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ORIGINAL ARTICLE

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Toxicity of intracranial and intraperitoneal O6-benzyl guanine in combination with BCNU delivered locally in a mouse model

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Abstract *Purpose:* The DNA-repair protein, O6-alkylguanine-DNA alkyl transferase, may account for resistance of CNS tumors to DNA-alkylating drugs, such as bis-(2-chloroethyl)-1-nitrosourea (BCNU). The therapeutic effects of BCNU can be potentiated by inhibiting the repair protein with an alkylated guanine analog, O6-benzyl guanine (O6BG). To investigate potential toxicity of this inhibition, we examined the effects of O6BG in mice treated with intracranial (i.c.) BCNU given via a biodegradable polymer. *Methods:* Mice were treated with escalating doses of BCNU chronically delivered i.c., and with chronically delivered O6BG. The O6BG was delivered via a 7-day intraperitoneal (i.p.) or i.c. osmotic minipump. Toxicity of the combination therapies was measured from survival data. Bone marrow response was estimated from white blood cell counts. *Results:* Combining systemic (i.p.) O6BG with locally (i.c.) delivered BCNU resulted in a decrease in the maximum tolerated dose (MTD) of local BCNU. With local delivery of O6BG, the MTD of BCNU in combination with O6BG was increased. *Conclusions:* Based on the results of this study, a dose escalation study will be necessary when combining systemic O6BG with the higher doses of i.c. BCNU.

Keywords O6-benzyl guanine (O6BG) · O6-alkylguanine-DNA alkyl transferase (AGAT, MGMT) · Bis-chloroethyl nitrosourea (BCNU)

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Introduction

Nitrosoureas are among the few drugs to benefit brain tumor patients. Thus it is important to maximize the therapeutic potential of these agents [12, 15, 17, 23]. Nitrosoureas induce mutations and cell death by alkylating DNA at the O-6 position of guanine [6, 18, 19]. Tumors resist damage by expressing a dealkylating repair protein, O6-alkylguanine-DNA alkyl transferase (frequently abbreviated "MGMT") [4, 11, 18, 24]. Generally, tumors that have high levels of MGMT (mer^+ phenotype) resist alkylating agents, while tumors with low levels (mer^-) are sensitive to these drugs [13].

Local delivery tools overcome blood-brain barrier limitations and reduce the toxicity associated with systemic therapy [1, 2]. The benefit-to-risk ratio of BCNU increases significantly with intracranial (i.c.) delivery [25]. Clinical trials confirm that local therapy increases survival in brain tumor patients [3]. Secondly, in vitro studies have shown that mer^+ cells become sensitive to alkylating agents after MGMT is inhibited with small molecular weight guanine analogs [20]. In vivo studies using the MGMT inhibitor O6BG have shown promising results. Mice with mer^+ tumors survive longer when treated systemically with a nitrosourea plus O6BG compared to controls treated with BCNU alone [9, 14]. Phase I clinical trials with O6BG and BCNU have shown no toxicity attributable to O6BG alone; bone marrow suppression is the primary toxicity in combined therapy [10, 23].

We have used sustained-release local delivery technology to further study the effects of O6BG-nitrosourea therapy. In a 9L glioma brain tumor model, O6BG given systemically along with BCNU delivered locally in the brain via a polymer resulted in a significant prolongation of median survival when compared to BCNU polymer alone [21]. In this study, we evaluated the toxicity of combinations of O6BG given i.c. and intraperitoneally (i.p.) with increasing doses of BCNU given i.c.

Materials and methods

BCNU was obtained from Bristol-Myers Squibb (Princeton, N.J.). O6-Benzyl guanine was obtained from Dr. Mary Wolpert, National Cancer Institute, (Bethesda, Md.). Cell culture supplies were obtained from Gibco (Rockville, Md.). Poly-[1,3-bis(carboxyphenoxy)propane-*co*-sebacic acid] anhydride was supplied by Guilford Pharmaceuticals Corporation (Baltimore, Md.).

