Modulation of chemotherapy resistance in regional therapy: a novel therapeutic approach to advanced extremity melanoma using intra-arterial temozolomide in combination with systemic O^6-benzylguanine

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Abstract

This study investigated whether the therapeutic index of regional melanoma therapy using parenteral temozolomide could be improved by chemomodulation with O^6-benzylguanine (O^6BG), an inhibitor of the DNA repair enzyme O^6-alkylguanine-DNA alkyltransferase (AGT). Using a nude rat s.c. human melanoma xenograft model of the extremity, tumors were analyzed for AGT level 2 to 3 hours after the i.p. injection of 3.5 to 70.0 mg/kg O^6BG to inhibit AGT activity. Survival studies were conducted using animals that were treated with a 15-minute isolated limb infusion with 10% DMSO in PBS (control), temozolomide alone, or temozolomide in conjunction with single or multiple doses of i.p. O^6BG. Tumor volume and toxicity level were monitored every other day. Administration of 3.5 mg/kg O^6BG depleted tumor AGT activity by 93.5% ($P < 0.01$). Groups treated with regional temozolomide alone (350 mg/kg), systemic temozolomide with O^6BG, or vehicle combined with O^6BG showed no significant tumor responses compared with controls. Whereas use of regional temozolomide alone at a higher dose (750 mg/kg) showed some degree of tumor response, regional temozolomide given in conjunction with multiple dosages of O^6BG showed a marked ($P < 0.01$) reduction in tumor growth with minimal toxicity. Our findings suggest that AGT modulation by the administration of O^6BG in combination with temozolomide regional chemotherapy leads to a significant improvement in melanoma antitumor responses. Clinical trials using chemotherapy modulation may improve response rates in future regional infusion and perfusion drug trials. [Mol Cancer Ther 2006; 5(3):732–8]

Introduction

Regional failure in patients with extremity melanoma is a significant problem. Aggressive therapy in the form of amputation suggests that 25% to 35% of these patients may have disease confined to the extremity (1). Isolated limb infusion or perfusion with cytotoxic agents is frequently used for this clinical problem. Melphalan (L-phenylalanine mustard) has been accepted as the most widely used drug in the regional setting for advanced extremity melanoma although many cytotoxic drugs have been studied. Recently, however, we have shown in a clinically relevant animal model of regionally advanced melanoma that regional temozolomide has better antitumor activity than either systemic temozolomide or regional melphalan (2).

Attempts at modulation of mechanisms that interfere with the optimal effectiveness of chemotherapeutic agents have been explored previously *in vitro*, but *in vivo* experiments have been limited due to increased systemic toxicity when chemotherapy and modulators are used together (3). Utilization of these strategies in a regional setting has the advantage of minimizing the systemic side effects of combination therapy while maximizing the effect regionally on the tumor. We have previously investigated modulation of putative resistance mechanisms in the context of regional therapy for melanoma and showed marked improvements in the therapeutic index of melphalan by interfering with the glutathione detoxification system (3–5). The specific aim for this project was to define if the therapeutic index of regional melanoma therapy with temozolomide can be enhanced using systemic inhibition of O^6-alkylguanine-DNA alkyltransferase (AGT), the predominant mechanism of resistance to this methylating agent (6), by administration of the drug O^6-benzylguanine (O^6BG) in an animal model that parallels the clinical situation of in-transit disease in humans.

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Materials and Methods

Animals and Tumor Inoculation

Female athymic nude rats (age, 5–7 weeks; RHU-M, Harlan, Indianapolis, IN) were housed in a temperature-controlled room with a 12-hour light-dark cycle. A standard laboratory diet and water were provided *ad libitum*.

Within 3 days after irradiation at a dose of 500 cGy to facilitate growth of human melanoma xenografts, the rats were injected s.c. in the right distal hind limb just proximal to the ankle with 5×10^6 culture human melanoma cells (DM6; ref. 3). Xenografts were measured every other day with vernier calipers in two perpendicular dimensions and tumor volume was calculated according to the following formula: $[(\text{length}) \times (\text{width})^2] / 2$ (5). All aspects of the experimental protocol were approved by the Durham Veterans Affairs Medical Center Institutional Animal Care and Use Committee.

O^6BG Dosing Study

We routinely prepared fresh O^6BG stock solution of 2 mg/mL in 40% polyethylene glycol-400 (PEG) in PBS. The O^6BG solution of interest was then made by further dilution of O^6BG stock solution with 40% PEG in PBS according to the given dosing regimen. A solution of 40% PEG in PBS was used as the vehicle control for the O^6BG experiments.

