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ORIGINAL ARTICLE

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A phase II trial of O^6-benzylguanine and carmustine in patients with advanced soft tissue sarcoma

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Abstract *Purpose*: Tumor resistance to alkylating agents such as carmustine (BCNU) has been found to be associated with intracellular expression of O^6-methylguanine-DNA methyltransferase (MGMT). Administration of O^6-benzylguanine (O^6-BG), a substrate that inactivates MGMT, may help overcome chemotherapy resistance. We performed a phase II study to explore the activity of O^6-BG in combination with BCNU in patients with advanced soft tissue sarcoma. *Experimental design*: Informed consent was obtained from patients with metastatic soft tissue sarcoma naïve to systemic chemotherapy (adjuvant chemotherapy allowed). Patients received O^6-BG 120 mg/m^2 I.V. followed by BCNU 40 mg/m^2 I.V. Treatment was repeated every 6 weeks until disease progression or development of unacceptable toxicity. *Results*: No objective responses were observed in 12 enrolled patients. Four patients exhibited stable disease lasting 11–25+ weeks. The median overall survival was 16.9 months (95% CI, 2.9–NR). The most common grade 3–4 toxicities were neutropenia, thrombocytopenia, and anemia. Depletion of MGMT activity was demonstrated in peripheral blood mononuclear cells. Immunohistochemical estimation of MGMT expression from archival tissue ranged from 20 to 99% positive staining cells. Conclusions: Observed toxicities were consistent with previous studies of O^6-BG plus BCNU. The degree of MGMT expression was variable in this small sample of heterogeneous sarcomas. Further development of this regimen and dose for the treatment of soft tissue sarcoma is not warranted due to the lack of objective responses.

Keywords Sarcoma · Carmustine · O^6-benzylguanine · DNA repair · Drug resistance

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Introduction

Soft tissue sarcomas are rare neoplasms accounting for only 1% of all malignancies. The term "soft tissue sarcoma" encompasses a wide range of histologic subtypes with differing biology and sensitivity to systemic therapy. Because of their individual rarity, these varying tumors have historically been studied together in clinical trials investigating chemotherapeutic agents. The choice of drugs for treatment of metastatic disease is limited, with anthracyclines and ifosfamide remaining the most-active agents [1–3]. Development of new treatments for soft tissue sarcomas remains a priority.

Carmustine (BCNU) is a nitrosourea that forms DNA cross-links by chloroethylation of a nucleophilic DNA site [4]. Its clinical utility is established in the treatment of brain cancers, lymphomas, and melanoma. The few studies that have investigated the use of nitrosoureas as treatment for soft tissue sarcomas have reported only limited activity [5–7]. A small study of whole body hyperthermia in combination with BCNU reported several responses in chemotherapy-resistant sarcoma patients [5].

Nimustine, a water-soluble nitrosourea, was studied by the EORTC Soft Tissue and Bone Sarcoma Group and 3 (9%) responses were observed in 28 previously treated soft tissue sarcoma patients [7]. A phase II study of fotemustine, a lipophilic nitrosourea, reported no responses [6].

Tumor resistance to alkylating agents has been found to be associated with the intracellular expression of O^6-methylguanine-DNA methyltransferase (MGMT, also known as O^6-alkylguanine-DNA alkyltransferase), a protein that mediates repair of O^6-guanine DNA adducts [8]. Silencing of the gene encoding MGMT by promoter methylation has been associated with improved survival in glioblastoma patients receiving alkylating agents such as BCNU and temozolomide [9, 10]. Lack of MGMT protein expression as measured by immunohistochemistry has also been associated with responses to temozolomide in malignant gliomas [11].

Overcoming MGMT-associated chemotherapy resistance by depleting tumor MGMT levels is a strategy that may be possible with the administration of O^6-benzylguanine (O^6-BG), a substrate that inactivates MGMT [12, 13]. Administration of O^6-BG has been shown to deplete MGMT levels in malignant gliomas [14, 15]. Optimal depletion of MGMT activity has been demonstrated in a variety of solid tumors using a dose of 120 mg/m^2 O^6-BG [16, 17]. Phase I trials of O^6-BG combined with BCNU demonstrated that reduced dosing of BCNU was required because of dose-limiting myelosuppression [18, 19]. The combination of BCNU plus O^6-BG has been explored principally in the treatment of malignant gliomas, but exploration in other potentially susceptible tumors is warranted [20].