Osmotic pumps (capacity 200 μl) and brain cannula were from ALZA Corporation (Palo Alto, Calif.). The O6BG solutions were prepared by mixing 20 mg with 0.2 ml ethanol and 1.8 ml propylene glycol (Sigma, St. Louis, Mo.). The mixture was sonicated at 50–55°C until the O6BG was evenly dispersed. The maximum solubility of O6BG in this system was approximately 10 mg/ml. Dilutions were prepared in propylene glycol. Pumps were loaded with O6BG solutions and stored for 4 h in phosphate-buffered saline at 37°C before i.c. or i.p. implantation. The ultraviolet spectra of O6BG in saline and in propylene glycol for 14 days at 37°C were compared to the respective spectrum of the fresh compound. The similarities between the spectra from the fresh and the stored compounds indicated no loss of drug activity under the experimental conditions.

Graded amounts of BCNU were blended with polymer and 2 ml HPLC-grade methylene chloride (Fisher Scientific, Fair Lawn, N.J.). In a typical experiment, 20 mg BCNU was mixed with 180 mg polymer to afford a 10% (w/w) BCNU polymer. Blank polymers were prepared without BCNU. The solution was protected from light, evaporated to dryness at room temperature, and stored in a desiccator for 1–2 days. Disc-shaped polymers 1.5 mm in diameter, 0.5 mm high and 5 mg in weight were pressed and stored desiccated at –70°C for up to 4 days before use. Polymers containing 0, 3.8, 10, and 20% BCNU were prepared. A 20% polymer thus contained 1 mg BCNU. All BCNU polymers were implanted i.c.

Tail blood was used for hematology studies. White blood cell (WBC) counts were measured in 5-μl aliquots diluted in 200 μl 2% acetic acid. Erythrocytes were measured in EDTA-anticoagulated blood. Histology was conducted on 4% buffered formalin-fixed specimens. Non-surviving animals were examined for signs of infection and hematomas at the polymer and pump sites. Balb/C female mice 6–12 weeks old were from Harlan (Indianapolis, Ind.). Mice were housed and treated in accordance with Johns Hopkins University Animal Care and Use Committee policies. Animals with i.c. pump implants were housed in individual cages.

Mice were anesthetized with 0.2 ml of a solution containing 8 mg/ml ketamine hydrochloride, 0.8 mg/ml xylazine, and 4.7% ethyl alcohol in saline. Surgical surfaces were shaved, washed with 70% ethyl alcohol and with Betadyne. To place i.c. polymers and osmotic pump cannulas, a midline cranial incision was made and sutures identified. A 2-mm burr hole was made centered over the left parietal lobe. Pump cannulas were placed to a depth of 3 mm in the burr hole, and fixed in place with surgical glue. The body of the pump was implanted subcutaneously on the back of the anesthetized mouse slightly posterior to the scapulae in a pocket created by inserting a hemostat into a mid-scapular incision, and by spreading the subcutaneous tissue by opening the jaws of the hemostat. The pocket was large enough to allow some free movement of the pump, i.e. 1 cm longer than the pump. The pump with sufficient catheter tubing to pass through a tunnel created under the skin of the back and neck to the site of the burr hole was inserted. The anterior end of the catheter was connected to the brain infusion cannula. Polymers were placed in an incision 2–3 mm deep made with a scalpel blade. Wounds were closed with 4.0 vicryl or surgical staples. When i.c. pump and polymer therapy were combined, the polymer was placed in a burr hole left of midline, and the pump cannula was placed to the right of the midline.

For i.p. pump use, the pump was implanted slightly posterior to the scapulae in a pocket as described above. A 1-cm incision was made into the peritoneal space through a tented abdominal surface. Approximately 5 mm proximal the tip of a catheter was secured with a purse string suture and passed through the viscera under the skin to connect to the mid-scapular pump body.

Pumps were removed under ketamine-xylazine anesthesia 2–3 days after their delivery schedule, and examined to verify that the solution had been discharged. The pump pocket was examined for signs of infection. Catheter lines were sealed with a suture and left in place.