To evaluate the inhibitory effect of O^6-benzylguanine (O^6BG; Sigma Chemical Co, St. Louis, MO) on AGT activity in the regional nude rat human melanoma xenograft model, we compared tumor and muscle AGT levels from four groups of rats with a similar size xenograft by giving 3.5, 17.5, 35, or 70 mg/kg O^6BG i.p. ($n = 3$ in each group). Two to three hours after the administration of O^6BG, both tumor and muscle tissues were harvested, immediately snap frozen, and analyzed for AGT. Samples were removed under general anesthesia to prevent the loss of enzyme activity. To measure the baseline AGT activity of tumor and muscle, tissues were harvested from four rats 2 to 3 hours after i.p. administration vehicle (40% PEG in PBS).

AGT Determination

Samples of harvested tumor xenografts were weighed and extracts were prepared by adding 2 mL of 50 mmol/L Tris (pH 7.5), 0.1 mmol/L EDTA, and 5 mmol/L DTT buffer per gram of tissue weight. Samples were homogenized for 1 minute, sonicated for 1 minute, and centrifuged at $14{,}000 \times g$ for 30 minutes. The assay for AGT activity was done as previously described by Domoradski et al. (7). Briefly, AGT activity was measured as the removal of O^6-[^{3}H]-methylguanine from a [^{3}H]-methylated DNA substrate (5.8 Ci/mmol) following incubation with tissue extract at 37°C for 30 minutes. The DNA was precipitated by adding ice-cold perchloric acid (0.25 N) and hydrolyzed by the addition of 0.1 N HCl at 70°C for 30 minutes. Following filtration using a microfilter apparatus, the modified bases are separated by reverse-phase high-performance liquid chromatography with 0.5 mol/L ammonium formate (pH 4.5) containing 5.5% methanol. Protein is determined by the method of Bradford and the amount of O^6-methylguanine released from the DNA substrate per mg of protein was calculated. All AGT levels are expressed as mean ± SE.

Isolated Limb Infusion Technique

Isolated limb infusion was done as previously described (2). Briefly, under general anesthesia, the right femoral artery and vein were isolated. The proximal femoral artery and vein were then ligated. The femoral artery was cannulated and the arterial catheter was attached to a peristaltic pump. The femoral vein was then cannulated. Once both cannulas were in place, the pump and tubing were primed with saline, the thigh tourniquet was tightened, and saline was initiated at a flow rate of 1.5 mL/min. After ensuring an appropriate flow, the limb was infused with the solution of interest. A 15-minute infusion was done at a flow rate of 1.5 mL/min, followed by a 1-minute wash-out infusion with saline at a flow rate of 3.0 mL/min. Following the wash, the arterial and venous cannulas were removed and the femoral artery and vein were ligated.

Temozolomide Treatment in the Absence and Presence of O^6BG

We have previously reported a dose-escalation study using regional temozolomide (350, 750, 1,000, 1,500, and 3,000 mg/kg) in the same animal model used in this study (2). In the present study, temozolomide was administered as a single dosage via isolated limb infusion at the doses of 350 or 750 mg/kg infused limb weight. Ten percent of the rat body weight was assumed as the limb weight.

We prepared fresh temozolomide stock solution of 4 mg/mL in 10% DMSO in PBS. Standard formulations of temozolomide were kindly provided by Dr. W. Robert Bishop (Schering-Plough Research Institute, Kenilworth, NJ). Infusate was made by further dilution of temozolomide stock solution into 22.5 mL of 10% DMSO in PBS according to the given dosing regimen for a 15-minute infusion.

Modulation on AGT activity was done with i.p. O^6BG following the results of O^6BG dosing study. Doses of O^6BG were prepared in the same manner as described above.

Once tumors reached 12 mm in greatest dimension, rats were randomly assigned to one of the following subgroups:

(1) Isolated limb infusion without modulation of AGT
  Isolated limb infusion with 10% DMSO
  Isolated limb infusion with 350 mg/kg temozolomide
  Isolated limb infusion with 750 mg/kg temozolomide
(2) Isolated limb infusion in combination with O^6BG
  Isolated limb infusion with 350 mg/kg temozolomide
  Isolated limb infusion with 750 mg/kg temozolomide
(3) Isolated limb infusion in combination with O^6BG every 24 hours for 5 consecutive days (A total amount of O^6BG per rat was 17.5 mg/kg.)
  Isolated limb infusion with 10% DMSO
  Isolated limb infusion with 350 mg/kg temozolomide
  Isolated limb infusion with 750 mg/kg temozolomide

(4) Systemic temozolomide in combination with O^6BG every 24 hours for 5 consecutive days

Isolated limb infusion following O^6BG administration was introduced two hours after the dosing.

