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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

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Abstract The prognosis for patients with malignant gliomas continues to be dismal. The high degree of resistance of gliomas to nitrosourea-based chemotherapy is one major factor in poor treatment outcome. The identification of O^6-alkylguanine-DNA alkyltransferase (AGAT) as a major determinant of nitrosourea resistance has resulted in the development of several agents to inactivate this repair protein and counteract tumor cell resistance. However, a major problem in preclinical trials has been the marked nitrosourea dose limitations imposed by the prior administration of AGAT-depleting agents. We investigated the AGAT depletion and selective enhancement of BCNU activity of intraarterial (i.a.) O^6-benzylguanine (O^6BG) in the human malignant glioma xenograft D-456 MG growing intracranially (i.c.) in athymic rats. Whereas i.a. O^6BG at 2.5 mg/kg produced 100% inhibition of D-456 MG AGAT i.c. activity 8 h after administration, intraperitoneal (i.p.) O^6BG at this dose produced only 40% inhibition, requiring dose escalation to 10 mg/kg to produce 100% AGAT depletion. Prior administration of i.p. O^6BG (10 mg/kg) and i.a. O^6BG (2.5 mg/kg) limited maximum tolerated intravenous (i.v.) BCNU doses (37.5 mg/kg when given alone) to 6.25 and 25 mg/kg, respectively. Higher doses of BCNU alone or in combination with O^6BG produced histopathologic evidence of cerebral and hepatic toxicity. Therapy experiments revealed a significantly improved median survival for rats treated with O^6BG i.a. (2.5 mg/kg) plus BCNU i.v. (25 mg/kg, days 61 and 59 in duplicate experiments) compared with saline (day 21, $P = 0.001$), O^6BG i.a. or i.p. (days 22 and 23, $P = 0.001$), BCNU i.v. (37.5 mg/kg, day 29, $P = 0.001$), and O^6BG i.p. (10 mg/kg) plus BCNU i.v. (6.25 mg/kg, day 37, $P < 0.001$). Therefore, O^6BG i.a., by virtue of rapid AGAT depletion and selective uptake into i.c. tumors, offers significant potential for regional chemomodulation of AGAT-mediated nitrosourea resistance in malignant human gliomas with concomitant reduction of systemic toxicity.

Key words BCNU · Chemotherapy · Drug resistance · Gliomas · O^6-benzylguanine

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Introduction

The dismal prognosis for patients with glioblastomas and anaplastic astrocytomas has seen little improvement [38, 44]. The primary reason for the failure of

most novel adjuvant therapies is the lack of availability of sufficiently active agents to effectively treat postsurgical tumor remnants. The nitrosoureas (particularly 1,3-bis(2-chloroethyl)-1-nitrosourea, BCNU) have long been the major agents in the chemotherapy of malignant gliomas because of their physicochemical properties, particularly their lipophilicity, favoring transport across the blood–brain barrier. The tumoricidal effect of the nitrosoureas has been proven to occur by the alkylation of guanine bases in DNA at the O^6 position followed by crosslinking of this residue to cytosine on the complementary strand of DNA. The crosslinking event, which is the major lethal lesion produced by these drugs, takes some time to consolidate [13, 14, 35, 46]. Resistance to such injury is mediated by a specific repair protein, O^6-alkylguanine-DNA alkyl transferase (AGAT) which effects a stoichiometric transfer of the alkylating unit from the guanine residue in DNA onto a cysteine residue at the active site of the repair protein before the crosslinking has occurred, an action leading to permanent inactivation of AGAT [37, 48].

AGAT is found in high levels in the liver, kidney, and spleen, but only low levels are detected in normal brain [22, 39]. An analysis of several glioma and other brain tumor lines has revealed a high level of AGAT in these tumors [23, 47], suggesting that the AGAT-mediated DNA repair may be a major mechanism of resistance to nitrosoureas. The suicidal action not only necessitates continued protein synthesis to replenish activity [35], but also suggests that AGAT inhibition may be a specific method to reverse resistance. Methods explored to inhibit the AGAT protein have included the use of DNA-methylating agents [19, 20] and a number of substrate analogs which competitively deplete the enzyme (6–8, 30). O^6Benzylguanine (O^6BG) is the prototype AGAT inactivator. However, the use of O^6BG is accompanied by an enhancement of BCNU toxicity, necessitating a large dose reduction to minimize these adverse effects [17]. It is therefore worthwhile to explore alternate routes of delivery of O^6BG and/or BCNU to enhance their therapeutic efficacy.

Intraarterial (i.a.) therapy has long been considered a potential method to improve selective therapy of gliomas. Theoretical studies using computer modeling predicted 200–500% higher drug levels in the brain with i.a. versus intravenous (i.v.) administration and reduced systemic toxicity due to both the decrease in drug dose that would be required and the effect of "first pass" metabolism [12, 16]. The relatively localized growth pattern of gliomas and the proximity of the two major trunk vessels that supply blood to the brain, the carotid and the vertebral arteries, make i.a. delivery more advantageous than i.v. therapy [45]. Many preclinical studies and clinical trials to date have examined the effect of i.a. delivery of therapeutic agents. Examples include effective preclinical experiments in immunosuppressed and athymic rats with arterial BCNU [4], 4-hydroperoxycyclophosphamide [42], and melphalan [24]. In all cases, dose-limiting toxicity has been in the normal rat brain and eye tissue. In a novel strategy, we decided to examine the possibility of achieving selective reversal of drug resistance using the i.a. route of delivery. Conceptually, a drug-resistant tumor in a localized compartment such as the CNS may become exquisitely susceptible to the effect of systemic chemotherapy by the compartmental use of resistance modulators. It should then be possible to utilize lower doses of a systemic agent with a consequent decrease in extraneural toxicity and enhanced antitumor activity.

