Phase II Trial of the O^6-Alkylguanine DNA Alkyltransferase Inhibitor O^6-Benzylguanine and 1,3-Bis(2-Chloroethyl)-1-Nitrosourea in Advanced Melanoma

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Abstract

Purpose: 1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU) induces DNA damage via a chloroethyl adduct at the O^6 position of guanine, which can be repaired by O^6-alkylguanine DNA alkyltransferase (AGT) expressed in melanoma. We postulated that the addition of O^6 benzylguanine (O^6BG), a potent inactivator of AGT, would improve the clinical response to BCNU in melanoma.

Experimental Design: Patients had measurable disease, adequate organ function, and a corrected Diffusing capacity of the lung for carbon monoxide (DLCO) of ≥70% predicted. They were accrued into two cohorts based on prior chemotherapy. O^6BG (120 mg/m^2) was administered i.v. followed by BCNU (40 mg/m^2) on an outpatient basis. Peripheral blood mononuclear cells (PBMC) were collected pre- and 18 hours post-O^6BG to analyze AGT depletion. Treatment was every 6 weeks, and clinical response was assessed after every two cycles.

Results: Forty-two patients were enrolled, 22 of these patients were chemotherapy-naïve. In the chemotherapy-naïve cohort, there was a patient with a complete response (CR), 4 with stable disease (SD), 13 with progressive disease (PD), and 4 nonevaluable patients; the median time to progression was 80 days and the median survival was 211 days. In the prior-chemotherapy cohort, there were no responses, 3 SD, 15 PD, and 2 nonevaluable patients; median time to progression was 54 days and median survival was 120 days. AGT was depleted from PBMC in the 15 patients tested. Grades 3 to 4 myelosuppression was seen in 57% of patients; toxicities were similar between the two cohorts.

Conclusions: O^6BG/BCNU was successfully administered on an outpatient basis and depleted AGT from PBMC. However, significant myelosuppression was observed and the clinical outcome was not improved. Alternative mechanisms of resistance to melanoma cell death need to be investigated.

The incidence of malignant melanoma is steadily increasing. More than 59,000 new cases and >10,000 deaths were estimated in 2004 (up from 54,000 and 7,600, respectively, in 2003). Dacarbazine and interleukin 2 remain the only Food and Drug Administration–approved drugs for metastatic melanoma, each with response rates of ~15%. Clearly, more effective therapeutic approaches are needed. Several alkylating agents are active in melanoma, including Temozolomide, cisplatin, and 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU). However, the overall response rates are ≥15%, and combination chemotherapy has not been shown to be superior to the single agent dacarbazine (1). These results suggest that mechanisms of chemotherapy resistance are dominant in this disease, which has prompted continued research to uncover treatable mechanisms of melanoma escape from the actions of cytotoxic chemotherapy.

It has been shown that BCNU induces its cytotoxic effect largely through the generation of a chlorethyl adduct at the O^6-position of guanine in DNA that subsequently undergoes an intramolecular rearrangement to produce an unstable ethanoguanine intermediate, which subsequently reacts with cytosine on the opposite DNA strand (2, 3). An N^1-guanine-N^3-cytosine-ethanol cross-link results. Development of the interstrand cross-links can be prevented by the DNA repair protein, O^6-alkylguanine DNA alkyltransferase (AGT), which removes the initial O^6-chloroethyl adduct or binds to the O^6 portion of the ethanoguanine adduct. The first reaction removes the alkyl group leaving intact DNA, whereas the second reaction leaves a protein-DNA cross-link. Several recent studies have shown that AGT is expressed at high levels in melanoma (4), arguing that efficient repair of DNA damage induced by BCNU might be responsible for the low-level clinical activity observed.

The use of compounds that deplete AGT activity can potentiate the cytotoxic effect of certain alkylating agents.

