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Phase I Trial of Temozolomide Plus O^6-Benzylguanine for Patients With Recurrent or Progressive Malignant Glioma

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ABSTRACT

Purpose
We conducted a two-phase clinical trial in patients with progressive malignant glioma (MG). The first phase of this trial was designed to determine the dose of O^6-BG effective in producing complete depletion of tumor AGT activity for 48 hours. The second phase of the trial was designed to define the maximum tolerated dose (MTD) of a single dose of temozolomide when combined with O^6-BG. In addition, plasma concentrations of O^6-BG and O^6-benzyl-8-oxoguanine were evaluated after O^6-BG.

Patients and Methods
For our first phase of the clinical trial, patients were scheduled to undergo craniotomy for AGT determination after receiving a 1-hour O^6-BG infusion at 120 mg/m^2 followed by a continuous infusion at an initial dose of 30 mg/m^2/d for 48 hours. The dose of the continuous infusion of O^6-BG escalated until tumor AGT was depleted. Once the O^6-BG dose was established a separate group of patients was enrolled in the second phase of clinical trial, in which temozolomide, administered as a single dose at the end of the 1-hour O^6-BG infusion, was escalated until the MTD was determined.

Results
The O^6-BG dose found to be effective in depleting tumor AGT activity at 48 hours was an IV bolus of 120 mg/m^2 over 1 hour followed by a continuous infusion of 30 mg/m^2/d for 48 hours. On enrolling 38 patients in six dose levels of temozolomide, the MTD was established at 472 mg/m^2 with dose-limiting toxicities limited to myelosuppression.

Conclusion
This study provides the foundation for a phase II trial of O^6-BG plus temozolomide in temozolomide-resistant MG.

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INTRODUCTION

Resistance to chemotherapy remains the central reason for the failure to cure patients with a diverse spectrum of malignancies. Malignant glioma is a neoplasm with particularly dismal attributes in that virtually all tumors display marked de novo or acquired drug resistance and ultimate lethal growth. Conventional treatment with surgery, radiotherapy, and alkylnitrosourea-based chemotherapy cures a minority of patients with anaplastic astrocytoma (AA) and few patients with glioblastoma multiforme (GBM).[1-3] temozolomide (Temodar; Schering-Plough, Kenilworth, NJ), a novel alkylating agent, has shown significant activity in recurrent malignant glioma.[4-11] Unfortunately, the majority of patients with

malignant glioma treated with alkylators, including temozolomide, demonstrate de novo or acquired resistance with subsequent tumor progression.

A series of preclinical studies conducted predominantly, but not exclusively, for non-CNS tumors has demonstrated that resistance to temozolomide is mediated in part through the DNA repair protein AGT.[12-15] AGT removes chlorethylation or methylation damage from the O^6 position of DNA guanines before cell injury and cell death. Although it is clear that AGT is not the only mechanism of resistance to temozolomide in malignant glioma,[13,16] it is certainly a highly important one.

The high incidence of AGT activity in human CNS tumors,[17] as well as recent clinical trials in malignant glioma using temozolomide that show an inverse relationship between response and AGT levels[18] and survival and inactivation of the *AGT* gene by promoter methylation,[19] provides the rationale for strategies designed to deplete tumor AGT levels during therapy with temozolomide.

O^6-benzylguanine (O^6-BG) is an AGT substrate that inactivates AGT and enhances the cytotoxicity of temozolomide both in vitro[14,15,20] and in vivo.[12,21] The dose of O^6-BG required to inactivate AGT and the duration of this inactivation in surgically removed tumor have proven to be controversial. Four clinical trials addressing this issue have been performed thus far. Spiro et al[22] and Dolan et al[23] found undetectable AGT levels in solid tumors, none of which were malignant gliomas, 18 hours and 16 $\pm$ 4 hours, respectively, after O^6-BG administration. Friedman et al[13] found a dose of 100 mg/m^2 O^6-BG to be adequate in depleting AGT activity to undetectable levels in malignant gliomas 18 hours after administration. On the other hand, Schold et al[24] found 120 mg/m^2 O^6-BG effective in suppressing AGT 6 hours but not 18 hours after administration.

We now report a two-phase clinical trial in patients with recurrent or progressive malignant glioma. The first phase of this trial was designed to determine the dose of O^6-BG effective in producing complete suppression of tumor AGT activity for 48 hours. The second phase of the trial was designed to define the MTD of a single dose of temozolomide when combined with O^6-BG. In addition, plasma concentrations of O^6-BG and O^6-benzyl-8-oxoguanine (8-oxoBG) were evaluated after O^6-BG.

PATIENTS AND METHODS

Objectives

The primary objectives were three-fold: (1) to determine the dose of O^6-BG administered as an intravenous bolus and subsequent 48-hour infusion effective in producing complete suppression of tumor AGT activity, (2) to determine the maximum tolerated dose (MTD) of temozolomide when administered after O^6-BG and thus characterize any toxicity associated with this combination, and (3) to determine the plasma concentration of O^6-BG and metabolites after this dose schedule of O^6-BG. Our secondary objective was to observe patients for antitumor response.

