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Treatment of subcutaneous and intracranial brain tumor xenografts with O^6-benzylguanine and 1,3-bis(2-chloroethyl)-1-nitrosourea

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Abstract. O^6-Alkylguanine-DNA alkyltransferase (AT) is a cellular protein that protects cells from the cytotoxic effects of nitrosoureas by repairing alkyl lesions at the O^6 position of guanine. We have studied the ability of O^6-benzylguanine to deplete AT activity in brain tumor xenografts and thereby increase the sensitivity of these tumors to 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU). In toxicity studies, pretreatment of athymic mice with O^6-benzylguanine increased the toxicity of BCNU significantly. After i.p. injection of O^6-benzylguanine into athymic mice carrying subcutaneous (s.c.) D341MED, a human medulloblastoma xenograft with a high AT activity, the AT activity of the tumors became undetectable within 1 h and remained depleted until 36 h. In s.c. xenografts to D341MED, treatment with O^6-benzylguanine followed 1 h later by BCNU produced a significantly greater growth delay (14.8 days) than was seen with BCNU alone (2.3 days). A lower pretreatment dose of O^6-benzylguanine produced a significantly smaller therapeutic effect. Delaying the administration of BCNU until 36 h after O^6-benzylguanine resulted in a growth delay (1.2 days) that was not significantly different from that produced by the control or BCNU alone. In athymic mice with intracranial (i.c.) xenografts of D341MED, pretreatment with O^6-benzylguanine followed 1 h later by BCNU produced a significantly increased survival as compared with that of the control, BCNU alone, O^6-benzylguanine alone, and O^6-benzylguanine followed 36 h later by BCNU. In experiments with s.c. xenografts of D245MG, a human glioma xenograft with undetectable AT activity, pretreatment with O^6-benzylguanine 1 h prior to BCNU produced a significantly greater effect than was seen with BCNU treatment alone. The combination regimen, however, was not as effective as an equitoxic dose of BCNU alone. These studies suggest that O^6-benzylguanine may be a useful adjuvant to nitrosourea therapy in human malignancies that exhibit a range of AT activities and that dose and timing are important variables in achieving therapeutic success. These data also indicate that therapeutic potentiation of BCNU by O^6-benzylguanine can be achieved in i.c. tumors. As a result, this approach may be useful in the treatment of neoplasms of the central nervous system.

Introduction

Alkylating agents are one of the few classes of drugs that have shown efficacy against neoplasms of the central nervous system [18]. Among the most commonly used are the chloroethylnitrosoureas, such as BCNU, which produce their tumoricidal effects through chloroethylation at the O^6 position of guanine in DNA. This is followed by intramolecular rearrangement to a cyclized intermediate, N^1,O^6-ethanoguanine, which cross-links with the complementary cytosine of the opposite strand to form the cytotoxic lesion [10, 11, 21, 26]. A cellular repair protein, AT, protects cells against such alkylation damage by removing these chloroethyl adducts before cross-linking can occur [22, 28]. This occurs through a stoichiometric transfer of the chloroethyl group to the cysteine moiety within the active site of the alkyltransferase protein [28]. This reaction permanently inactivates the AT such that subsequent repair of further alkylation damage depends on new protein synthesis [22]. AT has been found in almost all human tissues, with liver being among the highest and brain among the lowest [17]. Generally, central nervous system neoplasms have higher AT activity than normal brain, although there is significant variability among

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Abbreviations: AT, O^6-alkylguanine-DNA alkyltransferase; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; GD, growth delay; LD_{10}, dose lethal to 10% of the population; DTT, dithiothreitol; BG, O^6-benzylguanine

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different tumor types [12, 27]. Tumors with high levels of AT (termed mer+) have been shown to be resistant to the effects of alkylating agents, while those lacking AT activity (mer–) are sensitive to these drugs [1, 6, 25].

Attempts have been made to inhibit the AT repair system by pretreatment with methylating agents such as streptozotocin, but the increased toxicity and the potential for secondary mutagenesis have made this approach problematic [13, 14]. O^6-Methylguanine has been shown to decrease AT activity significantly in vitro, but the high doses of O^6-methylguanine required and its limited solubility present significant practical difficulties in in vivo studies [4, 5, 16, 30]. O^6-benzylguanine and its analogues, however, have been shown to deplete AT activity effectively and to increase the cytotoxic effect of nitrosoureas in both cultured cells and xenografts [7–9, 19]. In the present studies, we evaluated the ability of O^6-benzylguanine to deplete AT activity in human brain tumors growing as s.c. xenografts in athymic mice. We examined the effect of O^6-benzylguanine pretreatment on the sensitivity of s.c. brain tumor xenografts to BCNU and evaluated the effect of variation in the dosing and timing of treatment. We also examined the effect of this combination treatment on survival in athymic mice with i.c. xenografts.