Tumor Measurement and End Point

Tumors and any ulcer or full-thickness eschar were measured as described above. Tumor volume was calculated according to the same formula mentioned above and the volume of any ulcer or eschar was subtracted from the total tumor volume. Animals were euthanized either when a 500% change in tumor volume occurred or after 60 days.

Evaluation of Tumor Response and Toxicity

Time (in days) taken to reach a quintupling of tumor volume was calculated from tumor volume measurements using linear regression. The average tumor quintupling was then calculated and compared. Response to treatment was also assessed by growth delays and number of regressions. Growth delay (in days) was defined as the difference in tumor quintupling time in treated versus control animals. Tumor regression was defined as a tumor of which the volume decreased over two consecutive measurements (8).

Rats were also monitored for general well-being, weight, and functional ability of leg following surgery to access the toxicity. Intra-animal weight gain or loss was calculated as a percentage of its baseline weight before surgery (day 0), and the mean nadir weight loss was calculated for each group. Toxicity was defined by assigning each rat a toxicity score as follows: grade 1, no subjective or objective evidence of reaction (normal walking pattern); grade 2, slight erythemia and/or edema; grade 3, considerable erythemia and/or edema (rodent does not use its hind limb in a useful manner but stands on it when rising); grade 4, obvious damage to the deep tissues (rat drags its hind limb without any function); grade 5, reaction that leads to autoamputation; and grade 6, animal death.

Statistical Analysis

The therapeutic indexes of the various treatments were assessed by ANOVA. When significant differences were detected in ANOVA, differences between means were checked by Bonferroni's method. Fischer's exact test was done for tumor regressions. $P < 0.05$ was considered significant.

Results

O^6BG Dosing Study

The activity of AGT before and after exposure to O^6BG is shown in Fig. 1. The control values show AGT levels after treatment with 40% PEG in PBS, which was the vehicle used to solubilize O^6BG. These values are no different than AGT values seen in the tumors of untreated animals (data not shown). Complete depletion of AGT activity in both tumors ($P < 0.01$) and muscle tissues ($P < 0.05$) within 2 to 3 hours was achieved by the administration of 17.5 and 35 mg/kg O^6BG. Use of a low dose of O^6BG (3.5 mg/kg) was also effective and suppressed the activity of AGT in tumors by 93.5% ($P < 0.01$). All rats that received 70 mg/kg O^6BG and one of the three rats treated with 35 mg/kg O^6BG died before tissue harvest. In view of the toxicity at higher dosing, the lowest dose tested that showed efficacy (3.5 mg/kg) was used in the remaining survival studies with only the frequency of dosing being changed.

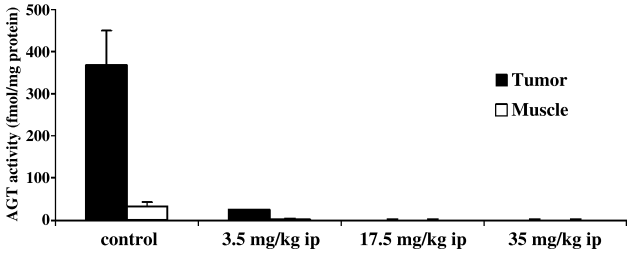

Figure 1. AGT inhibition by O^6BG in tumors and muscle tissues. Tumor and muscle AGT levels were measured in four groups of rats by giving 3.5, 17.5, 35, or 70 mg/kg O^6BG i.p. ($n = 3$ in each group). Two to three hours after the administration, samples were harvested and used for AGT determination. The administration of 17.5 and 35 mg/kg O^6BG showed complete depletion of AGT activity in both tumors and muscle tissues. A low dose of O^6BG (3.5 mg/kg) also suppressed the activity of AGT in tumors by 93.5%; however, it did not significantly inhibit the activity of AGT in muscle tissues. *Bars,* SE.

Improvement of Therapeutic Index with Temozolomide in Conjunction with O^6BG

Regional Temozolomide without O^6BG. Regional temozolomide at a concentration of 350 mg/kg without modulation of AGT was ineffective at delaying tumor growth although a small increase in the number of regressions compared with the group treated with regional 10% DMSO in PBS (Table 1) was seen. Using a dose of 750 mg/kg regional temozolomide alone, a slight increase in quintupling time was observed, which was not significantly different from the 10% DMSO control.