DNA alkylating agents are reasonable for study in soft tissue sarcoma given that dacarbazine (DTIC) is an agent with established activity that exerts its cytotoxic effect primarily through the alkylation of DNA at O^6-guanine by its metabolite monomethyltriazenoimidazole carboxamide [21]. However, the conversion of DTIC to its active methylating species is inhibited in the presence of O^6-BG, possibly by the inhibition of cytochrome P450 isoenzymes, making the combination of O^6-BG and DTIC unlikely to result in therapeutic benefit [22]. We conducted a phase II study of O^6-BG with BCNU in patients with advanced soft tissue sarcoma to determine the activity of this treatment and to characterize MGMT expression in these tumors.

Methods

Patient eligibility

Patients 18 years and older were eligible if they had a histologically or cytologically confirmed soft tissue sarcoma, either recurrent, metastatic or locally advanced, judged incurable by surgery or radiation therapy. Prior chemotherapy was not allowed, unless given in the neoadjuvant and/or adjuvant setting and unless at least 4 weeks had elapsed since the last chemotherapy. Patients were required to have bi-dimensional measurable lesion(s), at least 1 cm×1 cm. Prior radiation therapy was allowed provided at least 3 weeks had elapsed from completion of the radiation therapy and all signs of toxicity had resolved. Any sites of disease located within the radiation therapy port required evidence of progression within the radiation port to qualify as measurable lesions. Adequate organ function was required, defined as: DLCO (corrected carbon monoxide diffusing capacity) ≥ 80% predicted, WBC ≥ 3,000/μl, ANC ≥ 1,500/μl, platelets ≥ 100,000/μl, total bilirubin WNL (unless due to Gilbert's syndrome with normal direct bilirubin), SGOT and/or SGPT ≤ 2 × upper limit of normal, serum creatinine ≤ 1.5 mg/dl or measured 24 h creatinine clearance ≥ 60 ml/min. Patients must have had an ECOG performance status of 0–2 and a life expectancy of ≥12 weeks. All patients provided written informed consent approved by all the participating centers' institutional review board. A negative pregnancy test was required for women of child-bearing potential, breastfeeding was not allowed while on study, and both women and men of reproductive potential agreed to use contraception while on study. Significant underlying medical or psychiatric illness that would interfere with protocol treatment was not allowed. Patients with uncontrolled symptomatic brain metastases were not eligible. Patients with a prior history of other malignancy within the past 5 years were not eligible except for those with curatively treated non-melanoma skin cancer, carcinoma in situ of the cervix, or superficial bladder cancer.

Treatment plan

Patients received O^6-BG 120 mg/m^2 I.V. in 150 ml D5W over 1 h followed 1 h later by BCNU 40 mg/m^2 I.V. in Q.S.150 ml D5W over 15 min. Anti-emetic premedication with ondansetron 24 mg p.o. and dexamethasone 10 mg p.o. was given 30–60 min prior to BCNU. Treatment was repeated every 6 weeks and each 6 week period was considered 1 cycle. Patients underwent a history and physical exam on the first day of each cycle, as well as 1 and 4 weeks after chemotherapy administration. Complete blood counts and a chemistry panel were obtained weekly. A chest X-ray as well as tumor measurements obtained by appropriate imaging were performed after every 2 cycles of treatment. Repeat DLCO was performed after every 2 cycles of treatment. A 25% dose reduction of BCNU was mandated for patients who developed grade 3–4 non-hematologic toxicity. Patients continued on treatment until disease progression, unresolved toxicity, or a ≥25% decline in DLCO from baseline.

Assessment of response was performed with appropriate radiographic studies prior to each 6 week cycle. A partial response was defined as ≥50% decrease from baseline in the sum of the products of two perpendicular diameters of all measurable lesions. Objective

progression was defined as ≥25% increase in the product of the perpendicular diameters of any measured lesion over the smallest sum observed, or reappearance of any lesion which had disappeared, or appearance of any new lesion/site.