Materials and methods

Animals. Homozygous adult nu/nu BALB/c athymic mice derived from an independent breeding colony at Duke University were used in these experiments.

Drugs. BCNU (carmustine, Bristol Laboratories, Evansville, Ind.) was purchased commercially. O^6-Benzylguanine was synthesized by one of the authors (R.C.M.) as previously described [22]. BCNU was dissolved in ethanol (100 mg/3 ml) and diluted in normal saline. It was given by intraperitoneal (i.p.) injection in a volume of 30 ml/m^2. O^6-Benzylguanine was dissolved in 10% cremaphor EL (Sigma) and normal saline and given by i.p. injection in a volume of 90 ml/m^2. BCNU was given as a single dose (38 mg/m^2) that represented 38% of its LD_{10} as determined in animals derived from our colony. O^6-Benzylguanine was given at doses of 300, 225, or 150 mg/m^2.

Tumor lines. D341MED is a human brain tumor xenograft derived from a human medulloblastoma. It has a high level of AT activity (1,134 fmol/mg protein). The treatment experiments described herein were done between the 35th and 40th animal passage levels. D245MG is a human brain tumor xenograft that was established in athymic mice at Duke University after direct transplantation from a resected cerebral glioblastoma. It is highly sensitive to nitrosoureas and procarbazine and has an undetectable level of AT activity using our technique. The treatment experiments reported herein were done between the 20th and 30th animal passage levels.

AT depletion experiments. Mice with s.c. D341MED xenografts measuring between 500 and 1000 mm^3 were treated i.p. with O^6-benzylguanine at doses of 300 or 225 mg/m^2, and tumors were removed at various times up to 48 h. Tumors were excised and quick-frozen in liquid nitrogen, and the AT activity of the tumors was determined. Each time point represents the mean of the AT level from three animals.

Treatment experiments. The treatment of s.c. tumor xenografts in athymic mice has been described previously [2, 24]. Briefly, 50 µl of tumor suspension was injected into the right flank of 50–60 animals. Tumors were measured twice weekly with calipers, and tumor volume was estimated by the formula: volume = (length × width^2)/2. When the median tumor volume of all animals had exceeded 200 mm^3, animals were randomized into treatment groups such that there were 8–12 animals/group, with the average tumor size among the groups being approximately equal. Tumor volumes were measured three times per week until each tumor's volume exceeded 5 times its volume on the day of treatment. The treatment of i.c. xenografts has been described previously [23]. Briefly, animals were anesthetized i.p. with sodium pentobarbital; 5 µl of tumor suspension that contained 50% methylcellulose was injected through a 25-gauge needle into the right cerebral hemisphere to a depth of 4 mm. Animals were treated on the 15th day after tumor implantation, with the day of implantation being taken as day 1.

Evaluation of response. For s.c. treatment experiments, the therapeutic response was measured in two ways. First, the number of days required for each tumor to reach 5 times its treatment volume was determined. These values were compared among groups by Wilcoxon rank-sum analysis. Growth delay was calculated as the difference in days between the median of treated tumors and the median of control tumors. Second, the number of tumor regressions in each group was determined. A tumor regression was defined as any posttreatment volume less than the volume recorded on the day of treatment. These values were compared among groups using the Fisher exact test. For i.c. treatment experiments, the day of death of each animal was recorded and these values were compared among groups by the Wilcoxon rank-sum test.

AT assay. Subcutaneous xenografts (500–1000 mm^3) were excised and rapidly frozen in liquid nitrogen. For analysis, the frozen tumor was weighed and suspended in 4 vol. of buffer that contained 0.05 *M* TRIS-HCl (pH 7.5), 0.002 *M* ethylenediaminetetraacetic acid (EDTA), 0.1 *M* NaCl, 0.001 *M* dithiothreitol (DTT), 0.02% sodium azide, 0.2 m*M* phenylmethylsulfonyl fluoride, and 20 trypsin-inhibition units of apoprotinin/l. The tumor was homogenized and sonicated for 30 s. The extract was centrifuged for 60 min at 35,000 rpm. The supernatant was removed and frozen at –70° C. Protein was quantified using a Bio-Rad assay kit (Bio-Rad Laboratories, Richmond, Calif.).