Here we describe a series of experiments that demonstrate the selective modulation of BCNU resistance in intracranially (i.c.) implanted high AGAT-expressing malignant human glioblastoma xenografts in athymic rats by compartmental (i.a.) administration of O^6BG. This strategy resulted in the restoration of tumor sensitivity to BCNU (as predicted by the complete depletion of AGAT from tumors) using as little as 25% of the O^6BG dose that would be required with systemic administration. The dose reduction of O^6BG so obtained enabled, in turn, a fourfold dose escalation of systemic BCNU in relation to doses that can be administered with systemic O^6BG. Therapy of rats with i.c. tumors with the optimal i.a. O^6BG and i.v. BCNU doses determined, resulted in median survival prolongations up to threefold compared with saline-treated controls, and over twofold compared with the optimal intraperitoneal (i.p.) O^6BG and i.v. BCNU dose combination. We suggest, based on these results, that phase I trials be designed with the administration of i.a. O^6BG and i.v. BCNU in the therapy of patients with malignant human gliomas.

Materials and methods

Tumor and animal models

The human glioma xenograft D-456 MG (childhood glioblastoma multiforme) was used for all experiments. This tumor, grown as subcutaneous (s.c.) xenografts in athymic mice, is known to have a doubling time of approximately 5 days in these animals. Tumors removed from mice were finely minced and homogenized, and, after removal of necrotic debris, the homogenate was passed through a tissue sieve into Richter's zinc option medium [40] (ZO; Gibco, Grand Island, N.Y.). The resulting cell suspension was then passed through 25-gauge needles, centrifuged at 500 *g* for 3 min, mixed with an equal volume of methyl cellulose, and loaded into a 500-µl Hamilton syringe. Male nude athymic rats weighing approximately 300 g, bred and cared for at the Duke Cancer Center Isolation Facility as previously described [18], were inoculated in the right caudate nucleus with 20 µl of this mixture using a Kopf stereotactic instrument (David Kopf Instruments, Tujunga, Calif.) with coordinates 1 mm anterior, 2 mm lateral to the bregma. At this point, a hole was drilled through the skull using a 19-gauge needle. The needle was advanced downward 5 mm below the dura, and injections were given. The skull holes were sealed with bone wax, and the skin was closed with surgical staples.

Table 1 AGAT depletion in i.c. D-456 MG xenografts in athymic rats treated with O^6-BG i.p. or i.a. (*a*, *b*, *c*, three separate experiments from which the date were compiled; *NT* not tested). Tumor AGAT values in control rats (100% residual AGAT in tumors) treated with vehicle (40% PEG in saline) were 12.3 ± 4.2 (a), 19.5 ± 4.4 fmol/mg (b), and 31.4 ± 10.8 fmol/mg (c). Four or five animals in all groups tested

O^6BG dose (mg/kg)	Tumor AGAT with O^6BG i.p. (fmol/mg)	% Control residual AGAT (approx.)	Tumor AGAT with O^6BG i.a. (fmol/mg)	% Control residual AGAT (approx.)
80	0[a]	0[a]	NT	
40	0[a]	0[a]	NT	
20	0[a]	0[a]	NT	
10	0[a]	0[a]	NT	
5	2.1 ± 3.0[a]	30[a]	**0[b]**	0[b]
2.5	11.1[b]	60[b]	0[b]	0[b]
1.0	13.7 ± 2.6[b]	80[b]	10.4[c]	33[c]

Intraarterial injections

Rats anesthetized with an appropriate dose of a 6:5 ketamine-xylazine mixture were placed on their dorsal surface with appropriate limb restraint and suitable measures to ensure an open airway. A 3-cm longitudinal midline incision was made extending between the mentum and the sternal notch. The right sternomastoid and paratracheal muscles were retracted laterally and medially, respectively, to expose the mylohyoid muscle and the hyoid bone. The course and branches of the right carotid arterial system were then isolated by sharp dissection after division of the mylohyoid muscle and removal of the lateral third of the hyoid bone. The external carotid was tied distally after cauterization of its superior thyroid, occipital and ascending pharyngeal (occasional) branches. Deep dissection of the internal carotid artery was carried out, followed by cauterization of its pterygopalatine branch, to create a continuous conduit from the external to the internal carotid artery. Finally, the external carotid was catheterized distoproximally using a PE-10 polyethylene catheter, into which the injections were given. After injections were complete, the external carotid was cauterized, and the skin was closed with surgical staples.

Intravenous injections

Rats anesthetized as above were inoculated i.v. through a right femoral venous cutdown.

