Carmustine and Streptozocin in Refractory Melanoma: An Attempt at Modulation of \(O^6\)-Alkylguanine-DNA-alkyltransferase

David C. Smith, Stanton L. Gerson, Lili Liu, Sandra Donnelly, Roger Day, Donald L. Trump, and John M. Kirkwood

Division of Medical Oncology, Department of Medicine, University of Pittsburgh School of Medicine [D. C. S., D. L. T., J. M. K.] and University of Pittsburgh Cancer Institute [D. C. S., S. D., R. D., D. L. T., J. M. K.]. Pittsburgh, Pennsylvania 15213, and Hematology/Oncology Division, Cancer Research Center, Case Western Reserve University, Cleveland, Ohio 44106 [S. L. G., L. L.]

ABSTRACT

The activity of the enzyme \(O^6\)-alkylguanine-DNA-alkyltransferase (AGAT) protects cells from the cytotoxic effects of alkylating agents. This Phase II trial was designed to assess the efficacy of a strategy designed to modulate the resistance to carmustine (BCNU) mediated by AGAT using streptozocin (STZ) in patients with advanced refractory melanoma. Seventeen patients who had failed prior chemotherapy were treated with STZ at 500 mg/m\(^2\) daily for 4 days with BCNU at 150 mg/m\(^2\) on day 3. Peripheral blood lymphocytes for assay of AGAT activity levels were collected prior to therapy and following the third dose of STZ. There were two partial responses in the 15 patients evaluable for response (13%). Most patients received only a single cycle of therapy due to rapidly progressive disease. Two patients developed fatal pulmonary toxicity, and one developed myelodysplasia. Other toxicities included transient rises in liver function tests. AGAT levels decreased by a mean of 53% in 9 patients but actually increased over baseline in 3 patients while on therapy. Based on these data, BCNU and STZ are not an effective combination for the therapy of advanced refractory melanoma, and pulmonary toxicity due to this combination appears to be increased compared with BCNU alone. STZ is not an effective modulator of AGAT activity when given on this schedule. New strategies designed to deplete AGAT activity using \(O^6\)-benzylguanine or temozolomide should be explored with careful attention to the possibility that this approach may potentiate both the toxicity and efficacy of BCNU.

INTRODUCTION

Alkylating agents are among the most potent antineoplastic drugs in experimental systems and clinical practice. The nitrosoareas are classic alkylating agents, which have shown substantial promise in preclinical evaluations (1). They have been incorporated into a variety of chemotherapeutic regimens used in the treatment of lymphoma, myeloma, lung and colon cancer, melanoma, and brain neoplasms (2). Unfortunately, most tumors eventually develop resistance to the cytotoxic effects of the nitrosoareas. One mechanism of this resistance is mediated by the enzyme AGAT (3, 4). The \(O^6\) position of guanine is a preferred site of activity for the chloroethyl nitrosoareas such as BCNU, which initially attaches an alkyl group at the \(O^6\) position, followed several hours later by the formation of a cross-link with the cytidine of the complementary DNA strand (5, 6). During this period, the alkyl group is vulnerable to the activity of AGAT. This enzyme acts as a suicide enzyme, covalently binding alkyl and methyl groups attached to the \(O^6\) position and removing them (7). In doing so, the enzyme is depleted, resulting in increased vulnerability of cells to the cytotoxic activity of alkylating agents. Levels of AGAT are variable in tumor tissues, and expression can be induced by exposure to alkylating agents (3, 8).

One strategy to improve the efficacy of alkylating agents is based on this observation. Agents such as STZ efficiently methylate the \(O^6\) position of guanine (9). This methyl group can be removed from DNA by AGAT, depleting enzyme activity. \textit{In vitro}, BCNU-resistant HT-29 cells are sensitized by pretreatment or concurrent exposure to STZ via this mechanism (10, 11). STZ at noncytotoxic levels decreases AGAT activity by 75%, with subsequent exposure to BCNU resulting in a 3–4-log increase in cell killing compared with BCNU alone. The AGAT activity can be reduced by up to 90% following a single large dose of STZ in this system, with >50% depletion maintained for 24 h. Peripheral blood lymphocyte levels of AGAT have been used as intermediate markers of drug effect. These levels are reduced by 40% by a single 500-mg/m\(^2\) dose of STZ and by 75% by three doses (12). Based on these data, Phase I trials of the combination of BCNU and STZ have been conducted by Micetich \textit{et al.} (13), who gave STZ at 2 g/m\(^2\) as a single dose, followed 20 min later by BCNU, and Panella \textit{et al.} (14), using STZ at 500 mg/m\(^2\)/day for 4 days with BCNU after the third dose of STZ. The maximum tolerated doses of BCNU in these trials were 125 and 150 mg/m\(^2\), respectively. A third Phase I study using a single 2-g/m\(^2\) dose of STZ followed 6 h later by escalating doses of BCNU showed a maximum tolerated dose of 130 mg/m\(^2\) (15).