AT activity was determined by quantifying the ability of the protein extract to remove tritiated methyl groups from the O^6-methylguanine in an oligonucleotide substrate [20]. Aliquots of 10–100 µl of tumor extract were added to the assay buffer, which contained 0.01 *M* TRIS-HCl (pH 7.5), 0.002 *M* EDTA, 0.001 *M* DTT, 10% glycerol, and 0.02% sodium azide, such that the total volume was 190 µl. The addition of 10 µl DNA substrate that contained 1.1 pmol O^6-[^{3}H]-methylguanine (specific activity, 1.75 Ci/mmol; prepared by reacting calf thymus with [^{3}H]-methylnitrosourea) initiated the reaction. After the reaction mixture had been incubated at 37° C for 30 min, the addition of 500 µl of 5% (w/v) trichloroacetic acid (TCA) stopped the reaction. The reaction mixture was hydrolyzed for 30 min at 80° C and cooled for 10 min at 4° C. Then, 100 µg of bovine serum albumin was added as a carrier protein. Precipitated protein was collected on 4.25-cm GF/F Whatman filters and washed with 30 ml of 5% TCA and 15 ml of 95% ethanol using a Millipore vacuum filtration apparatus. After the filters had been allowed to dry overnight, 200 µl of Amersham NCS tissue solubilizer was added to each filter, followed by 10 ml of scintillation liquid. Radioactivity was determined by scintillation counting. The AT activity was expressed as in femtomoles of [^{3}H]-methyl transferred per milligram of protein as determined by regression analysis.

Results

Toxicity studies were performed to determine the toxicity of the combination of O^6-benzylguanine and BCNU in our animal population (Table 1). Pretreatment with 300 mg/m^2 of O^6-benzylguanine increased the toxicity of BCNU significantly, such that the dose of BCNU that produced no mortality with combination therapy was 50% of the maximally tolerated dose of BCNU alone. Notably, the combi-

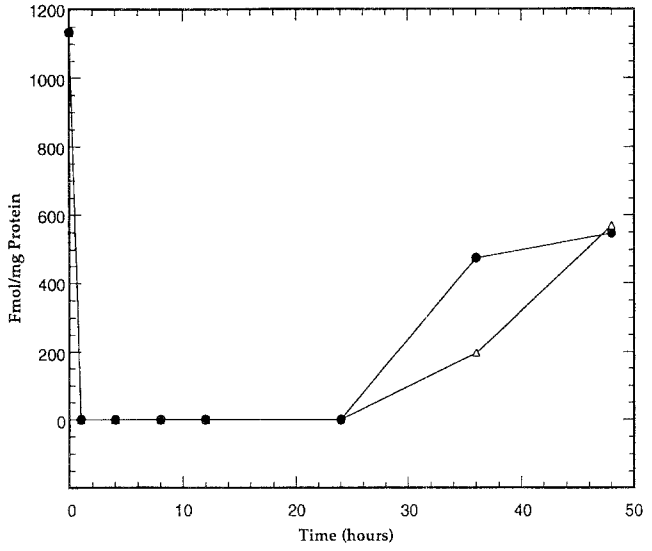

Fig. 1. AT inhibition in s.c. D341MED xenografts by O^6-benzylguanine. Athymic mice carrying D341MED xenografts received i.p. injections of 300 mg/m^2 (△) or 225 mg/m^2 (●) O^6-benzylguanine. Tumors were excised at the indicated times following injection. Data points represent the mean of 3 samples