Drugs

O^6-BG was synthesized as previously described [46]. A stock solution (4 mg/ml) was first prepared in 40% polyethylene glycol-400 (PEG-400) in saline. Briefly, 40 mg O^6BG was dissolved completely in 2 ml PEG-400. Dissolution was facilitated by mild heating to 40 °C and continuous stirring. Normal saline (3 ml) was then added, and heating and stirring were continued until the drug was completely dissolved. For ease of delivery into the carotid artery for rats given i.a. O^6BG, appropriate doses, once withdrawn into a tuberculin syringe just prior to administration, were diluted twofold (to a final concentration of 4 mg/ml) with saline. This method of achieving final dilutions in the syringe just prior to injection allowed a less viscous solution to be administered, while still retaining the O^6BG completely dissolved at the time of injection.

BCNU was obtained from the Drug Synthesis and Chemistry Branch of the National Cancer Institute. BCNU (300 mg) was dissolved in 3 ml 100% alcohol. A stock solution of 10 mg/ml BCNU was prepared by the addition of 27 ml sterile saline. The drug was then filtered through a 22-μm filter. BCNU was then dispensed into sterilized amber vials and snap-frozen in liquid nitrogen. In all experiments, stock solutions were prepared on the day before usage.

Table 2 Toxicity of O^6BG i.a. or i.p. and BCNU i.v. Toxicity was defined as the occurrence of death or $>25\%$ group weight loss. Pooled controls (two saline, two O^6BG i.a., and one O^6BG i.p.) all lived and steadily gained weight

BCNU dose (mg/kg)	BCNU	O^6BG 10 mg/kg i.p. + BCNU	O^6BG 2.5 mg/kg i.a. + BCNU
50	Toxic (2/4 deaths)	Not done	Not done
37.5	Tolerated ($n = 3$)	Not done	Toxic (2/4 deaths)
25	Tolerated ($n = 3$)	Not done	Tolerated ($n = 3$)
12.5	Not done	Toxic (4/4 deaths)	Tolerated ($n = 3$)
6.25	Not done	Tolerated ($n = 4$)	Not done

Appropriate quantities were injected i.v. into rats based on their individual body weights.

Alkyltransferase depletion experiments

Groups of four or five rats were used for these experiments. On day 18 after tumor implantation, rats were given O^6BG i.a. or i.p. (see Table 1). Rats were sacrificed by anesthetic overdose 8 h after O^6BG administration. The brains were rapidly dissected, and tumors were removed. Tumor and contralateral brain were submitted for AGAT analysis. Tumor dissection was facilitated by the i.v. administration of 2% Evans Blue dye in saline 30 min prior to sacrifice. Tumors were frozen in liquid nitrogen prior to storage at −70 °C. AGAT activity was measured as the removal of [^{3}H]methyl groups from the O^6 position of guanine in a methylated DNA substrate. Methylated DNA was prepared from the reaction of [^{3}H]methylnitrosourea and purified calf thymus DNA [11, 46]. Protein content was determined by the method of Bradford [3].

Toxicity experiments

In initial toxicity experiments, nontumor-bearing athymic rats were randomized by weight into groups of three or four and given BCNU i.v. alone or in combination with O^6BG i.a. or i.p. (see Table 2). For formal toxicity experiments, rats randomized by weight into groups of ten were given BCNU i.v. alone, O^6BG i.a. or i.p., the two drugs in

Table 3 Histopathologic evaluation of O^6BG and BCNU in D-456 MG-bearing rats (ten rats in each group)

Group[a]	Median day of death	Tumor incidence	Hemorrhage	Necrosis	Demyelination	Edema	Fibrosis	Liver
Saline	21	10/10	0/10	0/10	0/10	0/10	0/10	None
O^6BG 2.5 i.a. or 10 i.p.	22/23	20/20	0/20	0/20	0/20	0/20	0/20	None
BCNU 37.5	27	10/10	1/10	2/10	0/10	0/10	0/10	4/10 acute passive congestion
O^6BG 10 i.p. + BCNU 6.25	37	10/10	1/10	5/10 (3 focal parenchymal)	0/10	0/10	0/10	2/10 acute passive congestion
O^6BG 2.5 i.a. + BCNU 25	62	10/10	0/10	1/10	1/10	0/10	0/10	2/10 mild hepatic necrosis

[a] All dosages in mg/kg; all BCNU given i.v.

combination, or saline alone (see Table 3). After drug administration, animals were weighed daily. A complete autopsy was performed at the time of death for all animals.

Therapy experiments

Rats were randomized by weight into groups of eight to ten at 9 days after tumor implantation. Rats were given BCNU i.v. 1 h after treatment with O^6BG 2.5 mg/kg i.a. or 10 mg/kg i.p. For the rats given O^6BG i.a., BCNU was administered at 25 mg/kg, and for rats treated with O^6BG i.p., BCNU was administered at 6.25 mg/kg. Control rats were treated with O^6BG i.a or O^6BG i.p and saline. Animals were weighed on alternate days. A complete autopsy was performed at death for all animals.

Histopathologic examination

All animals were checked for mortality twice a day, and autopsies were performed on animals that died. No animals underwent autopsy more than 12 h after death. At autopsy, the head was separated and immersed in formalin. Internal organs were grossly examined. One week after autopsy, the skulls were immersed in decalcifying solution for 12–16 h. After adequate decalcification, representative sections of the brain were submitted for histopathologic examination. These included coronal tissue blocks taken at the levels of the nucleus caudatus putamen, the dentate gyrus, and the cerebellum. A representative section of the liver was also examined. Sections 5-μm thick were cut, deparaffinized, and stained with hematoxylin and eosin-Luxol fast blue (brain sections) or with hematoxylin and eosin (liver sections) as previously described. Slides were then examined by a histopathologist blinded to the treatment groups.