Patient Eligibility Criteria

For entry into the study, patients were required to have a histologically confirmed diagnosis of primary intracranial, infratentorial, or supratentorial WHO grade 3 or greater astrocytic, oligodendroglial, or mixed glial tumor. In the first phase of the study, patients with either newly diagnosed or recurrent/progressive disease were eligible. However, in the second phase of the study, patients were required to have recurrent/progressive disease. All patients were required to have residual disease on contrast-enhanced magnetic resonance imaging (MRI) study or computed tomography (CT) scan when MRI was medically contraindicated. Patients were required to be at least 18 years of age, have a Karnofsky performance status $\geq$ 60%, and have a life expectancy of greater than 12 weeks at study entry. Patients must have recovered from toxicity of prior treatment, and an interval of at least 4 weeks since prior chemotherapy (6 weeks for a nitrosourea-based regimen) had to have have elapsed for the patient to be enrolled into the clinical trial. The number or type of prior chemotherapy treatments or failures did not limit eligibility. Prior failure of temozolomide was not a requirement. Additional enrollment criteria included adequate pretreatment bone marrow function (total granulocyte count $\geq$ 1,500/μL; platelet count $\geq$ 100,000/μL), renal function (serum creatinine $\leq$ 1.5 mg/dL or creatinine clearance greater than 60 mL/dL and serum urea nitrogen $\leq$ 25 mg/dL), and hepatic function (serum glutamic oxalacetic transaminase $\leq$ 2.5$\times$ the upper limit of normal, and total serum bilirubin within normal limits). For patients on corticosteroids, a stable dose for 2 weeks before entry into the study was required, if clinically possible. Patients of reproductive potential were required to take effective contraceptive measures for the duration of the study and for 2 months after completing the study. All patients were informed of the investigational nature of the study and were required to provide signed informed consent as approved by the institutional review board.

The following patients were excluded from the study: pregnant women, nursing women, potentially fertile women or men who were not using an effective contraception method and patients recovering from surgery.

Treatment Design and Statistical Considerations

First phase: Testing biologic activity within a dose level of O^6-BG. In establishing the O^6-BG dose effective in completely depleting tumor AGT activity, patients received a fixed dose of O^6-BG at 120 mg/m^2 over 1-hour IV followed by a continuous infusion at an initial dose of 30 mg/m^2/d for 48 hours. At hour 48, patients underwent craniotomy, and tumor was snap-frozen for quantitation of AGT. If tumor AGT was not depleted, where tumor AGT depletion was defined as AGT levels less than 10 fmol/mg of protein, the dose of the continuous infusion of O^6-BG was escalated in the next cohort of patients. The dose escalations were as follows: dose level 1, 30 mg/m^2; dose level 2, 40 mg/m^2; dose level 3, 50 mg/m^2; and dose level 4, 60 mg/m^2.

O^6-BG was supplied by the National Cancer Institute (Bethesda, Maryland) in a dual pack with diluent. The O^6-BG was provided as a 100-mg vial of lyophilized powder with 670 mg of mannitol United States Pharmacopeia (USP) and sodium hydroxide. The diluent was provided as a 30-mL vial containing a sterile solution of 40% polyethylene glycol 400 in pH 8 phosphate buffer (106 mg of dibasic sodium phosphate and 102 mg of monobasic

potassium phosphate in sterile water for injection USP). The 30-mL diluent was added to the O^6-BG vial. Completely in solution, the O^6-BG was further diluted to 0.04 mg/mL with 0.9% saline and given intravenously.

The prevalence of AGT at the time of craniotomy was used in this two-stage study to determine at each O^6-BG dose level whether it was the optimal biologic dose. The null hypothesis used in this design anticipated that approximately 20% of tumors will have undetectable AGT in the absence of any treatment with O^6-BG. In particular, the hypothesis tested at each dose level differentiated between an undesirable (20%) and desirable (90%) level of AGT prevalence. In the first stage, a maximum of 10 patients was accrued. During the first stage, if AGT levels at any time were detectable in three or more patients, the patient accrual in that cohort was terminated and the dose of O^6-BG was escalated to the next dose level for subsequent patients. Otherwise, an additional four patients was added. If $\geq$ 11 of the 14 total patients at that dose had undetectable AGT levels, then the biologic modulatory dose of O^6-BG was reached. If less than 11 of 14 patients had detectable enzyme levels, then another cohort of patients were treated at a higher dose. The biologic modulatory end point of the study was the lowest dose of O^6-BG that gave undetectable AGT levels ($<$10 fmol/mg of protein) in $\geq$ 11 of 14 patients.

The decision rule concerning dose escalation satisfied the following criteria[1]: The chances of concluding that O^6-BG inhibited AGT (ie, percentage of patients without AGT at craniotomy is 90%) when in reality O^6-BG was not effective (ie, percentage of patients without AGT at craniotomy is 20%) was extremely small—much smaller than traditionally used error probabilities. A probability level of approximately 0.000004 was arbitrarily chosen.[2] The chance of concluding that O^6-BG inhibited AGT when in reality O^6-BG did inhibit AGT was greater than 95%.

Second Phase: Determination of MTD for Temozolomide With Optimal Biologic Dose of O^6-BG

After definition of the dose of O^6-BG that produces the target depletion of tumor AGT, eligible patients with recurrent malignant glioma were enrolled on a classic phase I trial of temozolomide plus O^6-BG. Cohorts of three to six patients were treated with O^6-BG at a dose of 120 mg/m^2 over 1 hour IV, followed in the next hour by temozolomide, using a single dose regimen (cycles repeated every 28 days), at an initial dose of 100 mg/m^2, followed by a continuous infusion with O^6-BG at 30 mg/m^2/d for 48 hours. Additional cohorts of three to six patients were treated with escalating doses of temozolomide until dose-limiting toxicity (DLT) was observed. The first three assessable patients at a dose level had to be followed for 4 weeks after the temozolomide/O^6-BG without experiencing DLT before entry of patients at the next dose level. Succeeding dose levels of temozolomide were as follows: 100, 200, 267, 355, 472, and 628 mg/m^2.