Immunohistochemistry for MGMT

The method for immunohistochemistry for MGMT used in this study has been previously described [11]. Paraffin sections (5-μm thick) were brought to water, washed three times in absolute alcohol, then incubated in 1.85% H_2O_2 in methanol to block endogenous peroxidase, followed by rehydration in dH_2O and two 1-min washes in dH_2O. Antigen retrieval was accomplished by microwave heating (Panasonic No. NN-S666; Matsushita Electronic Corporation, Secaucus, NJ, USA) at medium power for 10 min in Biogenex AR-10 buffer (20 ml stock AR-10 in 180 ml dH_2O; Biogenex, San Ramon, CA, USA), followed by cooling at room temperature for 30 min. After cooling, the slides were washed in running dH_2O and two changes of phosphate-buffered saline (PBS; 150 μM sodium chloride, 8.3 sodium phosphate dibasic; 2.2 μM sodium phosphate monobasic) for 2 min each. Slides were then incubated at room temperature in a moisture chamber for 15 min with 5% normal goat serum (NGS) to block protein binding. After removing the excess NGS by tipping the slides and blotting around the tissue, slides were incubated overnight at 4°C with either anti-MGMT (clone mT3.1 5 μg/ml; Chemicon International, Temecula, CA, USA) or mouse IgG1 (5 μg/ml; DAKO Corporation, Carpinteria, CA, USA). The next morning, the slides were allowed to acclimate to room temperature for 1 h, followed by twice PBS washes of 2 min each. The tissues were then incubated for 30 min with a Super Sensitive Multi-Link HRP detection system (Biogenex, San Ramon, CA, USA) according to the manufacturer's instructions with diaminobenzidine as the chromogen. Counter-staining was achieved with Harris Modified hematoxylin (Fisher Scientific, Pittsburgh, PA, USA). Normal tonsil was used as the positive control in each run. Immunoreactivity was quantified by counting staining tumor cell nuclei over 1,000 cells in regions considered to be the most immunoreactive for the antigen to determine the labeling index (LI) of percent positive nuclear reactivity. Cytoplasmic-only reactivity and granular nuclear reactivity were regarded as negative.

O^6-methylguanine-DNA methyltransferase activity of peripheral blood mononuclear cell (PBMC)

Blood (30 ml) was collected prior to beginning the O^6-BG infusion and at 24 h post-infusion. Within an hour of collection, RPMI medium was added to bring the volume to 40 ml and the diluted blood was layered on Ficoll-Paque (Histopaque 1077). After centrifugation at 1,500×g for 30 min, the lymphocyte layer was removed and resuspended in 15 ml PBS and centrifuged at 300×g for 10 min at room temperature. Red blood cells were lysed by the addition of 6 ml of deionized water for 15 s after which 2 ml of 3.6% NaCl and 15 ml of PBS were added. The samples were centrifuged at 300×g for 10 min. Final pellets were resuspended in 50 mM Tris, pH 7.5, 0.1 mM EDTA, 5 mM dithiothreitol. MGMT activity was determined as described previously [23]. Briefly, the cell extracts were incubated with [^{3}H]-methylated DNA substrate. 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 min. The modified bases were eluted on a C-18 reverse phase column using 10% methanol/0.05 M ammonium formate pH 4.5, at 37°C. The MGMT assay was performed with a positive control (HT29 cell extract) and negative control (H80 cell extract). Protein concentration was determined by the method of Bradford [24]. The results were expressed as fmol of O^6-methylguanine released from DNA per mg of protein. Assays were performed in duplicate or triplicate when there was adequate sample. Three pre O^6-BG samples were assayed only once due to small sample size.

Statistics

The primary endpoint of the study was to determine the objective response rate of O^6-BG with BCNU in patients with advanced soft tissue sarcoma. A Simon's two-stage, phase II design was employed to determine an overall response probability of not less than 10% versus an alternate response rate of ≥30%, a response rate that has been reported with combined anthracycline and ifosfamide therapy [1, 3]. Study termination would occur if 1 or fewer responses occurred among the first 12 patients. If at least two responses were observed, an additional 23 patients would be evaluated in a second stage. Only if six or more total responses were observed would the null hypothesis (that the underlying response rate is less than 10%) be rejected. The α error probability of this study design was 0.10 with a power of 90%.

Results

This study was performed through the University of Chicago Phase II Consortium. Between May 2000 and January 2001, 12 patients with soft tissue sarcoma were enrolled on this protocol at four separate institutions (University of Chicago, Chicago, IL, USA; Lutheran General Hospital, Park Ridge, IL, USA; Evanston Hospital, Evanston, IL, USA; Central Illinois Hematology Oncology Center, Springfield, IL, USA). Baseline patient characteristics are shown in Table 1. The most common histology was leiomyosarcoma with four subjects enrolled. Two subjects had gastrointestinal stromal tumor (GIST) and were treated on this study before the availability of imatinib. The median number of cycles received was 2 (range 1–4). No dose reductions were necessary. Treatment was discontinued for progressive

disease in ten patients, and electively by the investigator in two patients with stable disease because of toxicity that did not meet the protocol-specified withdrawal criteria (myelosuppression, supraventricular tachycardia).