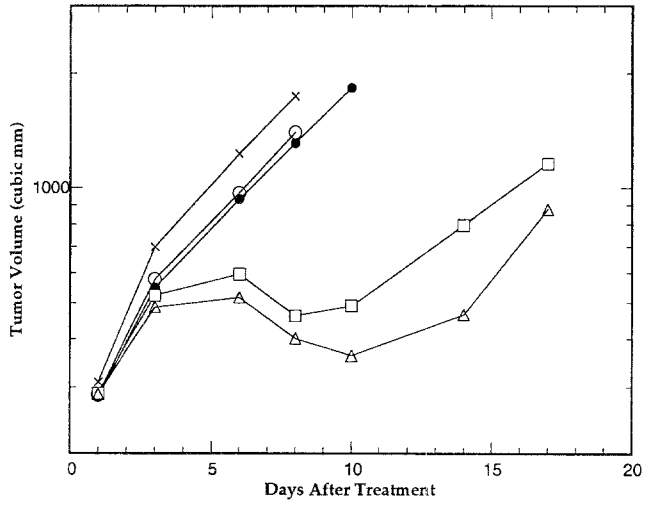

Fig. 2. Tumor growth inhibition in s.c. D341MED xenografts by O^6-benzylguanine (BG) and BCNU. Animals were treated with vehicle (X), BCNU (○), 300 mg/m^2 BG followed in 36 h by BCNU (●), 225 mg/m^2 BG followed in 1 h by BCNU (□), or 300 mg/m^2 BG followed in 1 h by BCNU (△). All BCNU doses were 38 mg/m^2. Each data point represents the mean of 10 animals. The difference in growth delay between pretreatment with 225 mg/m^2 BG and 300 mg/m^2 BG was statistically significant (P <0.01)

nation dose used in these treatment experiments, 300 mg/m^2 of O^6-benzylguanine followed 1 h later by a single treatment with 38 mg/m^2 of BCNU, produced no death in 44 animals. The LD_{10} of BCNU in our animals had previously been determined to be approximately 100 mg/m^2.

In s.c. D341MED xenografts from animals treated with 300 mg/m^2 of O^6-benzylguanine, AT activity was undetectable after 1 h and remained undetectable until 36 h after injection, reaching approximately 50% of the value in controls by 48 h. Similar inhibition was seen in animals treated with 225 mg/m^2 of O^6-benzylguanine (Fig. 1). We did not detect any significant difference in AT inhibition between the two different O^6-benzylguanine doses.

In treatment experiments using s.c. D341MED xenografts (Table 2, Fig. 2), there was little variability in tumor size within treatment groups, allowing the use of mean tumor volumes for data analysis. Pretreatment with 300 mg/m^2 of O^6-benzylguanine 1 h prior to 38 mg/m^2 of BCNU resulted in a growth delay (GD) of 14.8 days and in 6 of 10 regressions; these values were significantly greater (P <0.01) than the effect of treatment with 38 mg/m^2 of BCNU alone (GD, 2.3 days; 0 of 10 regressions). This response was also significantly greater (P <0.01) than that observed following pretreatment with the same dose of O^6-benzylguanine 36 h prior to BCNU (GD, 1.2 days; 0 of 10 regressions), which was not significantly different from the response to either the same dose of BCNU alone or the control. Pretreatment with 225 mg/m^2 of O^6-benzyl-

Table 1. Toxicity of BCNU and O^6-benzylguanine in athymic mice

O^6-Benzylguanine (mg/m^2)	BCNU (mg/m^2)	Mortality
0	100	1/10
0	75	0/10
300	100	18/18
300	75	21/28
300	50	4/14
300	38	0/44

Athymic mice were given i.p. injections of O^6-benzylguanine followed 1 h later by BCNU as indicated. Mortality: number of animal deaths 15 days after injection/number of treated animals

Table 2. Effect of O^6-benzylguanine and BCNU on brain tumor xenografts growing s.c. in athymic mice

Xenograft	BG dose (mg/m^2)	BCNU dose (mg/m^2)	Days (5X)	GD (days)	Regression
D341MED	0	0	5.5±2.1	–	0/10
	0	38	7.8±1.7	2.3	0/10
	300	38 (1 h)	20.3±3.0	14.8*	6/10**
	225	38 (1 h)	15.3±2.5	9.8*	4/10***
	300	38 (36 h)	6.7±1.1	1.2	0/10
D245MG	0	0	8.2±3.7	–	0/20
	0	38	22.2±2.6	14.0*	9/24**
	0	75	45.1±3.3	35.9*	8/8**
	300	0	7.5±2.1	–0.7	0/14
	300	38 (1 h)	30.4±6.6	22.2*	18/24**
	150	38 (1 h)	24.3±2.3	17.3*	7/14**