Survival was the end-point of the toxicity studies.

Results

The maximum tolerated doses of sustained-release local therapies were measured in normal mice. For reference, BCNU and O6BG dose equivalents based on an average mouse body weight (20 g) and body surface area are given in Table 1. BCNU was delivered i.c. as a polymer-bound drug. O6BG was delivered in a single dose i.p., and as a chronic infusion i.p. or i.c. Because of its limited solubility, 2 mg O6BG (0.2 ml of a 10 mg/ml solution) was the maximum dose tested.

As shown in Table 2, Balb/C mice implanted i.c. with 3.8% BCNU polymers survived a single i.p. injection of 1 mg O6BG for at least 150 days. Intracranial infusions of 1 and 2 mg O6BG given together with a blank polymer had limited toxicity. One mouse in the 1-mg i.c. dose group died on day 29. The cause of death was unknown. No deaths occurred in groups of five mice treated i.c. with 3.8% BCNU polymers and i.c. osmotic pumps containing carrier solution (0 mg O6BG) and 1 mg O6BG (70 μg per day). On day 15, there was one death in the group dosed with 2 mg (140 μg per day) O6BG. An autopsy of the animal that died in the 2-mg group was unremarkable. Single i.p. injections and chronic i.c. infusions of O6BG in mice with 10% and 20% BCNU polymers resulted in greater toxicity. Deaths in the 10% and 20% groups occurred as early as day 4 and day 5 (Table 2).

The bone marrow response of combined therapy was estimated from peripheral WBC counts. Pilot studies revealed that i.p. bolus injections of 1 mg BCNU led to leukopenia on day 3 or 4. The WBC count rebounded and frequently was elevated on day 8 before returning to normal levels by day 16. Bolus i.p. injections of 1 mg BCNU and 1 mg O6BG led to a

Table 1. O6BG and BCNU dose equivalents (from reference 5, pp 292–293)

Drug	Delivery	mg	mg/kg	mg/m^2
BCNU	i.c. polymer (%)			
	3.8	0.19	9.5	28.5
	10	0.50	25.0	75.0
	20	1.0	50.0	150
O6BG	i.c. or i.p. pump (mg)			
	0.5	0.5	25.0	75.0
	1.0	1.0	50.0	150
	2.0	2.0	100	300
	Single i.p. injection			
	1.0	1.0	50.0	150

Table 2. Toxicity of combined O6BG plus BCNU therapy in normal Balb/C mice. Five mice were tested in each group. O6BG pumps and i.p. bolus injections were given at the time of BCNU-polymer implantation

BCNU i.c. polymer (%)	O6BG		Survival 150 days	Day of death
	i.p. bolus (mg)	i.c. pump (mg)		
0 (blank)	1		5/5	
3.8	1		5/5	
10	1		2/5	6, 8, 25
20	1		1/5	5, 14, 21, 25
0 (blank)		1 (70 μg/day)	4/5	29
0		2 (140 μg/day)	5/5	
3.8		0 (blank)	5/5	
3.8		1	5/5	
3.8		2	4/5	15
10		1	4/5	7
10		2	3/5	4, 4
20		1	1/5	5, 6, 9, 11
20		2	1/5	4, 7, 8, 10

severe leukopenia on day 3 or 4, which remained depressed on day 8. These animals failed to thrive and were killed. The WBC counts of mice treated with combinations of 3.8% BCNU polymers and i.p. pumps delivering 1 mg O6BG (70 μg/day) were similar to the counts observed in mice given a single i.p. bolus injection of BCNU. Counts were decreased on day 4, elevated on day 8 and within normal limits on day 16 (Table 3). However, unlike the mice that received bolus i.p. doses of combined therapy, the mice that received combination sustained-release therapy appeared healthy and survived at least 60 days. Groups of mice treated with i.c. polymers containing10% and 20% BCNU combined with i.p. pumps containing 1 mg O6BG had decreased WBC counts on day 4. Two mice died in the 10% BCNU group before day 6. One mouse died in the 20% polymer group on day 17. Significantly less toxicity was observed from the combined i.c. treatment with 3.8% BCNU polymer and up to 2 mg O6BG (Table 4). These animals had normal WBC counts on day 3. Counts remained within normal