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One such agent is O^6-benzylguanine (O^6BG), which has been shown to potentiate the activity of BCNU in preclinical studies (5–11). Phase I studies defined the optimal dose of O^6BG for depletion of AGT activity in tumors at 120 mg/m^2 (12, 13). The half-life of O^6BG was short, predominantly due to intracellular metabolism to the biologically active metabolite O^6-benzyl-8-oxoguanine (14). The half-life of O^6-benzyl-8-oxoguanine was longer than that of O^6BG, and sustained levels at >200 ng/mL seemed to correlate with tumor AGT depletion. O^6BG alone was very well tolerated, with transient lymphopenia being the only drug-related side effect observed.

A phase I study of the combination of O^6BG and BCNU was done in which the BCNU dose was started at 10 mg/m^2 and escalated in combination with a fixed dose of O^6BG at 120 mg/m^2 administered 1 hour prior. The maximally tolerated dose of BCNU was 33 to 40 mg/m^2 when combined with O^6BG (13, 15). Dose-limiting toxicities were thrombocytopenia and neutropenia; no nonhematologic dose-limiting toxicities were observed. Based on these preclinical data, phase I results, and the high levels of AGT reported in melanoma, we undertook a phase II study of the combination of O^6BG and BCNU at 40 mg/m^2 in patients with advanced melanoma who were either chemotherapy-naïve or who had received previous chemotherapy.

Patients and Methods

Study design. This was an open label, multi-institutional phase II trial of O^6BG and BCNU in patients with advanced melanoma, and was approved by the University of Chicago Institutional Review Board. The study was conducted through the University of Chicago Phase II consortium,[4] and O^6BG was provided through the Cancer Treatment Evaluation Program of the National Cancer Institute. The end points were assessment of toxicity, clinical response rate, time to progression, and depletion of AGT activity from peripheral blood mononuclear cells (PBMC) in both chemotherapy-naïve and in chemotherapy-experienced patients.

Eligibility. Adult patients ≥18 years of age with histologically confirmed melanoma and documented measurable metastases (including nonresectable in transit metastases) were allowed. Patients with treated brain metastases were allowed entry into the study if neurologically stable off steroids and anticonvulsants. For the chemotherapy-naïve cohort, no more than two prior immunotherapy regimens were allowed, including adjuvant IFN-α. For the chemotherapy-experienced cohort, no more than one prior chemotherapy was additionally allowed, including BCNU. Additional inclusion criteria included life expectancy of ≥12 weeks, Eastern Cooperative Oncology Group performance status of ≤2, disease measurable by Response Evaluation Criteria in Solid Tumors, and adequate organ function (defined as WBC ≥3,000/μL, ANC ≥1,200/μL, platelets ≥100,000/μL, total bilirubin ≤1.5 mg/dL, transaminases ≤3× upper limit of normal, serum creatinine ≤1.5 mg/dL or CrCl ≥60 mL/min), prothrombin time within the upper limit of normal, and pulmonary function testing with a corrected DLCO of ≥70% predicted.

Patients with any of the following findings were excluded from the study: antimelanoma treatment within the previous 4 weeks (6 weeks for BCNU and mitomycin C); active second malignancy except curatively treated nonmelanoma skin cancer, carcinoma *in situ* of the cervix, or superficial bladder cancer; pregnancy or unwillingness to use contraception while on study; the presence of only brain metastases; or any psychiatric illness that would interfere with patient compliance or informed consent.

Treatment plan. All patients signed written informed consent. Treatment was done on an outpatient basis. Patients received 120 mg/m^2 of O^6BG administered i.v. over 60 minutes, followed immediately by 40 mg/m^2 of BCNU administered i.v. over 60 minutes. Treatment was repeated every 6 weeks, and disease reevaluation was done every two cycles. Patients were allowed to continue unless there was disease progression, treatment interruption for >4 weeks, intolerable adverse effects, pregnancy, unresolved grades 3 to 4 toxicity, treatment with other investigational agents, radiation therapy to any lesion, or a decline in corrected DLCO to <50% of that predicted.