Results of treatment experiments in athymic mice with s.c. D341MED and D245MG xenografts. Drugs were given i.p. as indicated. Parentheses after BCNU indicate the time lapse between injections. D245MG data represent the combination of 2 separate experiments. Results were determined as indicated in materials and methods. Days (5X), number of days required to reach 5 times the original tumor volume (range given indicates SD); GD, growth delay

* P <0.01 vs control by Wilcoxon rank-sum analysis

** P <0.01 vs control by Fisher exact test

*** P <0.05 vs control by Fisher exact test

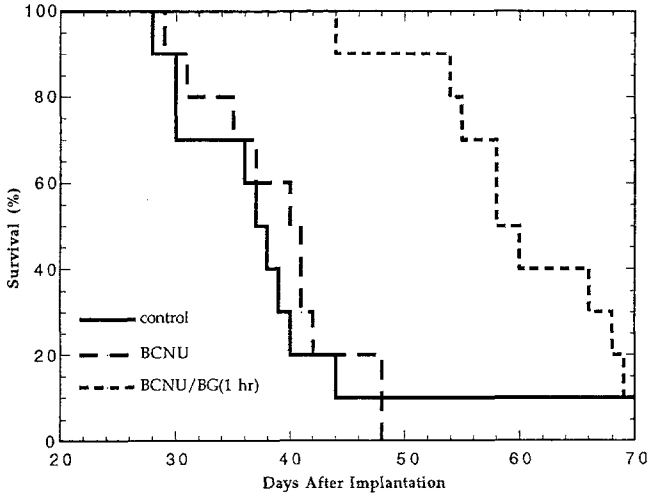

Fig. 3. Survival of athymic mice bearing i.c. D341MED xenografts after treatment with O^6-benzylguanine (BG) and BCNU. All BG doses were 300 mg/m^2. All BCNU doses were 38 mg/m^2. Animals received either drug vehicle, BCNU alone, or BG followed in 1 h by BCNU

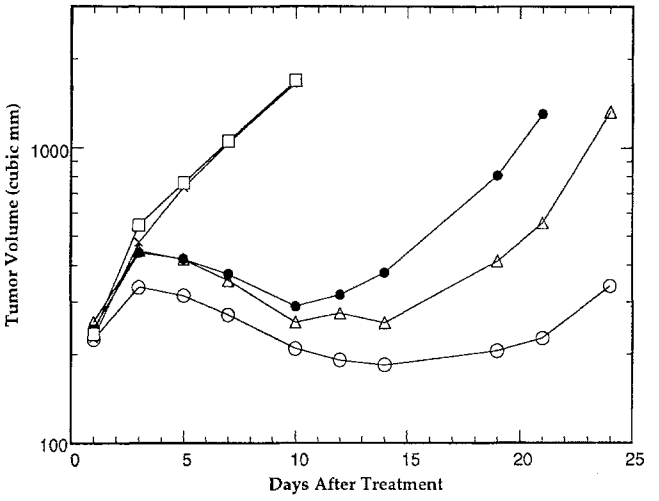

Fig. 4. Tumor growth inhibition in s.c. D245MG xenografts by O^6-benzylguanine (BG) and BCNU. Animals were treated with vehicle (X), 300 mg/m^2 BG (□), BCNU (●), 150 mg/m^2 BG followed in 1 h by BCNU (△), or 300 mg/m^2 BG followed in 1 h by BCNU (○). All BCNU doses were 38 mg/m^2. Each data point represents the mean of 10–14 animals

guanine 1 h prior to 38 mg/m^2 of BCNU gave an intermediate response (GD, 9.8 days; 4 of 10 regressions), which was significantly more effective than the control of BCNU alone and was less effective than pretreatment with 300 mg/m^2 of O^6-benzylguanine (P <0.01 in all cases, except that P <0.05 when tumor regressions were compared for 225 mg/m^2 of O^6-benzylguanine + BCNU vs BCNU alone). Notably, both of the combination regimens with a 1-h delay between drug administrations were much more effective in these tumors than in a separate experiment performed with 100 mg/m^2 of BCNU alone, which produced a growth delay of only 0.5 days and no regressions

Table 3. Effect of O^6-benzylguanine and BCNU on brain tumor xenografts growing i.c. in athymic mice

