4, 7, 9, 11, 15, 19] and is consistent with results obtained in cell culture. The role of AGT in removing BCNU-induced DNA adducts prior to conversion to cytotoxic DNA interstrand crosslinks provides an explanation for these results. However, enhancement of BCNU activity in Mer^- xenografts following treatment with O^6-BG, previously reported twice in the literature and extended with our current studies, may be more complex [9, 21].

The current results, in concert with Felker et al. [9] and Wedge and Newlands [21], clearly demonstrate that BCNU-induced tumor growth delays and tumor regressions are enhanced, despite undetectable tumor AGT levels, following treatment with O^6-BG. A possible explanation for this could be the presence of a small population of Mer^+ tumor cells interspersed in a larger Mer^- background. However, quantitation of cells staining for AGT using MT 3.1 [20] showed virtually no positive cells prior to or after treatment with BCNU ± O^6-BG (data not shown) in either D-245 MG or D-54 MG. Alternatively, since Mer^- cells do have the gene for AGT, it is possible that Mer^- tumors demonstrate AGT levels that, although below the level of detection using current assays, are still susceptible to further reduction (and subsequent enhancement of BCNU activity) following use of O^6-BG. Finally, it is possible that O^6-BG is depleting AGT levels in murine stromal cells. However, a direct relationship to antitumor activity of BCNU against the xenografts is not intuitively obvious. Nevertheless, enhancement of BCNU activity against Mer^- xenografts with O^6-BG is apparently the rule rather than specific for tumors expressing AGT.

Current studies reduced the dose of BCNU to avoid toxicity seen with O^6-BG pretreatment. Nevertheless, the toxicity of BCNU plus O^6-BG was greater than that produced by BCNU, as evidenced by the greater median weight loss seen with combination therapy. However, we have not seen antitumor effects at median weight loss $<25\%$ produced by ineffective albeit toxic chemotherapeutic agents. Full-dose (1.0 LD_{10}) BCNU showed two patterns of results. Treatment of D-54 MG with full-dose BCNU was equivalent to the activity of BCNU (0.38 LD_{10}) plus O^6-BG. However, full-dose BCNU was considerably less effective against D-245 MG compared with BCNU (0.38 LD^{10}) + O^6-BG ($P < 0.05$), suggesting a therapeutic advantage above that seen with BCNU alone.

The enhancement of BCNU activity with O^6-BG in Mer^- tumors has several implications for the clinical use of this combination. O^6-BG has been shown to effectively deplete malignant glioma AGT levels in patients with newly diagnosed or recurrent tumors [14]. A current phase I trial of O^6-BG plus BCNU in patients with recurrent malignant glioma will soon define the maximum tolerated dose (MTD) of BCNU given 1 h after O^6-BG (100 mg/m^2). The target population for the subsequent phase 22 trials could be selected using biochemical (tumors with measurable AGT levels) or clinical (tumor growth despite BCNU use) criteria. We believe BCNU plus O^6-BG should be evaluated in patients whose tumors do not respond to therapy with a nitrosourea, since this appears to be a common mechanism of resistance to these agents [11], irrespective of tumor AGT levels. Although there are other non-AGT mechanisms of resistance to nitrosoureas which contribute to BCNU resistance [1, 13], AGT depletion may restore at least partial nitrosourea sensitivity.

We plan, following completion of the phase I trial of BCNU plus O^6-BG, to conduct a phase II trial of O^6-BG plus BCNU in patients with recurrent malignant glioma who did not respond to treatment with a nitrosourea within the previous 6 weeks. Although a tumor sample will be obtained at the time of recurrence to document active tumor (as opposed to radiation necrosis) and quantitate tumor AGT levels, eligibility will not be determined based on these levels. However, it will be possible to correlate tumor AGT levels with response to O^6-BG plus BCNU. This could potentially identify Mer^- tumors that have failed prior therapy with a nitrosourea due to a mechanism of resistance distinct from AGT. Demonstration of restoration of at least partial sensitivity to O^6-BG plus BCNU in this setting could be an important observation for future use of these agents.

The use of O^6-BG plus BCNU in patients with newly diagnosed malignant glioma is more problematic. Should this approach be restricted to patients with Mer^+ tumors? We believe that this therapy should be randomized versus BCNU alone without considering tumor AGT levels, since enhancement of BCNU activity is seen both in Mer^- and Mer^+ tumors. Furthermore, it is possible that use of O^6-BG may delay emergence of AGT-expressing tumor cells resistant to BCNU. Future rational use of BCNU plus O^6-BG must include not only evaluation of toxicity and antitumor activity, but quantitation of tumor AGT levels before use of this combination and at the time of tumor progression following its use.

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