Regional Temozolomide with Single Dose of O^6BG. Using a dose of 350 mg/kg regional temozolomide in conjunction with a single dose of 3.5 mg/kg O^6BG showed increases in tumor growth delay and quintupling time that did not reach statistical significance. No increase in the number of regressions was seen over temozolomide alone (Fig. 2A; Table 1). However, 750 mg/kg of regionally administered temozolomide with a single dose of O^6BG systemically led to a significant prolongation of tumor growth delay ($P < 0.01$; Fig. 2B) and increased number of tumor regressions ($P < 0.05$; Table 1).

Regional Temozolomide with Multi-Dose of O^6BG. When O^6BG was administered systemically for 5 consecutive days, rats treated using regional isolated limb infusion with 350 or 750 mg/kg temozolomide showed significant prolongation of tumor growth delay ($P < 0.01$; Fig. 2A and B) and increased number of tumor regressions ($P < 0.01$; Table 1) compared with the group treated using isolated limb infusion with 10% DMSO in conjunction with the multi-dose course of O^6BG for 5 days. In contrast, animals who received systemic temozolomide combined with multiple dosages of O^6BG systemically showed no

Table 1. Effects of temozolomide and O^6BG on DM6 melanoma xenografts

Group	Quintupling time, mean ± SE (d)	Growth delay (d)	No. regressions
No modulation			
ILI + 10% DMSO (control; n = 6)	18.8 ± 2.2	—	1/6
ILI + 350 mg/kg temozolomide (n = 6)	17.3 ± 2.4	−1.5	3/6
ILI + 750 mg/kg temozolomide (n = 6)	23.8 ± 4.3	5.1	3/6
ILI + 750 mg/kg temozolomide (n = 6) with 40% PEG in PBS systemically × 5 d	20.6 ± 3.1	3.3	2/6
Modulation with 3.5 mg/kg O^6BG × 1 d			
ILI + 350 mg/kg temozolomide (n = 6)	30.4 ± 4.8	11.7	3/6
ILI + 750 mg/kg temozolomide (n = 6)	42.5 ± 8.2*	23.8	5/6†
Modulation with 3.5 mg/kg O^6BG × 5 d‡			
ILI + 10% DMSO (control; n = 6)	17.3 ± 2.5	—	0/6
ILI + 350 mg/kg temozolomide (n = 6)	39.8 ± 4.5§	22.6	5/6§
ILI + 750 mg/kg temozolomide (n = 6)	50.2 ± 5.5§	32.9	6/6§
Systemic 35 mg/kg temozolomide (n = 6)	13.7 ± 3.2	−3.5	0/6

Abbreviation: ILI, isolated limb infusion.
*P < 0.01, versus ILI + 10% DMSO.
†P < 0.05, versus ILI + 10% DMSO.
‡ILI with temozolomide or 10% DMSO was done 2 hours after first O^6BG i.p. administration. O^6BG was given every 24 hours for the next 4 days.
§P < 0.01, versus ILI + 10% DMSO with 3.5 mg/kg O^6BG for consecutive 5 days.

significant tumor growth delay (Table 1). Finally, to confirm that the observed activity of O^6BG was not related to the vehicle (40% PEG in PBS) in which it was mixed, a series of experiments was also carried out using vehicle alone as a systemic modulator. As shown in Fig. 2B and Table 1, there is no difference in the antitumor activity of temozolomide when given in the presence or absence of 40% PEG in PBS.

Evaluation of Toxicity. As shown in Table 2, no significant difference was found among groups in mean nadir weight loss or in maximum weight gain who underwent regional therapy using intra-arterial temozolomide in conjunction with systemic O^6BG. There were no rats who died or developed an autoamputation secondary to drug toxicity using combination therapy. The group which received systemic temozolomide in conjunction with multi-dose of O^6BG for 5 days showed the most severe mean nadir weight loss.

Discussion

In a quest for new regional chemotherapeutic agents against advanced extremity melanoma, we started to explore temozolomide (temozolomide), which is a novel methylating agent and is one of the more effective single-agent therapies for metastatic melanoma (9, 10). However, because its i.v. formulation is not currently available, temozolomide has not been tried as a regional chemotherapy in humans. We initially examined dose-escalation studies using regional and systemic temozolomide in a clinically relevant animal model of regionally advanced melanoma. We found that regional temozolomide had an antitumor effect as its dose was increased whereas systemic temozolomide had little antitumor effect even at a dose exceeding the LD_{10} in this model (2). Furthermore, when we compared the highest equitoxic dosages of regional melphalan, a gold standard drug in regional therapy, without heat, to regional temozolomide without heat, we found that temozolomide was significantly more effective in causing regressions and delaying tumor growth than melphalan (2). These findings suggest that temozolomide might be a promising new regional therapeutic agent.