Temozolomide was commercially available from Schering-Plough Research Institute (Kenilworth, NJ). Temozolomide was supplied as a machine-filled, white opaque, preservative-free, two-piece, hard gelatin capsule available in 250-, 100-, 20-, and 5-mg strengths. Temozolomide was administered orally on an empty stomach with approximately 100% bioavailability. The dose was being rounded up to the nearest 5 mg.

In establishing the optimal temozolomide dose when combined with O^6-BG, a modified classic "3 + 3" dose escalation design was employed, which permitted up to three additional patients to be accrued at a given dose level as long as none of the first three patients enrolled at that dose level experienced a DLT. The dose level was escalated in successive cohorts of three patients as long as no DLT was observed. If one instance of DLT was observed among the initial three assessable patients, an additional three patients had to be treated at that dose level with no further DLT in order for dose escalation to proceed. If two instances of DLT were observed at a dose level, the MTD was determined to be surpassed, and a total of six patients were treated at the previous level to ensure its tolerability. The MTD was therefore the highest, causing DLT in no more than 1 of 6 patients at that dose level. Any patient who had stable or responding disease who developed DLT could continue to be treated at the next lowest dose level, provided the patient's toxicity resolved to grade 1 or lower and no more than 2 weeks were required for recovery. However, the patient was removed from study if DLT occurred on the lower dose.

Dose-limiting toxicity was defined as grade 3 or greater nonhematopoietic toxicity or grade 4 hematologic toxicity if it occurred during the first cycle and was felt to be attributable to the study regimen. Furthermore, failure to recover from any non-DLT to no greater than grade 1 toxicity within 2 weeks of the end of the cycle (ie, 6 weeks from drug administration) was considered a DLT.

Pathologic Review

Tumor tissue obtained at the time of surgery was evaluated by the study pathologist with adjacent tissue submitted for AGT analysis.

Toxicity Evaluation

Toxicity was graded according to the National Cancer Institute's Common Toxicity Criteria version 2.0. Complete blood counts were obtained weekly, and measure of serum urea nitrogen/creatinine, liver function studies, and serum electrolytes were obtained every other week. Each patient underwent a physical examination and radiographic evaluation before initiating each cycle of therapy.

AGT Activity

Extracts were prepared from tumors by homogenization in 50 mmol/L Tris, pH 7.5, 0.1 mmol/L EDTA, and 5 mmol/L dithiothreitol. AGT activity was determined as described previously.[25] In brief, cell extracts were incubated with ^{3}H-methylated DNA substrate (5.77 Ci/mmol). The DNA was precipitated by adding ice-cold perchloric acid at a final concentration of 0.25 N and hydrolyzed in 0.1 N HCL at 70°C for 30 minutes. The modified bases were eluted on a C18 reverse phase column using 10% methanol/0.05 M ammonium formate, pH 4.5, at 37°C. Each assay was performed with a positive control cell line (DaOY cell extract) and negative control (Chinese hamster ovary cell extract). Protein concentration was determined by the method of Bradford.[26] The results were expressed as femtomoles of O^6-methylguanine released from DNA per milligram of protein. Assays were performed in triplicate when there was adequate sample.

Plasma Sampling and Pharmacokinetic Studies

In the second phase of the study, the steady-state plasma concentrations of O^6-BG and 8-oxoBG were measured. Whole blood samples were collected in sodium heparinized Vacutainers on day 1, week 1, of the first cycle, in the first 10 patients enrolled. Samples were obtained before administration of O^6-BG (120 mg/m^2 O^6-BG over 1 hour followed immediately by 30 mg/m^2 continuous IV for 48 hours) and at 25, 49, and 73 hours after starting the O^6-BG bolus. Plasma was obtained by centrifugation at 1,200 $\times$ *g* for 10 minutes. Plasma samples were coded and stored at $-$80°C before pharmacologic analysis. Total plasma concentrations of O^6-BG and 8-oxoBG were measured by high-pressure

liquid chromatography using methods that have been described previously.[27] Aliquots of plasma (750 μL) were spiked with 125 μL of O^6-(*p*-fluorobenzyl)guanine (internal standard), followed by extraction with 5 mL ethyl acetate. The samples were vortexed, centrifuged at 1,300 $\times$ *g* for 10 minutes, lyophilized, and resuspended in mobile phase for high-performance liquid chromatography analysis, the equipment for which consisted of a Waters Separation Module (model 2695) with a Waters μBondapak 125-Å phenyl precolumn and column (3.9 $\times$ 300 mm) connected to a Waters 996 Photodiode Array Detector in series with a Hitachi L-7485 Fluorescence Detector (Tokyo, Japan). Samples were monitored by UV at 280 nm and by fluorescence detection at λ_{exc} = 295 nm and λ_{emm} = 360. The separation occurred under isocratic conditions using 32% methanol/10 mmol/L potassium phosphate buffer, pH 7.5 at 1 mL/min and 30°C. Retention times were 23.7, 28.2, and 33.1 minutes for 8-oxoBG, O^6-BG, and O^6-(*p*-fluorobenzyl)guanine, respectively. The limit of detection for both O^6-BG and 8-oxoBG was determined to be 10 ng/mL.