Evaluation of toxicity and response. Toxicity assessments were done every 2 weeks according to the National Cancer Institute Common Toxicity Criteria scale (version 2.0). No dose modifications of O^6BG were allowed. Dose reductions of BCNU were done in the case of grade IV thrombocytopenia or neutropenia that lasted >5 days. In such cases, the dose was reduced to 33 mg/m^2, and sequentially to 26, 19, and 12 mg/m^2 on subsequent cycles if necessary. Clinical and imaging responses were recorded after every two cycles of treatment. Response Evaluation Criteria in Solid Tumors was used to assess clinical response.

Measurement of O^6-alkylguanine DNA alkyltransferase activity in peripheral blood mononuclear cells. Approximately 35 mL of blood was collected into heparinized tubes prior to and ~18 hours after the first dose of O^6BG. PBMC were isolated by Ficoll-Hypaque centrifugation, and cell pellets were frozen at −70°C until analysis. Samples were analyzed in batch fashion. Substrate [^{3}H]-methyl-DNA was prepared by reacting calf thymus DNA with [^{3}H]-methylnitrosourea. Protein (50-250 μg) from PBMC was incubated with this substrate for 60 minutes at 37°C, and the reaction was stopped with 7.5% trichloroacetic acid. The precipitate was washed, and methylated purines were liberated by hydrolysis. [^{3}H]O^6-methylguanine and [^{3}H]N^7-methylguanine (as an internal control) were separated by reversed-phase high-performance liquid chromatography and quantitated by liquid scintillation counting. Radioactivity (dpm) in each peak was determined, and background radioactivity was subtracted. AGT activity is expressed as O^6methylguanine removed from DNA (fmol/μg). The limit of detection of this assay is 0.05 fmol/μg DNA (16).

Statistical considerations. The study was planned using separate Simon (17) two-stage designs for each cohort. In the chemotherapy-naïve cohort, the study was designed to test the null hypothesis that the true response rate is <10% against the alternative that it is >30%. In the prior-chemotherapy cohort, the trial was designed to test the null hypothesis that the true response rate is <5% against the alternative that it is >15%. If 3 of the first 18 patients in the chemotherapy-naïve cohort, or 2 of the first 23 patients in the prior-chemotherapy cohort showed a clinical response, then the study would be opened to a second stage in that cohort to include an additional 17 patients. This design had a significance level of 0.05 and 90% power to detect a true response rate of >30% in the chemotherapy-naïve cohort, and a significance level of 0.05 and 78% power to detect a true response rate of >15% in the prior-chemotherapy cohort. When only a single clinical responder was seen in the first 42 total patients from both cohorts, the trial was closed and did not proceed to the second stage. Time to progression and overall survival estimates were calculated using the Kaplan-Meier method, and comparisons between cohorts were analyzed using the generalized Wilcoxon test. Nonevaluable patients were not included in the analysis as they lacked a formal posttreatment disease assessment. For AGT assays pre- and posttherapy, a paired Student's t test was employed.

Results

Patient characteristics. Forty-two patients with advanced melanoma were enrolled in this multicenter phase II study between August 2000 and March 2003. Twenty-two were

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Table 1. Patient characteristics

	Chemotherapy-naïve (n = 22)	Prior chemotherapy (n = 20)
Median age (range)	57.5 (42-83)	53 (34-77)
Gender (M/F)	15/7	14/6
Performance status		
0	13	10
1	9	9
2	0	1
Median	0	0
Prior therapies		
0	11	0
1	7	3
2	3	9
>2	1	8
Median	1	2

chemotherapy-naïve and 20 had received prior chemotherapy in the metastatic disease setting. Most of the chemotherapy-naïve patients had received a prior immunotherapeutic regimen. The median ages were 57.5 and 53 in the chemotherapy-naïve and prior-chemotherapy cohorts, respectively, and approximately two-thirds were male (Table 1). Most patients had a performance status of 0 to 1. The median number of prior systemic therapies (including adjuvant IFN-α) was 1 in the chemotherapy-naïve cohort and 2 in the prior-chemotherapy cohort. Only nine patients (21%) had not received prior systemic therapy of any sort.