The antitumor activity of temozolomide is a direct consequence of DNA methylation. The cytotoxic lesion is felt to be due to methylation of the O^6 position of guanine.

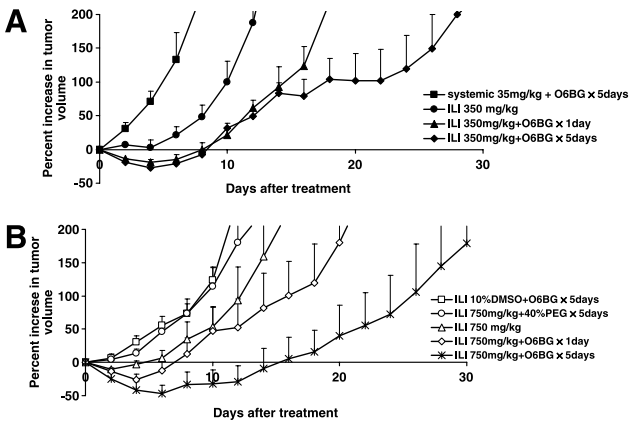

Figure 2. Effect of regional or systemic temozolomide with or without O^6BG. The average tumor growth inhibition with systemic 35 mg/kg temozolomide with 3.5 mg/kg O^6BG for 5 consecutive days and intra-arterial administration of 350 mg/kg temozolomide with or without O^6BG (**A**) and intra-arterial 10% DMSO (control) or 750 mg/kg of intra-arterial administration of temozolomide with or without O^6BG (**B**). *Y axis,* percent increase in tumor volume; *X axis,* days after treatment. *Bars,* SE.

Table 2. Adverse effects of temozolomide and O^6BG on body weight

Group	Mean nadir weight loss, mean ± SE (%)	Maximum weight gain, mean ± SE (%)
No modulation		
ILI + 10% DMSO (control; $n = 6$)	−6.2 ± 3.2	2.7 ± 2.0
ILI + 350 mg/kg temozolomide ($n = 6$)	−5.4 ± 1.0	1.2 ± 1.9
ILI + 750 mg/kg temozolomide ($n = 6$)	−3.3 ± 1.3	3.1 ± 1.3
Modulation with 3.5 mg/kg O^6BG		
ILI + 350 mg/kg temozolomide ($n = 6$)	−1.8 ± 0.8	3.5 ± 1.2
ILI + 750 mg/kg temozolomide ($n = 6$)	−5.3 ± 1.9	3.9 ± 1.5
Modulation with 3.5 mg/kg O^6BG × 5 d		
ILI + 10% DMSO (control; $n = 6$)	−3.3 ± 1.4	3.0 ± 1.5
ILI + 350 mg/kg temozolomide ($n = 6$)	−6.7 ± 2.9	7.3 ± 3.5
ILI + 750 mg/kg temozolomide ($n = 6$)	−7.5 ± 1.6	4.4 ± 1.6
Systemic 35 mg/kg temozolomide ($n = 6$)	−8.8 ± 2.9	1.6 ± 1.8

Unrepaired O^6-methylguanine (O^6MG) seems as an ''adenine'' base to the cell and it is paired with a thymine base (11). The function of a repair protein, AGT, in normal cells (as well as in tumor cells) involves removing chloroethylation or methylation damage from the O^6-position of guanine. If AGT is present during the repair, O^6MG bases are removed and DNA repair results in the correction of the mispairing that occurred due to the methylating agent (12). However, if AGT is not present, O^6MG:T mispaired bases trigger the process of DNA mismatch repair (13); then this repair process leads to a repetitive cycle of futile mismatch repair where thymine is repetitively mispaired with O^6MG and subsequently leads to growth arrest and apoptosis (14, 15).