Response Criteria

Response determination was based on measurable changes in tumor size as evidenced by CT or MRI, and clinical criteria including corticosteroid requirement and the results of the neurologic examination. Complete response (CR) was defined as the complete disappearance of all enhancing tumor and mass effect, off all corticosteroids (or receiving only adrenal replacement replacement doses), accompanied by a stable or improving neurologic examination, and maintained for at least 8 weeks (two sequential scans). Partial response (PR) was defined as $\geq$ 50% reduction in the tumor size by bidimensional measurement or volumetric MRI/CT, on a stable or decreasing dose of corticosteroids, accompanied by a stable or improving neurologic examination, and maintained for at least 8 weeks (two sequential scans). Progressive disease (PD) was defined as more than 25% increase in the bidimensional measurement or volumetric measurement on MRI/CT, or worsening neurologic status related to tumor progression or increasing dose of corticosteroids required to maintain stable neurologic status or imaging. Stable disease (SD) was defined as MRI/CT imaging meeting neither the criteria for PR nor PD, accompanied by a stable or improved neurologic examination, on a stable or decreasing dose of corticosteroids, maintained for at least 8 weeks (two sequential scans).

RESULTS

As detailed in Table 1, 15 patients with malignant gliomas were enrolled into the first phase of the study, where the dose of O^6-BG effective in producing complete depletion of tumor AGT activity was identified. Only one dose level of the 48-hour continuous infusion of O^6-BG was evaluated before identifying a dose of 30 mg/m^2/d as effective in depleting AGT at 48 hours when preceded by a fixed dose of O^6-BG at 120 mg/m^2 over 1 hour. Tumor AGT levels were not assessable in two of the 15 patients because of compromised tissue. These two samples were not analyzed because the pathologist found no viable tumor. One of the 13 assessable patients had AGT activity $\geq$ 10 fmol/mg of protein (11.6 $\pm$ 16.4 fmol/mg of protein). Twelve of the 13 assessable patients had AGT activity of less than 10 fmol/mg of protein, fulfilling our criterion for AGT depletion. Although statistically 14 assessable patients were recommended, only 13 assessable patients were enrolled once the AGT status of 12 of these patients was found to be undetectable. In other words, the AGT status of the fourteenth patient would not have changed the outcome, because only 11 patients with undetectable AGT levels were necessary to determine that the biologic modulatory dose of O^6-BG was reached.

Table 1. Tumor AGT Activity

Patient	AGT Activity (fmol $\pm$ SD)
1	0 $\pm$ 0
2	3.9 $\pm$ 5.5
3	0 $\pm$ 0
4	11.6 $\pm$ 16.4
5	NA
6	0 $\pm$ 0
7	0 $\pm$ 0
8	1.2 $\pm$ 1.7
9	0 $\pm$ 0
10	0 $\pm$ 0
11	0 $\pm$ 0
12	NA
13	0 $\pm$ 0
14	0 $\pm$ 0
15	0 $\pm$ 0

NOTE. For all patients, the dose of O^6-benzylguanine was 30 mg/m^2.
Abbreviations: SD, standard deviation; NA, nonassessable.

Patient Data

Between February 7, 2001, and August 19, 2002, a total of 38 patients with recurrent or progressive high-grade gliomas (WHO stage III-IV) were enrolled into the study (Table 2) at one of six dose levels of temozolomide: 100, 200, 267, 355, 472, and 628 mg/m^2. Consistent with the distribution of high-grade gliomas in adults, the majority of patients had a GBM (69%), whereas cases of AA were less frequent (26%), and AO was rare (5%). The median age was 43 years (range, 19 to 73 years), and there was a male predominance (76%). All patients underwent prior resection (82%), or biopsy (18%), radiotherapy, and chemotherapy (median of two different regimens [range, 1 to 5]). All patients but one (patient 21 in Table 4) had experienced prior chemotherapy treatment failure with temozolomide. In addition to radiotherapy, one patient received liquid brachytherapy with radioisotope-labeled monoclonal antibodies.

Toxicity Evaluation

Of the 38 patients enrolled into the study, 34 were assessable for toxicity (Tables 3 and 4). At the first temozolomide dose level of 100 mg/m^2, four of seven patients were assessable for toxicity. Of the three patients who were not assessable for toxicity, all failed to return for follow-up after study enrollment, one of whom died of cerebral herniation

Table 2. Patient Characteristics

Characteristics	No. of Patients	%
Total	38	
Age, years		
Median	43	
Range	19-73	
Sex		
Male	29	76
Female	9	34
Histology		
Glioblastoma multiforme	26	69
Anaplastic astrocytoma	10	26
Anaplastic oligodendroglioma	2	5
Prior therapy		
Radiotherapy	38	100
Chemotherapy	38	100
Temozolomide	38	100
Temozolomide failure	37	97
Monoclonal antibody	1	3
Resection	31	82