Statistical analysis

The Wilcoxon rank sum test was used to compare group median survival. A P-value <0.05 was considered statistically significant.

Results

Alkyltransferase depletion experiments

Initial experiments were directed at defining the minimum dose of O^6BG i.p. that was required to achieve complete depletion of AGAT in i.c. tumors. Groups of four to five rats bearing i.c. D-456 MG were given O^6BG i.p at several dosages (Table 1). AGAT activity was undetected in i.c. tumors at all doses except at 5 mg/kg, where approximately 20% (of control) residual AGAT activity was detected. Experiments were then performed to define the minimum dose of O^6BG i.a. that was required to achieve complete depletion of AGAT from i.c. tumors. In this set of studies, O^6BG was administered at 5 or 2.5 mg/kg i.a. Complete depletion was seen at both doses. Direct comparison in the same experiment with O^6BG 2.5 mg/kg i.p. revealed that, at this dose, residual AGAT levels in tumor were 60% of control. Further reduction of the O^6BG dose to 1.0 mg/kg i.a. in a third experiment resulted in incomplete inactivation of the protein, with tumor AGAT levels about 33% of control values.

Toxicity experiments

Two to four rats were used in all groups and were followed for a period of 6 weeks (Table 2). Toxicity-related deaths were noted in 2/4 rats given BCNU 50 mg/kg, 4/4 rats given O^6BG 10 mg/kg i.p./BCNU 12.5 mg/kg, and 2/4 rats given O^6BG 2.5 mg/kg i.a./BCNU 37.5 mg/kg. All groups in which deaths occurred had evidence of severe and precipitous weight loss from the third day after drug administration (data not shown); six of eight rats that died showed evidence

of jaundice prior to death. The maximum tolerated doses (MTDs, defined as less than 25% group mean weight loss) of BCNU alone, O^6BG i.a./BCNU, and O^6BG i.p./BCNU were 37.5, 2.5/25, and 10/6.25 mg/kg, respectively. The doses of O^6BG i.a and i.p. used in these experiments had been previously shown to produce complete depletion of tumor AGAT activity in i.c. D-456 MG-bearing rats. The present experiments were intended to achieve dose escalation of BCNU to MTDs in rats treated previously with these doses of O^6BG.

Histopathologic examination was performed on standard brain and liver sections as previously described [34, 42]. Massive cerebral necrosis was identified in all eight rats that died in this study (Fig. 1). In addition, hepatotoxicity was present in all four rats receiving O^6BG i.p./BCNU (Fig. 2).

Based on the previous experiments, groups of ten rats were given saline alone, BCNU i.v. alone, O^6BG alone i.a. or i.p., or O^6BG i.a. or i.p./BCNU (Fig. 3). Daily weight monitoring showed that these doses were well tolerated, with the nadir of group mean weight in rats given BCNU 37.5 mg/kg or the combination of O^6BG 2.5 mg/kg i.a./BCNU 25 mg/kg at 22% of mean pretreatment weight at 10–12 days posttreatment. There were no deaths in any group; all rats recovered well, and were steadily gaining weight at the time the experiment was terminated at 6 weeks after drug administration.

Therapy experiments

Eight to ten rats were used in all groups. The doses of O^6BG used i.a. or i.p. reflected the minimum dose that was required to completely deplete AGAT activity from i.c tumors by either route. The first therapy experiment (Fig. 4) included groups of rats that were given O^6BG i.a or i.p. combined with BCNU i.v. at the MTDs as determined above for each of these combinations. From the toxicity data, rats receiving O^6BG 10 mg/kg i.p. were limited to BCNU 6.25 mg/kg. To control for the survival effect from BCNU 6.25 mg/kg, we included groups of rats treated with BCNU alone at 6.25 mg/kg; and O^6BG 2.5 mg/kg i.a. with BCNU 6.25 mg/kg. Groups of rats that received the MTD of

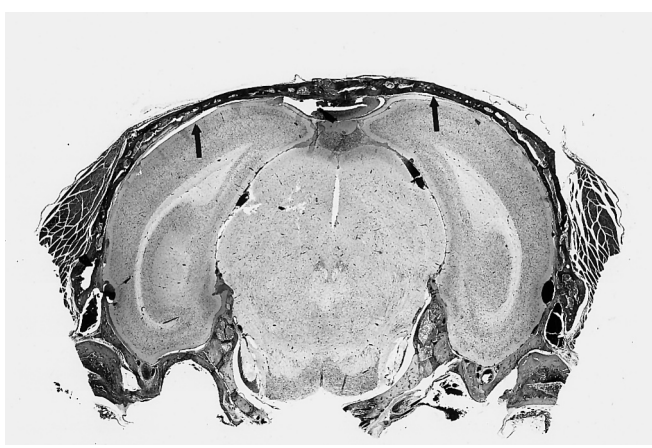

Fig. 1 The brains of four of four nontumor bearing rats receiving O^6BG 10 mg/kg i.p. /BCNU 12.5 mg/kg revealed massive cerebral edema as characterized by virtually complete obliteration of the subdural and subarachnoid spaces (*arrows*) and by the markedly swollen brain (hematoxylin and eosin-Luxol Fast Blue)