Treatment	n	Median survival (days)
Control	10	37.5±11.9
BCNU	10	40.5±14.8
BG	6	42.5±15.9
BG + BCNU (36 h)	6	42.5±11.6*
BG + BCNU (1 h)	10	59.0±8.2**

Results of treatment experiment in athymic mice with intracranial D341MED xenografts. Drugs were given i.p. as indicated. All O^6-benzylguanine (BG) doses were 300 mg/m^2. All BCNU doses were 38 mg/m^2. Parentheses after BCNU indicate the time lapse between injections. Results were determined as indicated in Materials and methods. The range given for survival indicates SD
* P <0.05 vs control by Wilcoxon rank-sum analysis
** P <0.01 vs control by Wilcoxon rank-sum analysis

while resulting in 1 death in the 10 animals treated (data not shown).

In experiments with animals bearing i.c. D341MED xenografts (Table 3, Fig. 3), animals receiving pretreatment with 300 mg/m^2 of O^6-benzylguanine 1 h prior to 38 mg/m^2 of BCNU had a median survival of 59.0 days, which was significantly greater (P <0.01) than that of animals receiving the control (37.5 days), BCNU alone (40.5 days), O^6-benzylguanine alone (42.5 days), or O^6-benzylguanine followed after 36 h by BCNU (42.5 days). Among these latter groups, only O^6-benzylguanine followed after 36 h by BCNU was statistically better than the control (P <0.05).

Animals bearing s.c. D245MG xenografts and receiving pretreatment with O^6-benzylguanine 1 h prior to treatment with BCNU showed a significantly greater growth delay than those treated with the same dose of BCNU alone (Table 2, Fig. 4). Treatment with 300 mg/m^2 of O^6-benzylguanine prior to 38 mg/m^2 of BCNU resulted in a growth delay of 22.2 days and in 18 of 24 regressions as compared with 17.3 days and 7 of 14 regressions for pretreatment with 150 mg/m^2 of O^6-benzylguanine and 14.0 days and 9 of 24 regressions for 38 mg/m^2 of BCNU alone. The differences in growth delay noted among all of these groups were statistically significant (P <0.01). The differences in tumor regression were statistically significant only for 300 mg/m^2 of O^6-benzylguanine + BCNU vs BCNU alone (P <0.01). However, none of the combination regimens was as effective as an equitoxic dose (75 mg/m^2) of BCNU alone, which produced 8 of 8 regressions and a growth delay of 35.9 days. No significant difference in growth delay was observed between animals receiving O^6-benzylguanine alone and those given the control (P >0.05), and no regression was seen in either group.

Discussion

These results demonstrate that O^6-benzylguanine administration depletes AT activity in brain tumor xenografts. Pretreatment with O^6-benzylguanine also significantly increased the sensitivity of these human brain tumor lines to

BCNU. We observed significantly increased growth delays and tumor regressions in tumor lines with both high (D341MED) and low (D245MG) AT activity at drug doses that produced no mortality in our animal population. This suggests that significant efficacy is possible with O^6-benzylguanine/BCNU regimens that exhibit very low toxicity. The mechanism of the therapeutic effect seen in D245MG with combination treatment is unclear, given that D245MG has no measurable AT activity as determined by our assay. This effect may be due to very low levels of AT activity that are undetectable by our techniques or could represent an additional unknown mechanism of BCNU resistance that is overcome by O^6-benzylguanine pretreatment. In tumor lines such as D245MG with extremely low levels of AT, combination therapy offers no advantage over equitoxic doses of BCNU alone. However, in tumor lines with high AT activity such as D341MED, O^6-benzylguanine treatment dramatically increases the therapeutic effect of BCNU in both s.c. and i.c. tumors. Notably, these results were obtained with only a single course of therapy. Multiple cycles of treatment could provide even more substantial results, although prolonged inhibition of AT activity could introduce additional toxicity as well.

In experiments in which the pretreatment dose of O^6-benzylguanine was varied, greater tumor inhibition was seen with 300 mg/m^2 than with 225 or 150 mg/m^2, despite the observation that a single injection of either 300 or 225 mg/m^2 of O^6-benzylguanine completely depleted the AT activity of D341MED xenografts for up to 36 h as determined by our AT assay. These findings suggest that differences between the AT inhibition produced by the two doses of O^6-benzylguanine may be more subtle than our assay could detect. Indeed, these data suggest that treatment experiments in athymic mice may be more sensitive to these subtle differences in residual AT activity than the AT assay itself.