Table 3. Effects of systemic O6BG and i.c. BCNU on WBC in normal Balb/C mice. Values are average ± SD counts ($n = 3$ mice/group)

BCNU i.c. polymer (%)	O6BG i.p. pump (mg)	WBC count (cells/μl ×1000)		
		Day 4	Day 8	Day 16
0 (blank)	0.0 (blank)	7.5 ± 2.2	5.2 ± 0.5	14.3 ± 2.6[c]
0 (blank)	1	6.4 ± 1.1	6.9 ± 0.8	4.8 ± 0.2
3.8	1	2.1 ± 0.3	17.6 ± 5.8	5.3 ± 2.0
10	1	1.7 ± 0.3[a]	3.1	5.2
20	1	2.3 ± 0.9	3.5 ± 0.3	4.1 ± 2.0[b]

[a]Two deaths on day 5
[b]One death on day 17
[c]Literature values for WBC counts in mice vary considerably [22]. Repeated sampling can lead to leukocytosis, an effect that has been attributed to an inflammatory response [22]

Table 4. Effects of i.c. O6BG and BCNU on WBC in normal Balb/C mice

BCNU i.c. polymer (%)	O6BG i.c. pump (mg)	WBC Count (cells/μl ×1000)			
		Day 3	Day 7	Day 16	Day 23
3.8	0.02	6.6	11.4	18.0	13.2
3.8	0.2	5.1	6.9	7.8	10.5
3.8	1	4.5	9.9	13.5	15.6
3.8	2	7.2	12.6	8.1	10.8

limits or had become elevated by day 7 or subsequently.

Our laboratory normal values for WBC differential counts in Balb/C mice are 10–15% neutrophils, 10–15% monocytes, and 70–80% lymphocytes. The percent monocytes/neutrophils routinely increased to 40–60% in mice treated with combination O6BG/BCNU doses. Red blood cell morphology and platelets in the O6BG plus BCNU-treated leukopenic groups appeared normal at the time the animals were killed.

The surviving animals treated with 10% and 20% BCNU polymers (Table 3) were killed on day 16 or 17 for pathology studies. Macrophages, giant cells, fragments of polymer and gemistocytic astrocytes were present at the site of the blank polymer implant. Other organs were within normal limits. There was necrosis of neutrophils adjacent to the polymer site in the BCNU groups. Minimal inflammation consisting of macrophages and a few neutrophils was observed. Occasional cells were seen with marked karyomegaly which were not identified. Marked frank necrosis with minimal inflammatory infiltrate consisting of a few neutrophils was seen in the BCNU plus O6BG group. In one animal in the 10% BCNU group there was severe multifocal necrosis of the liver, and necrosis of multinucleated cells in the bone marrow which were compatible with megakaryocytes. A focus of basophilia near the surface of the polymer site might have represented bacterial infection. All other organs were normal.

Discussion

Combination therapy with O6BG and BCNU has the potential to improve tumor control in patients with high-grade gliomas. The treatment strategy utilizing locally delivered intratumoral BCNU via a biodegradable polymer limits systemic exposure to BCNU. This strategy has been validated in several clinical trials [3, 26], and has been approved by the FDA. Combining locally delivered BCNU with O6BG has the potential to limit systemic side effects, because of the lower systemic BCNU levels, and to maximize the synergistic effect of the two drugs in the brain. This study explored the toxicity of BCNU plus O6BG delivered via systemic and local routes in a mouse model.

A 20% loaded BCNU polymer implanted in the brain is the MTD in this animal model [7, 8]. Clinically, a 3.8% loaded polymer is used [3, 26]. In this study, we found that as the BCNU load was increased from 3.8% to 20% in the presence of systemic O6BG, toxicity increased, such that with a 20% loaded dose only one of five animals survived. The toxicity and cause of death was systemic toxicity, evidenced by the WBC changes measured. The conclusion from these studies is that the systemic BCNU levels when the higher loaded polymers are implanted is significant.