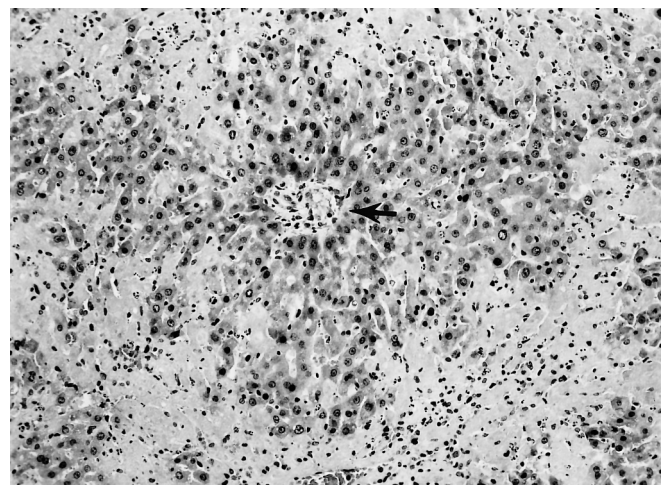

Fig. 2 Hepatotoxicity was found in four of four nontumor bearing rats receiving O^6BG 10 mg/kg i.p./BCNU 12.5 mg/kg and was characterized by confluent hepatocellular necrosis which bridged between central veins sparing the periportal regions (*arrow*) (hematoxylin and eosin, ×130)

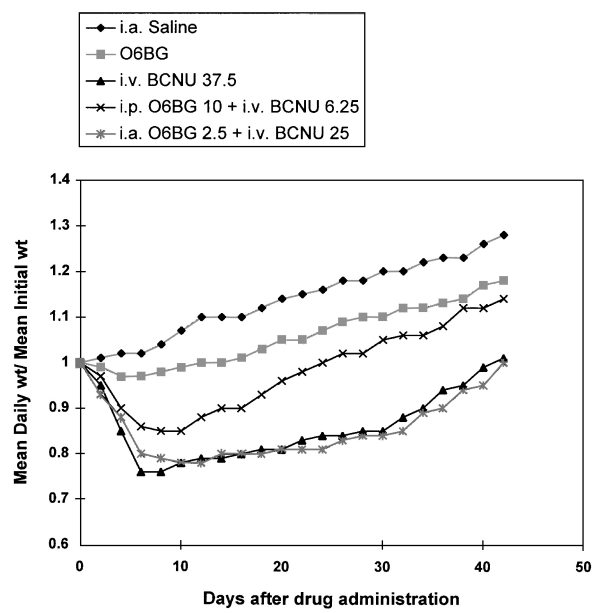

Fig. 3 Toxicity-induced weight profile of nontumor bearing athymic rats given O^6BG and/or BCNU ($n = 10$ in all groups). Dosages are in mg/kg

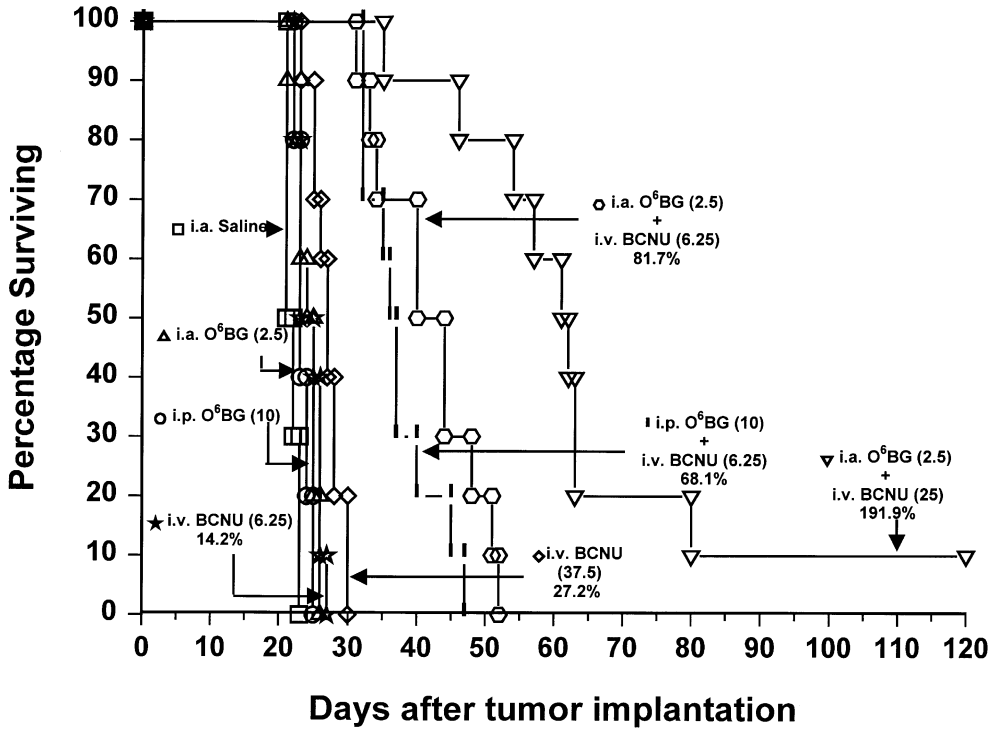

Fig. 4 Treatment of i.c. D-456 MG xenografts in athymic rats with O^6BG and BCNU, alone or in combination ($n = 10$ in all groups except in those given O^6BG 2.5 mg/kg i.a./BCNU 6.25 mg/kg, where $n = 9$). Numbers in brackets refer to drug doses in mg/kg, and percentage values to increase in survival in that group

BCNU 37.5 mg/kg and the optimum dose combination of O^6BG 2.5 mg/kg i.a./BCNU 25 mg/kg were also included. Control groups received O^6BG i.a, O^6BG i.p., or saline.