Grade 3–4 toxicities are summarized in Table 2. The most common grade 3–4 toxicities were hematologic. Six patients (50%) developed grade 3–4 neutropenia. There were no incidences of neutropenic fever. Thrombocytopenia was seen in the majority of patients, with nine patients (75%) developing grade 3–4 thrombocytopenia. Grade 3 anemia was seen in three patients (25%). One subject had an asymptomatic fall in DLCO from 79 to 57% after 2 cycles of therapy. One subject developed grade 2 supraventricular tachycardia.

There were no objective responses (Table 3). Stable disease for a period of 11+, 16, 22, and 25+ weeks was observed in four patients, respectively. One patient with stable disease successfully underwent resection of a large primary leiomyosarcoma from the chest wall after receiving 2 cycles of treatment. The median overall survival was 16.9 months (95% CI, 2.9–NR). The Kaplan–Meier curve for overall survival is shown in Fig. 1. The median progression-free survival was 2.5 months (95% CI, 1.2–4.9).

Peripheral blood mononuclear cells from whole blood samples were obtained prior to administration of O^6-BG in six subjects. All 6 samples demonstrated high levels of PBMC MGMT activity (185–958 fmol/mg protein).

Table 1 Patient characteristics

Subjects enrolled	12
Age (years)	
Median (range)	60.5 (42–79)
Gender	
Male	8
Female	4
ECOG performance status	
0	9
1	3
Histology	
Leiomyosarcoma	4
Liposarcoma	2
GIST	2
Spindle cell	3
Synovial sarcoma	1
Previous therapy	
Surgery	11
Radiation	7
Adjuvant chemotherapy	1

Table 2 Number of patients with grade 3–4 toxicities, worst overall ($N = 12$ subjects)

Adverse event	Gr. 3 no.	Gr. 4 no.
Neutropenia	3	3
Thrombocytopenia	7	2
Anemia (Hgb)	3	0
Nausea	1	0
Vomiting	1	0
Alk Phos	1	0
Injection site R×n	1	0

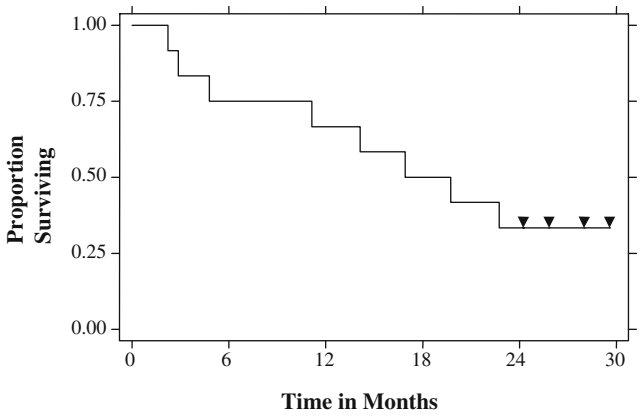

Fig. 1 Overall survival. *Tick marks* represent the censored patients

Table 3 Individual response data and baseline MGMT expression in tumor measured by immunohistochemistry

Patient no.	Histology	Best response	% + MGMT
1	Leiomyosarcoma	PD	20
2	Leiomyosarcoma	SD	30
3	Liposarcoma	PD	30
4	Liposarcoma	PD	30
5	Synovial cell sarcoma	SD	99
6	GIST	PD	99
7	Spindle cell sarcoma	SD	50
8	Spindle cell sarcoma	PD	Not done
9	Leiomyosarcoma	PD	95
10	GIST	PD	99
11	Spindle cell sarcoma	PD	99
12	Leiomyosarcoma	SD	80

PMBCs obtained 15 h post O^6-BG infusion were available in two subjects, and both samples demonstrated undetectable MGMT activity (Fig. 2).

Tumor levels of MGMT were measured by the immunohistochemistry staining of paraffin embedded tissue (Table 3). Previous studies by our group [11] have found that an MGMT LI of 20% can be used to dichotomize those who may respond to alkylating chemotherapeutic agents (LI < 20%) and those that will not respond (LI > 20%). We used an LI of 20% to dichotomize our groups of immunohistochemical results in statistical analyses. There was no obvious relationship between the level of MGMT immunohistochemical staining and any particular sarcoma subtype, but this was limited by the small sample size. All tumors measured for MGMT exhibited at least 20% positive staining cells. Two leiomyosarcomas exhibited a high level of MGMT staining (80–95%), while two did not (20–30%). Both GIST tumors expressed high levels of MGMT (99%) and both GIST patients developed progressive disease on the study treatment. High staining tumors are expected to be resistant to BCNU; however, O^6-BG was used as a means to deplete MGMT. Since MGMT activity was not measured post O^6-BG in tumors, we cannot report the degree of modulation by this agent.