Based on these results, we hypothesized that locally delivered O6BG may reduce the toxicity of the combination treatment. When O6BG was delivered i.c. with a 10% loaded polymer, the survival was improved compared to systemic O6BG and 10% loaded BCNU polymer. Toxicity was not reduced when combining i.c. delivery of O6BG with a 20% loaded polymer. Therefore, it appears that by delivering O6BG i.c. rather than i.p., the maximum polymer load can be increased from 3.8% to 10%.

In summary, in this animal model, combining i.p. O6BG with locally delivered BCNU resulted in a decrease in the MTD of the locally delivered BCNU. By changing the route of delivery of O6BG to i.c., the MTD of locally delivered BCNU in combination with O6BG was increased.

These results will be used to plan future clinical trials. Systemic O6BG plus 3.8% loaded BCNU polymer is in clinical trials currently [10, 23]. A dose escalation trial with the BCNU polymer has demonstrated that the MTD is 20% loaded BCNU polymer [16]. Based on the results of this study, a dose-escalation study will be necessary when combining systemic O6BG with the higher load of BCNU in the polymer. An additional approach to maximize this potential synergy would be to deliver both drugs locally in the brain.

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References

1. Bakhshi S, North RB (1995) Implantable pumps for drug delivery to the brain. J Neurooncol 26:133–139
2. Brem H (1990) Polymers to treat brain tumors. Biomaterials 11:699–701
3. Brem H, Piantadosi S, Purger PC, Walker M, Selker R, Vick NA, Black K, Sisti M, Brem S, Mohr G, Muller P, Morawetz R, Schold SC (1995) Placebo-controlled trial of safety and efficacy of intraoperative controlled delivery by biodegradable polymers of chemotherapy for recurrent gliomas. Lancet 345:1008–1012
4. Colvin M, Russo JE, Hilton J, Dulik DM, Fenselau C (1988) Enzymic mechanisms of resistance to alkylating agents in tumor cells and normal tissues. Adv Enzyme Regul 27:211–221
5. DeVita VT Jr (1985) Principles of chemotherapy. In: DeVita VT Jr, Hellman S, Rosenberg SA (eds.) Cancer. Principles and practice of oncology, vol 1. J.B. Lippincott, Philadelphia, pp 257–286
6. Engelbergs J, Thomale J, Galhoff A, Rajewsky MF (1998) Fast repair of O6-ethylguanine, but not O6-methylguanine, in transcribed genes prevents mutation of H-*ras* in rat mammary tumorigenesis induced by ethylnitrosourea in place of methylnitrosourea. J Biol Chem 95:1635–1640
7. Ewend MG, Williams JA, Tabassi K, Tyler BM, Babel KM, Anderson RC, Pinn ML, Brat DJ, Brem H (1996) Local delivery of chemotherapy and concurrent external beam radiotherapy prolongs survival in metastatic brain tumor models. Cancer Res 56:5217–5223
8. Ewend MG, Sampath P, Williams JA, Tyler BA, Brem H (1998) Local delivery of chemotherapy prolongs survival in experimental brain metastases from breast carcinoma. Neurosurgery 43:1185–1193
9. Felker GM, Friedman HS, Dolan ME, Moschel RC, Schold C (1993) Treatment of subcutaneous and i.c. brain tumor xenografts with O6-benzylguanine and 1,3-bis(2-chloroethyl)-1-nitrosourea. Cancer Chemother Pharmacol 32:471–476