All 42 enrolled patients received at least one cycle of therapy with O^6BG/BCNU. All were evaluable for toxicity, and all but six were evaluable for clinical response. Of the nonevaluable patients, one died from apparent disease progression, and the other five had declining performance status consistent with clinical disease progression and were removed from the study before completing two cycles of therapy.

Clinical response. In the chemotherapy-naïve cohort, there was one complete response that was confirmed 6 weeks later, and 4 of 18 patients evaluable showed stable disease. In the prior-chemotherapy cohort, there were no objective responses and 3 of 18 patients evaluable showed stable disease. One of the patients went on to have complete surgical resection of residual disease and has not yet had a recurrence. All other evaluable patients progressed.

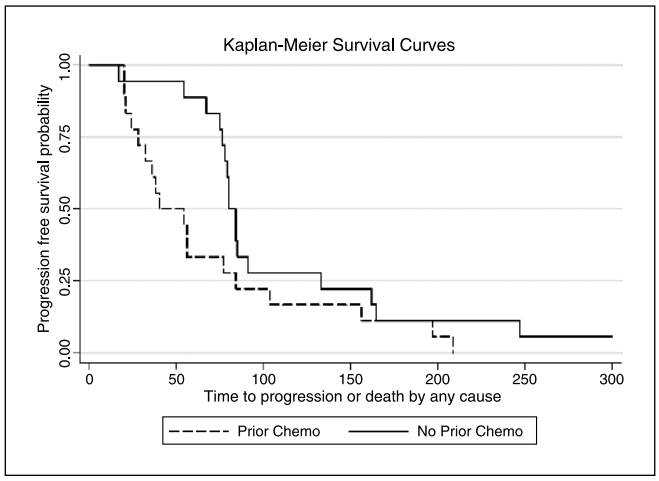

Fig. 1. Time to progression or death for the chemotherapy-naïve and the prior chemotherapy cohorts. Time to progression or death was determined using the Kaplan-Meier method. Comparisons were made using the generalized Wilcoxon test and showed significantly greater median time to progression in the chemotherapy-naïve cohort (P = 0.01).

The median time to progression or death was significantly longer in the chemotherapy-naïve cohort than in the prior-chemotherapy cohort (Fig. 1; P = 0.01). Similarly, the median overall survival time in these groups was 211 and 120 days, respectively. These results are consistent with more advanced disease in the prior-chemotherapy cohort, but also suggest that melanoma tumors that had progressed due to resistance to other chemotherapy agents are usually also resistant to O^6BG plus BCNU.

Toxicity. The chemotherapy-naïve and prior-chemotherapy cohorts showed a similar incidence and distribution of toxicities (Tables 2 and 3). Neutropenia, thrombocytopenia, and anemia were commonly seen, with grades 3 and 4 of at least one of these toxicities seen in 63% of chemotherapy-naïve patients and 55% of the prior-chemotherapy patients. Of note, although five patients showed a decline in pulmonary function DLCO testing following treatment, only one of these was a grade 3 event requiring discontinuation of therapy. Thus, O^6BG given at this dose and schedule does not seem to increase the incidence of BCNU-induced pulmonary toxicity.