before completing the first cycle. Although no DLTs were noted at this dose level, one patient was hospitalized 3 days after initiation of the first cycle for signs of cerebral herniation (grade 3 nausea, vomiting, headache, and decreased level of consciousness) confirmed on MRI and deemed not related to study drug. At the second temozolomide dose level of 200 mg/m^2, six of seven patients were assessable for toxicity. One patient was not assessable for toxicity because she died of cerebral herniation 18 days after enrollment. Although there were no DLTs at this dose level, one patient experienced a grade 3 leukopenia during the first cycle. At the third temozolomide dose level of 267 mg/m^2, all six patients were assessable for toxicity with none experiencing any toxicities. At the fourth temozolomide dose level of 355 mg/m^2, all six patients were assessable for toxicity. One of the six patients experienced a DLT of a grade 4 thrombocytopenia during the first cycle, followed by grade 3 anemia and vomiting after the first cycle. Two patients experienced an elevation in alanine aminotransferase (ALT) levels, both categorized as not dose limiting. One patient's ALT became elevated after the first cycle and the other patient's ALT remained elevated during the first cycle with the elevation proceeding study enrollment. At the fifth temozolomide dose level of 472 mg/m^2, all seven patients were assessable for toxicity. One patient experienced a DLT of a grade 4 neutropenia. At the sixth temozolomide dose level of 628 mg/m^2, all five patients were assessable for toxicity. Four of the five patients experienced DLTs consisting of grade 4 thrombocytopenia, grade 4 anemia, and grade 4 neutropenia along with grade 3 anemia and grade 3 thrombocytopenia, which were not dose limiting. Thus, after exceeding the MTD at a temozolomide dose of 628 mg/m^2, the MTD was established at 472 mg/m^2.

Antitumor Responses

Of the 38 patients enrolled into the study, 36 were assessable for response. Two patients were not assessable for response because they failed to return for follow-up after enrollment. Twenty patients demonstrated progressive disease as their best response (Table 4). Thirteen patients demonstrated stable disease for one or two cycles. Seven patients with stable disease continued on treatment, having received a median of five cycles (range, three to 12). One patient (8) demonstrated a complete response (Fig 1) and remains free of disease after 12 cycles of temozolomide and 33 months off therapy.

Pharmacokinetic Analysis

Plasma concentrations of O^6-BG and metabolites during and up to 73 hours after the initial O^6-BG infusion were collected on the initial 10 patients from the second phase of this trial; however, one patient was dropped from analysis because only a 25-hour post sample was collected (Fig 2). The number of patient samples collected for analysis at pre-O^6-BG and at 25, 49, and 73 hours after were n = 9, 7, 9, and 4. Previous clinical studies[13,27] demonstrated single bolus O^6-BG

Table 3. Toxicities

Temozolomide Dose (mg/m^2)	Ratio of Patients (assessable/total)	Toxicities (No. of patients)	
		Not Dose Limiting	Dose Limiting
100	4/7	0	0
200	6/7	1 (3 WBC)	0
267	6/6	0	0
355	6/6	1 (3 HGB, 3V)	1 (4 PLT)
		2 (3 ALT)	
472	7/7	0	1 (4 ANC)
628	5/5	1 (3 WBC)	1 (4 ANC, 4 PLT)
		1 (3 ALT)	1 (4 WBC, 4 PLT)
		1 (3 PLT)	1 (4 WBC, 4 ANC, 4 PLT)
			1 (4 PLT)

Abbreviations: WBC, white blood cell count; HGB, hemoglobin; V, vomiting; PLT, platelet count; ANC, absolute neutrophil count; ALT, alanine aminotransferase.

Table 4. Patient Demographics, Response and Toxicity

Patient	Diagnosis	Age (years)	Surgery	Prior Chemotherapy in Addition to Temozolomide	Temozolomide Dose (mg/m^2)	Cycles	Best Response	Non-Dose Limiting	Dose Limiting
1	GBM	45	Resection		100	1	NA	NA	NA
2	GBM	54	Resection	CCNU, VP-16, irinotecan	100	1	PD	NA	NA
3	GBM	56	Resection	CCNU, irinotecan, cyclophosphamide	100	1	NA	NA	NA
4	GBM	50	Resection	VP-16, irinotecan/BCNU	100	1	PD		
5	AO	44	Biopsy	PCV	100	2	SD		
6	GBM	37	Resection	BCNU, VP-16	100	1	PD		
7	AA	37	Resection	Irinotecan/BCNU	100	2	SD		
8	AA	33	Resection	CCNU	200	12	CR		
9	GBM	29	Resection	PCV, irinotecan/BCNU	200	1	PD		
10	GBM	52	Resection	PCV	200	2	SD		
11	GBM	53	Resection		200	1	PD	NA	NA
12	AA	51	Resection	PCV, carboplatin, irinotecan	200	1	PD		
13	GBM	73	Resection		200	1	PD	3WBC	
14	GBM	46	Resection	CCNU, VP-16, irinotecan/BCNU	200	9	SD		
15	GBM	32	Resection	Gliadel	267	1	PD		
16	GBM	40	Resection	BCNU	267	1	PD		
17	AA	42	Biopsy	Irinotecan	267	6	SD		
18	GBM	35	Resection	BCNU/cisplatin/VP-16, polifeprosan/ carmustine	267	1	PD		
19	GBM	47	Resection	Thalidomide, irinotecan/BCNU	267	1	PD		
20	AA	46	Resection	Thalidomide, irinotecan/BCNU	267	1	PD		
21	GBM	49	Resection	VP-16, irinotecan/BCNU	355	1	PD		
22	GBM	30	Biopsy	CCNU	355	3	SD	3HGB, 3V	4PLT
23	AO	41	Resection	PCV	355	9	SD	3ALT	
24	GBM	45	Resection		355	5	SD		
25	GBM	51	Resection		355	1	PD		
26	GBM	35	Resection		355	1	PD	3ALT	
27	GBM	40	Resection	CCNU	472	1	PD		
28	AA	40	Resection	Irinotecan, PCV	472	1	PD		
29	GBM	31	Resection	CCNU	472	1	PD		
30	GBM	19	Resection		472	2	SD		
31	AA	35	Resection		472	1	PD		
32	GBM	35	Biopsy	PCV	472	4	SD		4ANC
38	AA	51	Resection	BCNU	472	1	PD		
33	GBM	56	Resection	CCNU, polifeprosan/carmustine	628	2	SD	3WBC	4ANC, 4PLT
34	AA	36	Resection	PCV, carboplatin	628	1	PD		4WBC, 4PLT
35	GBM	40	Resection	CCNU, irinotecan	628	5	SD	3ALT	4WBC, 4ANC, 4PLT
36	AA	55	Resection	CCNU	628	6	SD	3PLT	4PLT
37	GBM	44	Resection	CCNU	628	1	PD		