10. Friedman HS, Pluda J, Quinn JA, Ewesuedo RB, Long L, Friedman AH, Cokgor I, Colvin OM, Haglund MM, Ashley DM, Rich JN, Sampson J, Pegg AE, Moschel RC, McLendon RE, Provenzale JM, Stewart ES, Tourt-Uhlig S, Garcia-Turner AM, Herndon JE, Bigner DD, Dolan ME (2000) Phase I trial of carmustine plus O6-benzylguanine for patients with recurrent or progressive malignant glioma. J Clin Oncol 18:3522–3528
11. Hongeng S, Brent TP, Sanford RA, Li H, Kun LE, Heideman RL (1997) O6-Methylguanine-DNA methyltransferase protein levels in pediatric brain tumors. Clinical Cancer Res 3:2459–2463
12. Keir ST, Dolan ME, Pegg AE, Lawless A, Moschel RC, Bigner DD, Friedman HS (2000) O6-Benzylguanine-mediated enhancement of nitrosourea activity in Mer$^-$ central nervous system tumor xenografts – implications for clinical trials. Cancer Chemother Pharmacol 45:437–440
13. Kokkinakis DM, Bocangel DB, Schold SC, Moschel RC, Pegg AE (2001) Thresholds of O6-alkylguanine-DNA alkyltransferase which confer significant resistance of human glial tumor xenografts to treatment with 1,3-bis(2-chloroethyl)-1-nitrosourea or temozolomide. Clin Cancer Res 7:421–428
14. Kurpad SN, Dolan ME, McLendon RE, Archer GE, Moschel RC, Pegg AE, Bigner DD, Friedman HS (1997) Intraarterial O6-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
15. Matthay KK (1997) Neuroblastoma: biology and therapy. Oncology 11:1857–1866
16. Olivi A, Barker F, Tatter S, Bruce J, Judy K, Burak E, Hilt D, Grossman SA, Fisher J, Piantadosi S (2000) Results of a phase I study of interstitial BCNU administration via wafers to patients with recurrent malignant gliomas. Proc Am Soc Clin Oncol 19:160a
17. Pech IV, Peterson K, Cairncross JG (1998) Chemotherapy for brain tumors. Oncology 12:537–553
18. Pegg AE, Dolan ME, Moschel RC (1995) Structure, function, and inhibition of O6-alkylguanine-DNA alkyltransferase. Prog Nucleic Acid Res 51:167–223
19. Petersdorf SH, Berger MS (1996) Molecular basis of chemotherapy for brain tumors. In: Raffel C, Harsh GR (eds) The molecular basis of neurosurgical disease, vol 8. Williams and Wilkins, Baltimore, pp 198–210
20. Pieper RO (1997) Understanding and manipulating O6-methylguanine-DNA methyltransferase expression. Pharmacol Ther 74:285–297
21. Rhines LD, Sampath P, Dolan ME, Tyler BM, Brem H, Weingart J (2000) O6-benzylguanine potentiates the antitumor effect of locally delivered carmustine against an intracranial rat glioma. Cancer Res 60:6307–6310
22. Schermer S (1967) The white mouse. The blood morphology of laboratory animals. F.A. Davis Company, Philadelphia, pp 61–74

23. Schilsky RL, Dolan ME, Bertucci D, Ewesuedo RB, Vogelzang NJ, Mani S, Wilson LR, Ratain MJ (2000) Phase I clinical and pharmacological study of O6-benzylguanine followed by carmustine in patients with advanced cancer. Clin Cancer Res 6:3025–3031
24. Silber JR, Bobola MS, Ghatan S, Blank A, Kolstoe D, Berger MS (1998) O6-Methylguanine-DNA methyltransferase activity in adult gliomas: relation to patient and tumor characteristics. Cancer Research 58:1068–1073
25. Storm PB, Brem H (1997) The treatment of brain tumors with drug-impregnated biodegradable polymers. In: Kornblinth PL, Walker MD (eds) Advances in neuro-oncology II. Futura Publishing, Armonk, pp 435–446
26. Valtonen S, Timonen U, Toivanen P, Kalimo H, Kivipelto L, Heiskanen O, Unsgaard G, Kuurne T (1997) Interstitial chemotherapy with carmustine-loaded polymers for high-grade gliomas: a randomized double-blind study. Neurosurgery 41:44–49