Assessment of O^6-alkylguanine DNA alkyltransferase depletion. To determine whether O^6BG achieved the biochemical end point of AGT depletion, AGT activity was measured from PBMC obtained before and 18 hours after the first dose of O^6BG. Samples were successfully processed at both time points

Table 2. Adverse events in the chemotherapy-naïve cohort (n = 22)

Toxicity	Grade			
	1	2	3	4
Anemia	7	4	5	0
Neutropenia	0	7	5	6
Thrombocytopenia	2	1	9	5
Lymphopenia	2	5	3	0
Hyponatremia	1	0	2	0
Hypokalemia	2	0	0	0
Hypercalcemia	2	0	0	0
Transaminases	5	1	0	0
Bilirubin	0	1	1	0
Fatigue	5	3	1	0
Nausea/vomiting	8	0	1	0
Fever	2	2	1	0
Neutropenic fever	0	0	1	0
Neurologic	1	1	0	0
Edema	0	1	0	0
Dyspnea	0	0	0	0
Anorexia	5	0	0	0
Diarrhea	2	0	0	0
Mucositis	2	0	0	0
Constipation	0	0	0	0
DLCO decline	3	0	1	0

Table 3. Adverse events in the prior-chemotherapy cohort ($n = 20$)

Toxicity	Grade			
	1	2	3	4
Anemia	2	9	5	0
Neutropenia	1	3	5	1
Thrombocytopenia	2	1	9	2
Lymphopenia	0	3	2	0
Hyponatremia	0	0	0	0
Hypokalemia	1	0	0	0
Hypercalcemia	0	0	0	0
Transaminases	4	0	0	0
Bilirubin	1	1	0	0
Fatigue	8	3	0	0
Nausea/vomiting	5	0	0	0
Fever	2	0	0	0
Neutropenic fever	0	0	1	0
Neurologic	2	0	0	0
Edema	1	0	0	0
Dyspnea	0	3	0	0
Anorexia	5	1	0	0
Diarrhea	1	0	0	0
Mucositis	2	0	0	0
Constipation	2	0	0	0
DLCO decline	1	0	0	0

from 15 patients. As shown in Fig. 2, AGT activity was significantly depleted in all patients tested ($P = 0.002$). The degree of inhibition was greater than the reported variability of the assay (12).

Discussion

Chemotherapy resistance is a major obstacle in patients with metastatic melanoma. Although clinical response rates in the 20% range have been reported in smaller scale phase II trials, a recent multicenter randomized study comparing dacarbazine to the combination of dacarbazine/cisplatin/vinblastine yielded a response rate of ~11% (1). The observation of high AGT expression in melanoma tumors (4) gave rise to the hypothesis that DNA repair following alkylating agent activity could represent a major mechanism by which melanoma escapes the effects of cytotoxic chemotherapy. This hypothesis became testable with the availability of O^6BG, a potent AGT inactivator, for investigational clinical use.

O^6BG successfully depleted AGT activity from PBMCs in all patients tested in our study. In addition, myelosuppression was significantly induced, to an extent that would not be expected with this dose of BCNU alone (40 mg/m^2). These observations suggest that O^6BG was indeed exerting the anticipated potentiating effect on BCNU-induced cytotoxicity *in vivo*. Nevertheless, clinical tumor regression was not apparently different from what might be expected in response to BCNU alone. There are several possible explanations for this result that are worth considering. First, although O^6BG augments the cytotoxic effect of BCNU in tumor cells, it also does so in normal cells, which necessitated giving a lower dose of BCNU when used in this combination to avoid unacceptable toxicity. Thus, a lower dose of BCNU with O^6BG may be biologically equivalent to a higher dose of BCNU alone (i.e., with a similar therapeutic index). Second, it is possible that when AGT is depleted, an alternative DNA repair mechanism compensates. Nitrosoureas have also been reported to induce *N*-alkylated purine lesions, and also induce double-strand DNA breaks. DNA damage other than O^6-guanine alkylation would not be repaired by AGT and thus is not affected by O^6BG. Third, we did not directly measure AGT levels in tumor biopsy material in our trial, as the logistical limitations of performing pre- and posttreatment biopsies would have limited accrual. It is formally possible that AGT activity was depleted from PBMCs but not from the tumor microenvironment. This is unlikely as the dose of O^6BG used in our study has previously been shown to effectively deplete AGT in several tumor types including that in two patients with melanoma (18). However, it is conceivable that some melanoma tumors could have properties that render the activity of O^6BG less effective. The quantity of O^6BG that distributes into the tumor may be lower, or the rate of AGT resynthesis may be greater compared with other tumors. On the other hand, in melanomas, the level of AGT activity has been reported to be lower than in other tumor types (19). Thus, other escape mechanisms from cytotoxic chemotherapy may be operational in this disease. Finally, AGT activity was not 100% depleted from PBMC in all of our treated patients, arguing that interpatient heterogeneity of O^6BG activity may be another contributing factor.