Abbreviations: GBM, glioblastoma multiforme; AO, anaplastic oligodendroglioma; AA, anaplasticastrocytoma; CCNU, lomustine; VP-16, etoposide; BCNU, carmustine; PCV, procarbazine/lomustine/vincristine; NA, nonassessable; PD, progressive disease; SD, stable disease; CR, completeresponse; LOC, depressed level of consciousness; V, vomiting; SZ, seizure; WBC, white blood cell count; HGB, hemoglobin; ALT, alanine aminotransferase; PLT, platelet count; ANC, absolute neutrophil count.

(120 mg/m^2) was rapidly oxidized to a longer-lived and equally effective alkyltransferase inactivator 8-oxoBG. Consistent with single bolus O^6-BG, higher concentrations (19× to 24×) of mean plasma concentrations (± standard deviation) of 8-oxo-BG than O^6-BG were observed at 25 and 49 hours after 120 and 30 mg/m^2 continuous IV.

DISCUSSION

Although considerable effort has gone into the creation of novel therapies for brain tumors, alkylating agents remain the mainstay of glioma therapy. Of the alkylating agents, temozolomide, an orally available DNA-methylating agent that readily crosses the blood-brain barrier, has shown efficacy against human gliomas in clinical trials.[4-11,28,29] Although, there have been attempts to improve the efficacy of temozolomide in anaplastic gliomas by combining it with carmustine [BCNU], these attempts have failed.[30,31]

The cytotoxic action of temozolomide, like that of other DNA-methylating agents, is dependent on the creation of O^6-methylguanine DNA adducts,[32] which initiates a futile recycling of the mismatch repair pathway,

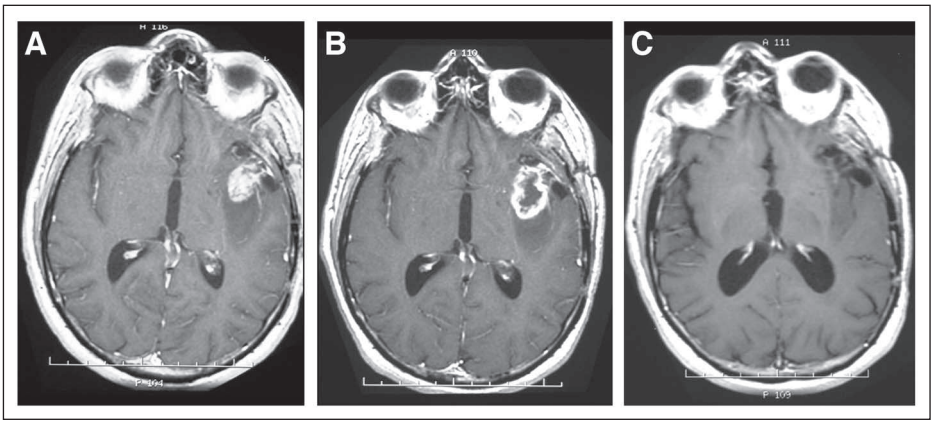

Fig 1. (A) Patient 8 with an anaplastic astrocytoma after failure of surgery, radiotherapy, and lomustine. (B) Decrease of tumor by more than 50% after two cycles. (C) Absence of tumor after six cycles.

causing DNA strand breaks and apoptotic cell death in mismatch repair-proficient cells.[33,34] The DNA repair protein AGT repairs these adducts in a suicide manner and reduces the cytotoxic action of temozolomide.[12-15]

The high incidence of AGT activity in human CNS tumors,[17] as well as the inverse relationship between procarbazine activity and alkyltransferase levels in human brain tumor xenografts,[35] support a role for this protein in mediating resistance to nitrosoureas in patients with CNS tumors and provide an approach for reversal of drug resistance. Furthermore, recent clinical trials have suggested that AGT levels in patients receiving BCNU therapy correlate with outcome. Belanich et al[36] reported that in 167 patients treated with BCNU for malignant glioma, low tumor AGT content correlated with enhanced survival and longer time to treatment failure, whereas patients with elevated tumor AGT had poorer outcomes. Likewise, Hotta et al[37] showed the same relationship in 42 patients with malignant glioma. Jaeckle et al.[38] have reported a Southwest Oncology Group trial also correlating survival of patients with malignant glioma with tumor AGT levels. Finally, Esteller et al[39] have also confirmed the relationship, albeit using methylation of the AGT promoter gene in lieu of quantitation of tumor AGT levels. The Brain Tumor Center at Duke has demonstrated the relationship between high AGT levels in GBM and failure to respond to preradiation temozolomide.[18] Furthermore, Hegi et al[19] have shown a relationship between inactivation of the AGT gene by promoter methylation and survival in patients with newly diagnosed glioblastoma multiforme treated with surgery, radiotherapy, and temozolomide. Therefore, depletion of tumor AGT might enhance subsequent BCNU or temozolomide therapy and patient survival.