As shown in Fig. 4, rats that received saline alone were the first group to succumb, with a median survival time of 21 days. Histopathology indicated that deaths were due to tumor in all cases. Rats administered O^6BG either i.a. or i.p. exhibited median survivals at 23 and 24 days, respectively ($P > 0.05$). Rats receiving BCNU 6.25 mg/kg all died by day 29, with a median survival of 25 days ($P > 0.05$). In contrast, rats receiving the MTD of BCNU 37.5 mg/kg showed a median survival of 27 days (Fig. 5, $P < 0.003$). Significantly improved survivals were encountered in the groups of rats that received O^6BG 10 mg/kg i.p./BCNU 6.25 mg/kg compared with either saline controls or BCNU 37.5 mg/kg ($P < 0.001$ in both cases). This improved survival was also recorded in the rats that received O^6BG 2.5 mg/kg i.a./BCNU 6.25 mg/kg ($P < 0.001$ in both cases). These groups showed median survivals of 37 days and 44 days, respectively, with no significant difference in survival between these groups ($P < 0.109$). The most prolonged survivals were noted in the group of rats that received O^6BG 2.5 mg/kg i.a./BCNU 25 mg/kg, where a median survival of 62 days was recorded. Survival in this group was significantly better than saline controls, BCNU alone at either dose, and O^6BG i.p./BCNU at its optimum dose ($P < 0.001$ in all cases).

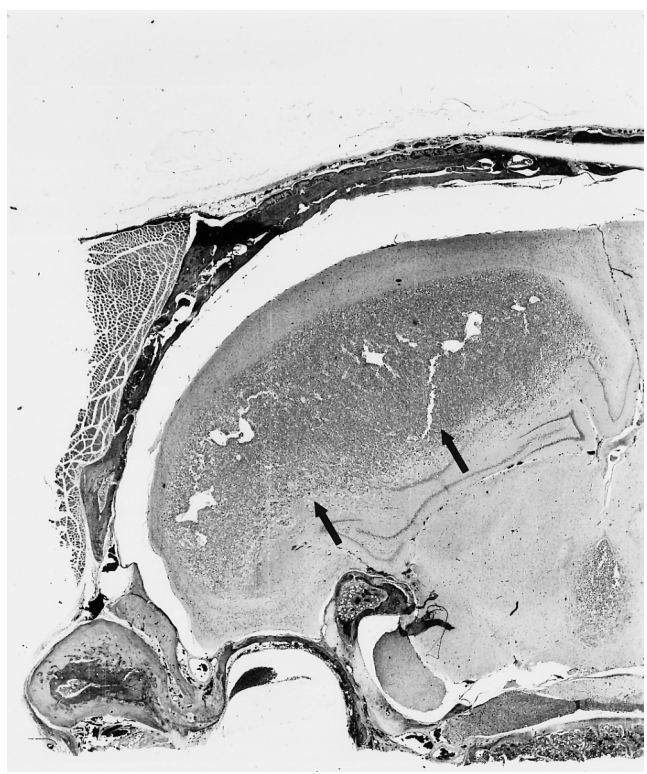

Fig. 5 A prominent intraparenchymal tumor (*arrows*) is evident in the brain of this D-456 MG-bearing rat treated with BCNU 37.5 mg/kg i.v. alone. No obvious response was noted in rats in this group compared with saline-treated controls (hematoxylin and Eosin-Luxol Fast Blue)

Again, histologic evaluation of all rats from this therapy experiment was performed as above, to ascertain any toxicity of the drug dosages used in these experiments. Our predictions from the preliminary toxicity studies were confirmed, with none of the brain sections examined from any of the therapy groups showing evidence of toxicity (Table 3). Histologic examination of liver sections did reveal some evidence of toxicity, with one of ten rats from the group treated with O^6BG 2.5 mg/kg i.a./BCNU 25 mg/kg showing mild multifocal, subconfluent hepatic necrosis. Of ten rats treated with O^6BG 10 mg/kg i.p./BCNU 6.25 mg/kg, three showed acute passive congestion, with two additional rats in this group also demonstrating mild multifocal, subconfluent hepatic necrosis.