Although we do not recommend further exploration of O^6BG/BCNU in the treatment of melanoma, there are several considerations for future development of AGT inhibition strategies. First, AGT inhibitors may be of greater use in tumors that show the highest level of AGT activity or methylation of the AGT gene locus (19, 20) and thus may be relying more heavily on this mechanism of chemotherapy resistance. Second, lentiviral transduction of hematopoietic cells to overexpress AGT is a potential strategy to render bone

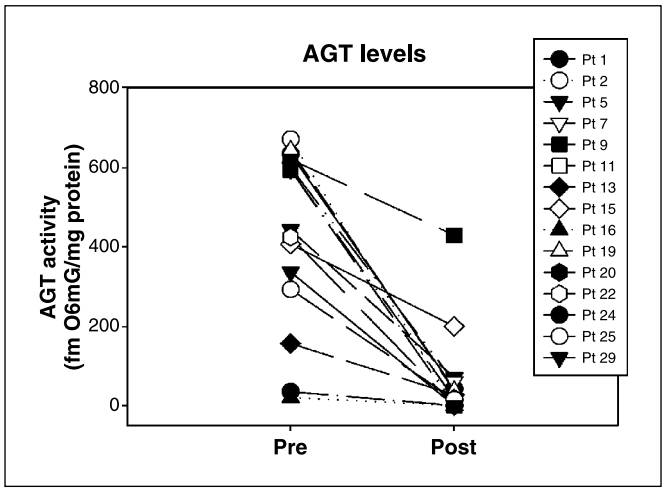

Fig. 2. Depletion of AGT activity from PBMC. AGT levels were measured pre- and post-O^6BG administration. Comparisons were made using a paired Student's t test and showed significantly reduced AGT activity posttreatment ($P = 0.002$).

marrow cells relatively resistant to alkylating agent chemotherapy, which may be of use for high-dose chemotherapy administration (21). Finally, in contrast to the situation with BCNU, there may be other alkylating agents that can be administered at full dose along with O^6BG, without an increase in toxicity to normal tissues. AGT inhibition has been shown to potentiate Temozolomide activity *in vitro* and *in vivo* (22), and Temozolomide is active in melanoma. In one ongoing clinical trial in primary brain tumor patients, Temozolomide is being administered in combination with O^6BG. Another consideration is the use of an altered schedule of administration of Temozolomide without O^6BG. Temozolomide has successfully been given at a dose of 75 mg/m^2 daily on a prolonged schedule, either continuously or for 6 to 7 weeks out of an 8-week cycle (23). This schedule has been shown to result in depletion of AGT levels *in vivo* (24), in part, associated with gene locus methylation (25), and thus could be advantageous for this reason. One might consider the combination of O^6BG with dacarbazine in melanoma. However, we have shown that O^6BG inhibits the metabolism of dacarbazine to its activating methylating species, thus making this combination less attractive (26). Finally, the ultimate mechanism of chemotherapy resistance in melanoma very likely is failed induction of apoptosis even when DNA damage or cell cycle arrest have occurred. Identifying strategies to potentiate melanoma cell death or restore normal apoptosis in response to therapy should receive a high degree of attention in future investigations.

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