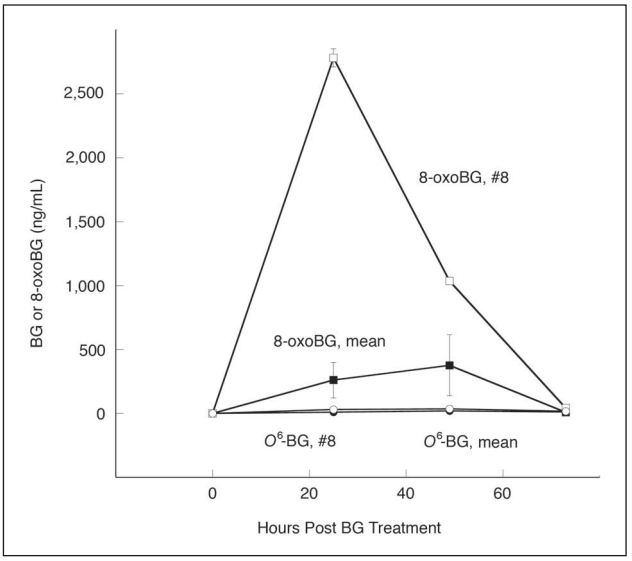

Fig 2. Mean plasma concentration of O^6-BG (●) and 8-oxoBG (■) of all patients evaluated (except patient 8) at various time periods after O^6-BG. Plasma concentration of O^6-BG (○) and 8-oxoBG (□) of patient 8 (complete responder) after O^6-BG.

Depletion of AGT activity by a selective inhibitor, O^6-BG, enhances the cytotoxicity of chloroethylators and methylators.[12,14,18,20,21,40-42] However, the main limitation in the clinical use of alkylating agents in combination with O^6-BG is their potential for dose-related acute toxicity to the hematopoietic system. In animal studies[43-45] of the combination of O^6-BG and BCNU, enhanced bone marrow toxicity has been noted. This may also be true in humans, because AGT activity in hematopoietic progenitor cells has been shown to be low.[46,47] Furthermore, O^6-BG pretreatment markedly sensitized hematopoietic progenitor colony-forming cells to BCNU.[47] To further explore the effect of O^6-BG on BCNU activity and toxicity we performed phase 1[48] and phase 2[49] clinical trials in which this drug combination was administered to patients with recurrent or progressive malignant gliomas resistant to nitrosoureas.

Our phase 1 trial established the MTD of BCNU to be 40 mg/m^2 when combined with a sufficient dose of O^6-BG (1-hour IV bolus of 120 mg/m^2) found to deplete AGT in gliomas 18 hours after administration. This trial demonstrated the need for a marked reduction in the dose of

BCNU compared with the usual dose of 200 mg/m^2 when BCNU is used alone. The myelosuppression seen in the phase 1 trial was also seen in the phase 2 trial. This profound reduction of BCNU may be the underlying factor in the failure of this drug combination to cause frank tumor regressions in our phase 2 trial.

Given the fact that O^6-BG seemed to enhance the toxicity of BCNU without a corresponding increase in activity, a clinical combination of temozolomide with O^6-BG may be preferred to a regimen involving BCNU and O^6-BG, simply because the chloroethylnitrosoureas are inherently more toxic, particularly to hematopoietic cells. Thus, we explored temozolomide in combination with O^6-BG for treatment of patients with malignant gliomas.

The results of this trial clearly indicate that a O^6-BG bolus dose of 120 mg/m^2 over 1 hour followed by a 48-hour continuous infusion of 30 mg/m^2/d can maintain tumor AGT levels at less than 10 fmol/mg of protein at 48 hours. Once this dose of O^6-BG was combined with a single dose of temozolomide, the MTD of temozolomide was established at 472 mg/m^2. Dose-limiting toxicity was limited to myelosuppression. Severe but reversible neutropenia, leukopenia, and thrombocytopenia were observed at a temozolomide dose of 628 mg/m^2, which required reduction to 472 mg/m^2 as the suggested dose for our phase 2 trial. Despite prior temozolomide failure, one patient demonstrated a complete response and currently remains disease-free 33 months after therapy completion.

The mean plasma concentrations of O^6-BG and 8-oxoBG over time after administration of 120 mg/m^2 O^6-BG for 1 hour have been reported[13,27]; however, this is the first report of plasma concentrations of drug and metabolite after a bolus dose of O^6-BG at 120 mg/m^2 with 30 mg/m^2 continuous infusion for 48 hours. Previous studies demonstrated the area under the concentration-time curve for 8-oxoBG were 12- to 29-fold greater than those of O^6-BG.[27] Our data are consistent with 8-oxoBG plasma concentrations higher than those of O^6-BG.