A series of laboratory studies has shown that at least two mechanisms of resistance seem to be operational in mediating resistance to temozolomide. The first of these mechanisms involves removal of the methyl adduct on the O^6-position of O^6MG by AGT, which has been shown in both cell culture and xenograft studies to produce resistance to temozolomide (16–20). The second mechanism of resistance is related to a deficiency in DNA mismatch repair. This mechanism has been shown to confer resistance to temozolomide *in vitro* (21) and *in vivo* (22). The futile cycle created by the inability of the DNA mismatch repair proteins to correctly fix the methyl adducts is needed for methylating agents to mediate their cytotoxic effect. The deficiency of this DNA mismatch repair process results in tolerance of O^6MG residues as well as other methyl adducts in DNA and, eventually, cell survival in the presence of persistent DNA damage. Additional mechanisms of resistance to temozolomide may be operational with recent work suggesting a role for enhanced DNA base excision repair (23–25) and alterations in cell signaling involving the apoptotic pathway (26, 27).

The presence of chemotherapy resistance pathways is associated with poor tumor responses and patient survival in a variety of tumors. In melanoma cells, increased but variable AGT activity is found (28–31). There is higher AGT activity found in tumor cells than in normal skin cells and higher activity in metastatic tumors than in primary lesions. In addition, expression of AGT activity is higher in melanoma metastasis after chemotherapy with alkylating agents (28, 32).

Depletion of AGT, either with methylating agents that generate O^6MG residues in DNA (33, 34) or with O^6-alkylguanine (16, 35–41), renders cells more sensitive to methylator-induced cytotoxicity, presumably by way of increased formation of interstrand cross-links or initiation of a lethal cycle of mismatch repair. Dolan et al. (39, 40, 42) have reported that the substrate analogue O^6-benzylguanine (O^6BG) inhibits AGT activity and potentiates the cytotoxicity of both chloroethylating agents and methylating agents. They showed that O^6BG binds AGT, transferring the benzyl moiety to the active-site cysteine (43). The reaction is very rapid and complete, making O^6BG one of the strongest inhibitors of AGT. O^6BG is not incorporated into DNA in living cells and reacts directly with both cytoplasmic and nuclear AGT. O^6BG is now being used in clinical studies to assess the ability of AGT inhibition to overcome chemotherapy resistance in a variety of tumors (44–46).

When O^6BG was given at concentrations of 10 to 30 mg/kg in mice, it has been reported that human tumor xenograft AGT is depleted within 30 minutes and depletion is maintained for 6 to 8 hours, after which endogenous regeneration of AGT occurs through synthesis of new protein (47, 48). In the present study, to determine an appropriate dose of O^6BG to maximize AGT depletion with less toxicity in this model, we conducted O^6BG dosing studies. The activity of AGT was markedly inhibited using a concentration of 3.5 mg/kg O^6BG. Repeated dosing of O^6BG has been shown to maintain depletion of AGT activity in tumors (49).

AGT depletion by O^6BG has been shown to increase the cytotoxicity of temozolomide *in vitro* (50) and *in vivo* (6, 49, 51). Wedge et al. (49) have shown that the combination of the two agents given systemically can result in an increased therapeutic index for temozolomide in a human xenograft model. However, there is no significant difference between the groups with or without treatment of O^6BG, even in the most pronounced effect in combination with 100 mg/kg systemic temozolomide. Whereas O^6BG is known to inhibit AGT activity and, in several animal models, improves temozolomide efficacy *in vivo* against melanoma, the magnitude of the action of temozolomide is frequently limited by the synergistic toxicity that occurs when both agents are given systemically.

No previous studies have been done in the setting of regional temozolomide administration using systemic O^6BG to inhibit AGT. Therefore, we then did a set of infusional treatments using two different temozolomide dosages and found marked prolongation of melanoma tumor quintupling time, increased tumor growth delay, and tumor regressions at both temozolomide dosages as compared with control treatments in animals not receiving O^6BG, as well as in animals who received O^6BG but had a sham (DMSO) infusion as shown in Table 1. Interestingly, there was no increase in limb toxicity seen in these animals.

In this report, we showed how the effect of regional temozolomide on an *in vivo* model having advanced extremity melanoma can be optimized using chemomodulation with O^6BG. Modulation of chemoresistance pathways improves the therapeutic index of regional temozolomide-based therapy in our animal model of isolated limb infusion. All of these studies were done without the addition of tumor hyperthermia. These observations further support our earlier findings, using melphalan, that modulation of the tumor resistance pathways for chemotherapies used in regional infusional treatments is an effective novel strategy to improve the therapeutic index of this treatment approach (5). Our findings may offer a new approach to improve the efficacy of treatment for melanoma patients with regionally advanced disease and pave the way for a series of temozolomide-based regional therapy clinical trials.

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