The timing of O^6-benzylguanine pretreatment is influenced by three factors: the rate of chloroethylation of guanine by BCNU, the rate of subsequent cross-linking with the complementary strand, and the rate of repair of these chloroethyl adducts by the AT. Although the chloroethylation at the O^6 position of guanine occurs rapidly, the subsequent cross-link that is the cytotoxic lesion may take from 6 to 12 h to form [3, 10]. Recent studies suggest that AT removes cross-link precursors very rapidly [19]. Any attempt at useful AT inhibition must therefore effectively deplete AT levels prior to BCNU administration and keep the AT level depleted for several hours until a sufficient number of cross-links have formed to result in cell death.

When the timing of O^6-benzylguanine pretreatment in our studies was altered from 1 to 36 h prior to treatment with BCNU, a significant decrease in therapeutic efficacy was seen in both s.c. and i.c. experiments. In s.c. studies, the results of treatment with BCNU 36 h after O^6-benzylguanine were not statistically different from those observed for the control or BCNU alone. This suggests that by 36 h, sufficient de novo synthesis of AT had occurred to protect cells from the cytotoxic effects of BCNU. Studies by Yarosh and colleagues [29] with cell line HT29 showed that after AT depletion by *N*-methyl-*N'*-nitrosoguanidine, de novo protein synthesis restored AT to pretreatment levels within 24 h [29]. In our studies, AT activity was slowly regained after the administration of 300 mg/m^2 of O^6-benzylguanine, reaching only 50% of pretreatment levels by 48 h, which is in agreement with the previous findings of Dolan et al. [8] in SF767 xenografts. The greater delay in the synthesis of new protein observed in our studies may be due to the greater ability of O^6-benzylguanine to deplete AT or could be due to differences in the rate of AT resynthesis among different tumor lines. AT levels of 50% of baseline appear to be adequate to protect cells from the cytotoxic effects of BCNU, perhaps because AT has greater affinity for chloroethyl adducts on DNA than for free O^6-benzylguanine. Although there is currently no direct experimental evidence to this effect, the available AT could therefore preferentially repair chloroethyl adducts, preventing cytotoxic cross-linking. Since AT depletion by O^6-benzylguanine is rapid (within 1 h in our studies), it would appear that the administration of BCNU within a few hours of O^6-benzylguanine treatment would be the optimal timing for maximal therapeutic effect. BCNU treatment must occur in the window between full AT inhibition and the resynthesis of a cytoprotective level of AT. It is not known whether additional treatment with O^6-benzylguanine after BCNU administration will enhance the effect. It is also not known whether O^6-benzylguanine administration has any effect on the pharmacokinetics of BCNU.

The data presented herein demonstrate that O^6-benzylguanine inhibits AT activity in vivo and thereby increases the efficacy of chloroethylnitrosoureas. O^6-Benzylguanine is more efficacious in this regard than either methylating agents or other alkylguanines [4, 5, 14, 16]. The close correlation between the results of our s.c. and i.c. studies suggests that O^6-benzylguanine crosses the blood-brain barrier in sufficient concentrations to be therapeutically effective against i.c. tumors. Indeed, this is the first demonstration of the potentiation of a therapeutic effect against an i.c. tumor by systemic administration of a noncytotoxic AT substrate.

Some limitations to this form of therapy exist. O^6-Benzylguanine administration increases the acute toxicity of the nitrosoureas in animals. In humans, the dose-limiting side effect of nitrosourea therapy is bone marrow suppression. Since normal human myeloid cells have very low levels of AT activity, it is possible that the addition of O^6-benzylguanine would introduce little additional hematologic toxicity, but this hypothesis would need to be tested [15]. Nitrosoureas, in addition to being cytotoxic, are also promutagenic. The inhibition of the AT repair system may increase the risk of secondary malignancy in other organs. These limitations emphasize the need for selectivity in the use of these combination treatments. Tumors with high levels of AT activity that are resistant to nitrosourea therapy alone could potentially be very susceptible to combination regimens. Differences in the timing of AT inhibition in tumors and other organs should be explored since these differences could potentially be used to direct the cytotoxic effects of therapy specifically at the tumors themselves. Despite its limitations, the potential of this approach as an adjuvant to nitrosourea therapy in human chemotherapy is significant and should be aggressively investigated.

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