We repeated these therapeutic trials at the optimum treatment doses for all routes of adminisration (Fig. 6). A median survival of 59 days was recorded in tumor-bearing rats given O^6BG 2.5 mg/kg i.a./BCNU 25 mg/kg, and this was significantly better than rats treated with saline alone (day 21, $P < 0.001$), O^6BG 2.5 mg/kg i.a. (day 22, $P < 0.001$), O^6BG 10 mg/kg i.p. (day 23, $P < 0.001$), BCNU 37.5 mg/kg (day 29, $P < 0.001$), or O^6BG 10 mg/kg i.p./BCNU 6.25 mg/kg (day 35, $P < 0.001$). As stated above, survival prolongation was also significantly improved in rats treated with O^6BG 10 mg/kg i.p./BCNU 6.25 mg/kg compared with groups treated with BCNU alone ($P < 0.003$) and saline alone ($P < 0.001$). Finally, treatment with i.v BCNU 37.5 mg/kg significantly prolonged median survival time in comparison with saline controls ($P < 0.001$), although the extent of such prolongation was relatively small in comparison with the optimally treated group (day 29 vs day 59).

Discussion

Chemotherapeutic agents for the therapy of malignant gliomas have traditionally been chosen based on their ability to cross the blood–brain barrier in sufficient concentrations to elicit therapeutic effects [43]. The relative ineffectiveness of nitrosourea-based chemotherapy in brain tumors is probably due to intrinsic tumor cell resistance rather than inadequate drug delivery to the i.c. site [17]. The findings by several studies of high AGAT levels in these and other tumors and the effective restoration of BCNU sensitivity in both human tumor cell lines and xenografts after interventions designed to deplete tumor cell AGAT suggest that this repair protein may be primarily responsible for a significant degree of drug resistance [36].

Based upon knowledge derived from the mechanism of action of AGAT, several agents have been developed and tested as potential mechanisms to deplete tumor cell AGAT to restore BCNU sensitivity. These include DNA methylators such as dacarbazine (DTIC) and streptozotocin. The severe combined toxicity of DTIC in combination with BCNU [29] and the significant toxicity with doses of streptozotocin required for

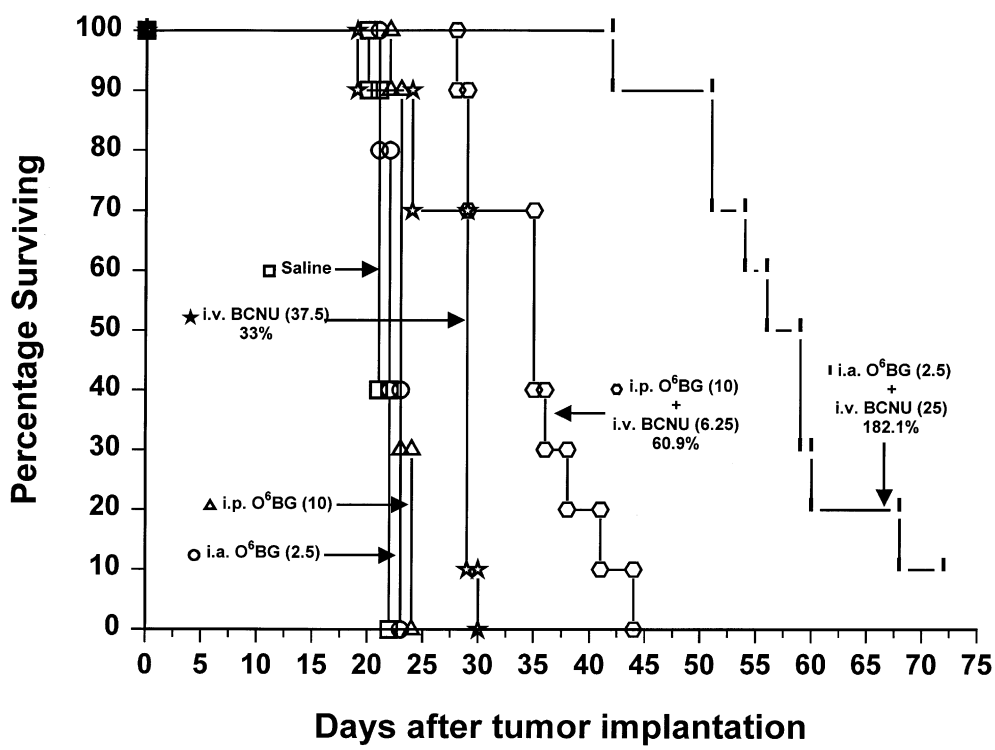

Fig. 6 Treatment of i.c. D-456 MG xenografts in athymic rats with the optimal dose combinations of O^6BG i.p. or i.a. and BCNU alone or in combination ($n = 10$ in all groups). Numbers in brackets refer to drug doses in mg/kg, and percentage values refer to increase in survival in that group

appreciable AGAT depletion render the safety of methylating agents as AGAT depletors questionable in clinical settings [26]. In phase I clinical trials with sequenced administration of streptozotocin and BCNU for nonhematologic solid tumors [26, 33] dose limitation of BCNU to 50% of the MTD of BCNU alone was necessary [26]. Drug-induced thrombocytopenia was dose-limiting in both studies, and 2/24 patients died from drug-induced toxic effects [26]. A separate phase I trial with sequenced administration of DTIC and BCNU in 42 patients with refractory solid tumors also resulted in dose-limiting hematologic toxicity [31]. Although AGAT activity was depleted to $65 \pm 14\%$ of baseline in the peripheral blood mononuclear cells at 24 h after DTIC infusion, no direct correlation was noted between DTIC dose and the extent of AGAT depletion [31]. In addition, the use of methylating agents presents a risk of introduction of promutagenic lesions with the potential for the development of new malignancies [35].