Although the 5-day dosing regimen of temozolomide (150 to 200 mg/m^2/d) is accepted as the most efficacious regimen for administration, it is far from clear that it is the most efficacious and least toxic regimen when combined with O^6-BG. Thus, we explored the 1-day temozolomide dosing schedule before exploring the 5-day schedule when combined with O^6-BG for the following four reasons. First, although experimentally temozolomide activity seems to be schedule-dependent,[50] with improved efficacy on a 5-day dosing regimen compared with a 1-day dosing regimen, no classic phase 1 or 2 trials have been performed to evaluate a single-day dosing schedule of temozolomide. Newlands et al[51] performed a dose escalation study using a single dose of temozolomide and reported that no clinical responses were seen on this regimen. However, most of the courses the patients received were at a dose $\leq$ 520 mg/m^2, far below what seems to be the MTD, somewhere between 750 and 1,000 mg/m^2. Second, if the schedule-dependent activity is truly real, then perhaps one explanation for improved activity for the 5-day regimen over the 1-day regimen is that prolonged administration of temozolomide causes greater depletion of AGT. Although this idea has yet to be proven in brain tumors, progressive depletion of AGT was observed with each consecutive dose of temozolomide in a 5-day schedule[52] in human peripheral blood mononuclear cells. Thus, if the effect of O^6-BG is to suppress AGT, it can be theorized that a shorter 1-day dosing schedule of temozolomide might produce fewer toxicities when combined with O^6-BG but be just as efficacious as the longer 5-day dosing schedule. Third, although in vitro studies suggest that a 5-day dosing schedule of temozolomide and O^6-BG may be more efficacious than a 1-day dosing schedule, in vivo studies have been far less convincing. An in vitro study by Wedge et al[42] found the potentiation of temozolomide cytotoxicity by O^6-BG to increase linearly on 5 consecutive days. However, when Wedge et al[53]explored the effect of single versus multiple administration of O^6-BG/temozolomide combination in human melanoma xenograft model, the results were not as convincingly supportive of the 5-day regimen over the 1-day regimen. Although Wedge concludes that prolonged administration of the combination can lead to an increase in the therapeutic index of temozolomide, no direct comparison of the 5-day to the 1-day regimen of temozolomide plus O^6-BG was made to support this conclusion. In fact, the efficacy and toxicity of these two regimens look remarkably similar if you compare the group that received a single dose of temozolomide at 200 mg/kg pretreated with 35 mg/kg of O^6-BG and the group who received a total dose of 200 mg/kg of temozolomide over 5 days pretreated each day with 35 mg/kg.

When designing this clinical trial, no preclinical information existed to address the optimum dosing schedule of O^6-BG when combined with temozolomide in glioma cells. However, it was theorized that suppression of AGT for 48 hours after temozolomide administration would provide the time required for temozolomide to push cells toward the apoptotic pathway through futile mismatch repair cycling. Since initiation of this clinical trial, a preclinical study was performed by Hirose et al[54] that addresses this exact issue. They report that maximal temozolomide sensitization in gliomas through prolonged AGT depletion with continuous O^6-BG exposure was achieved 2 to 3 days after temozolomide exposure. This would suggest that continuous depletion of O^6-BG for at least 48 hours, as performed in this study, would be preferred to intermittent depletion, as inferred from an alternative O^6-BG regimen used in a clinical trial in pediatric tumors.[55] This competing regimen uses a daily 60-minute IV infusion of O^6-BG at 120 mg/m^2 in combination with daily temozolomide, both of which were administered for 5 days. Although research[13] shows

depletion of AGT at 18 hours after a 120 mg/m^2 60-minute IV infusion of O^6-BG, it is highly unlikely and has never been proven that this depletion is continuous from 18 to 24 hours. Thus, the daily O^6-BG bolus regimen in which continuous AGT depletion is improbable becomes a less attractive alternative. Support of our O^6-BG regimen in providing levels of AGT activation can be found in the study by Gerson,[56] where patients who received a 1-hour infusion of 120 mg/m^2 O^6-BG followed by a 23-hour infusion of 40 mg/m^2 had persistent AGT inactivation.

Given the ubiquitous presence of AGT in all human organs and tissues, it was not surprising to find that temozolomide toxicity was enhanced and the dose of temozolomide defined as the MTD in this trial was significantly less (~50%) than when temozolomide was administered on the standard 5-day dosing schedule. However, it is far from clear whether the temozolomide dose has been reduced to an insufficient level at which antitumor activity will not be seen. Despite the reduced dose of temozolomide, our results suggest that O^6-BG was effective in restoring temozolomide sensitivity in one patient with a temozolomide resistant tumor who demonstrated a complete response and remains disease-free after 33 months off therapy.

The current trial of O^6-BG and temozolomide has defined the therapeutic approach for our current phase 2 trial of temozolomide plus O^6-BG. Patients with recurrent temozolomide-resistant malignant glioma, defined as tumor growth within 8 weeks of receiving temozolomide, will be enrolled onto this trial. This will provide the evidence needed to determine whether temozolomide plus O^6-BG can restore temozolomide sensitivity in patients for whom this agent has previously failed.

Authors' Disclosures of Potential Conflicts of Interest

Although all authors completed the disclosure declaration, the following authors or their immediate family members indicated a financial interest. No conflict exists for drugs or devices used in a study if they are not being evaluated as part of the investigation. For a detailed description of the disclosure categories, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.

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