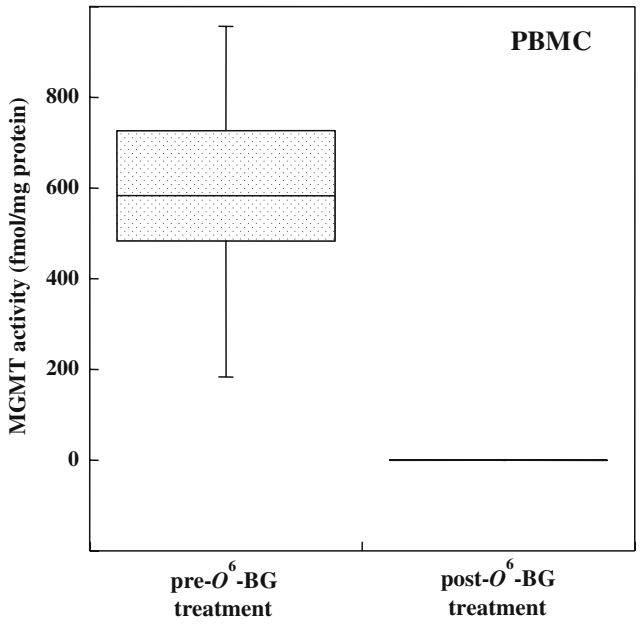

Fig. 2 Boxplot of PBMC MGMT activity from sarcoma patients before and 24 h following treatment with O^6-BG. PBMCs isolated from blood before and 24 h following 120 mg/m^2 O^6-BG were assayed for MGMT activity using [^{3}H]DNA. Pre O^6-BG represents the mean MGMT activity (fmol/mg protein) PBMCs from six patients and post O^6-BG represents the mean from two patients (both values were 0). Positive control cells (HT29; 358 fmol/mg protein) and negative control cells (H80; 0 fmol/mg protein) were included in the assay

Discussion

We did not detect any significant activity with O^6-BG combined with BCNU in this small series of chemotherapy-naïve soft tissue sarcoma patients. Further development of this regimen for soft tissue sarcoma cannot be recommended based on these results. While it is possible that certain sarcoma subtypes may be susceptible to treatment with BCNU and MGMT depletion, we were unable to detect any signal of activity in this 12 patient study.

The dose of BCNU used in this study was based on the results of a phase I study that reported myelosuppression as the dose-limiting toxicity and a maximal BCNU dose approximately one-third of the standard clinical dose when administered in the presence of O^6-BG [19]. The incidence of significant neutropenia and thrombocytopenia observed in our study is consistent with previous reports using this regimen. While no serious clinical consequences were associated with this myelosuppression, it provides confirmation that the doses employed are the maximum tolerable.

The same dosing of BCNU and O^6-BG was used in a phase II study in malignant glioma [20]. No responses were observed in 18 patients treated on that study. The low dose of BCNU mandated by co-administration with O^6-BG has been suggested as a reason for the low response rate seen in glioma, and may also be an explanation for the lack of activity observed in our study in soft tissue sarcoma. Another explanation is that O^6-BG did not effectively inactivate MGMT activity in the tumor. While we demonstrated the depletion of MGMT activity in PBMCs obtained from two subjects, depletion of MGMT activity in PBMC is not a reliable predictor for tumor tissue depletion [17, 25]. Our study did not include analysis of MGMT post O^6-BG in the tumor to ascertain its effectiveness as a modulator of MGMT in sarcomas. Although this dose has been effective at depleting MGMT activity in other tumor types, a systematic study in sarcomas has not been performed [16]. Another possibility is that the MGMT is depleted but is followed by rapid resynthesis of new protein in the tumor. Furthermore, immunohistochemistry is limited in this analysis because it provides us with a value for the percentage of cells expressing MGMT protein but not MGMT activity.

It is of historical interest that of the four long-term surviving patients, two carried a diagnosis of GIST. This is explained by the emergence of imatinib as therapy for this disease shortly after the conduct of this study. The fact that both GIST patients progressed on study treatment but were able to achieve prolonged survival suggests that "window" therapy prior to imatinib may be a reasonable strategy to test new agents. Unfortunately, survival for other metastatic soft tissue sarcomas remains poor and development of new therapies for these diseases remains a priority. Because of the toxicity and limited efficacy of standard drugs for soft tissue sarcoma, testing new agents in the chemotherapy-naïve setting is a reasonable strategy and such clinical trials should be offered to sarcoma patients, if available.

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