Another approach to address this problem has been the design of substrates for the AGAT protein. Of the several agents of this nature that have been examined, O^6-methylguanine and O^6BG have shown promising activity in vitro and in vivo [35]. However, O^6-methylguanine, while successful as a moderate potentiator of nitrosourea activity in glioma and medulloblastoma cell lines and xenografts, is limited by the large doses required to deplete AGAT and its limited solubility [5].

O^6BG, by virtue of greater affinity to AGAT, has been successfully used to deplete AGAT in a spectrum of human tumor cell lines and xenografts. Friedman et al. [17] have demonstrated partial and complete tumor regression in all athymic mouse s.c. tumor xenografts (D-341 Med and D-456 MG) treated with the combination of systemic O^6BG and BCNU, while Felker et al. [15] have shown significant growth inhibition and survival prolongation in athymic mice implanted s.c. and i.c. with D-341 Med xenografts. Mineura et al. [27] have demonstrated increased in vitro and in vivo sensitivity of the C6 and 9L rat gliomas to ACNU after treatment with O^6BG. The limiting factor in all these studies, however, is the drastic reduction required in the dose of BCNU (38% of the MTD of BCNU alone) after administration of O^6BG sufficient to achieve complete AGAT depletion in either the s.c or the i.c locations [15, 17].

The utility of O^6BG has also been demonstrated as an effective AGAT depletor in a wide variety of systemic malignancies. Exquisite sensitization to BCNU has been obtained by Macgull-Seltenreich and Zeller [25] after treatment of human ovarian cell lines with O^6BG, leading to the suggestion that regional (i.p) O^6BG/BCNU may be very effective for the treatment of advanced ovarian cancer. Although the combination O^6BG/BCNU considerably improves the therapeutic efficacy of BCNU-resistant s.c human colon tumor xenografts in mice [10, 21], both studies reported the necessity to reduce the BCNU dose two- to fourfold when combined with O^6BG to prevent toxicity-induced deaths. Further, although combination therapy is more effective than BCNU alone, an eightfold reduction in BCNU dose is required when given after O^6BG to treat prostatic carcinoma xenografts in rats [9]. It is likely that preclinical testing for other high AGAT-expressing malignancies will reveal similar requirements for dose limitations of BCNU after pretreatment with O^6BG.

Our studies, therefore, were designed to examine the feasibility of regional administration of O^6BG to minimize O^6BG doses required for AGAT depletion, while maximizing subsequently administerable BCNU doses. The rapid inactivation of AGAT by O^6BG suggests that the i.a route of administration may be ideal for obtaining regional modulation of i.c tumor AGAT. To the best of our knowledge, our experiments have shown for the first time, that indeed, a highly significant dose reduction (fourfold) of O^6BG can be obtained by i.a. delivery to obtain complete AGAT depletion in i.c. tumors compared with i.p. administration. This allowed dose escalation of BCNU up to fourfold in our model, raising subsequent BCNU doses to nearly 70% of the MTD of BCNU alone. There appears to be no histopathologic evidence of brain toxicity at the optimal effective therapeutic combination doses (Table 3), with the maximum pathologic changes evident in the group of rats that received BCNU alone at its MTD (37.5 mg/kg) in comparison with controls. Histologic evaluation of a constitutively high AGAT-containing systemic organ such as the liver revealed no differences in toxic effects between the optimal effective therapeutic combination in comparison with controls, although only slight elevation of BCNU (to 12.5 from 6.25 mg/kg, preliminary toxicity studies) with i.p. O^6BG resulted in severe hepatic and cerebral toxic effects (Figs. 2, 3).

The conceptual implications of this study are of further significance in view of the demonstration of the potentiating activity of O^6BG with other nitrosoureas, including BCNU, CCNU [1], ACNU [28], and also temozolomide [1]. Regional administration of O^6BG or other rapid AGAT inactivators may be adopted for many tumor sites, with potentially equivalent beneficial therapeutic effects. Selective cannulation of various relevant arteries is an obvious mechanism to apply our findings in a clinical setting. The degree of technical refinement already possible with such procedures should render it a relatively simple task to deliver O^6BG in a highly selective manner. For conditions such as neoplastic meningitis, intrathecal instillation of O^6BG should result in a similar "regional sensitizing" effect. Further, the use of stereotactic microinfusion techniques for direct intratumoral instillation may facilitate even more selective sensitization [2, 32]. These procedures, developed initially for the improved dispersion of macromolecules in the brain, may be ideal for promoting access of a smaller molecule such as O^6BG

to remote regions of intracerebral tumors. The observation that hypoxic regions of tumors exposed to O^6BG are more sensitive to BCNU [41] may further aid in the effective therapy of tumors such as glioblastomas, where spatial intratumoral hypoxia is a common finding.

To summarize, we have demonstrated in a preclinical human brain tumor model for the first time that it is possible to selectively enhance the effect of systemic nitrosourea chemotherapy by prior regional administration of O^6BG to deplete the enzyme AGAT and therefore restore chemosensitivity of resistant brain tumor cells to nitrosoureas. We recommend, based on these results, that clinical trials be considered to enable effective application of this approach to patients with brain tumors.

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