DTIC and BCNU are among the most active drugs in the therapy of melanoma (16). These agents share a common site of activity at the \(O^6\) position of guanine. Thus, a possible strategy...
for treating patients with refractory melanoma is to focus efforts on improving the response rate to these agents through modulation of resistance mediated by AGAT. To explore this approach, we conducted a Phase II trial of BCNU and STZ in patients with refractory advanced melanoma. The secondary objectives of this trial were to assess the degree of AGAT depletion in peripheral blood lymphocytes from patients treated with this combination and to correlate this depletion with response and toxicity.

PATIENTS AND METHODS

Patients. Patients eligible for this trial had pathologically confirmed, measurable stage 3 or 4 malignant melanoma, normal hematological (absolute granulocyte count ≥1500 mm$^3$; platelets ≥100,000 mm$^3$), hepatic (bilirubin ≤2.0; aspartate aminotransferase ≤4× normal), renal (creatinine ≤2.0 mg/dl), and pulmonary function (FEV1 > 1 liter/min in patients with symptomatic pulmonary disease), age 18 years or older, ECOG performance status ≤1, and no more than one prior chemotherapy regimen. Patients who had received prior irradiation, chemotherapy, or immunological therapy were required to wait 4 weeks prior to study entry. Patients with brain metastases or concurrent additional cancers were excluded from the study. This study was approved by the Biomedical Institutional Review Board of the University of Pittsburgh, and all patients provided written informed consent.

Treatment Plan. All patients underwent an initial screening evaluation consisting of a complete history and physical exam (including documentation of all measurable lesions) and laboratory evaluations. Patients who had symptomatic pulmonary disease underwent pulmonary function testing with measurement of the diffusion capacity. Eligible patients were treated with 500 mg/m$^2$ STZ as a 20-min i.v. infusion for 4 consecutive days. BCNU was administered at a dose of 150 mg/m$^2$ 4 h following the third dose of STZ. Treatment cycles were repeated every 6 weeks. Patients were eligible for retreatment following recovery of their laboratory parameters to the study entry criteria and if performance status remained ≤2 on the ECOG scale with no evidence of progressive disease.

Dose Modifications. The dose of BCNU for subsequent cycles was reduced by 25% for any patient developing grade 4 hematological toxicity, any symptomatic grade 3 hematological toxicity, or any grade 3 nonhematological toxicity, excluding nausea and vomiting. Any grade 4 nonhematological toxicity other than nausea and vomiting resulted in removal of the patient from the study. Colony-stimulating factors were used at the discretion of the treating physician.

Assessment of AGAT Levels. Peripheral blood lymphocytes for assay of AGAT levels were collected prior to the first dose of STZ and prior to the dose of BCNU in each treatment course. Mononuclear cells from 20 ml heparinized blood were isolated on Ficoll-Hypaque gradients. Cells were washed twice in PBS, resuspended in 1 ml HEPES buffer (pH 7.5–7.8), and stored at −70°C. This procedure represented a modification of the initial protocol for cell storage in which the cells were not resuspended. For the first seven patients, cells were simply pelleted after the PBS wash and stored at −70°C. The cell pellets were resuspended in 0.5 ml cell extract buffer (70 mm 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (pH 7.8), 1 mM EDTA, 5% glycerol, and 1 mM DTT). The assay of AGAT activity in the cell extracts was performed as described previously (17). In brief, the cell suspension was sonicated three times for 10 s at 4°C to complete cell disruption and centrifuged at 10,000 × g for 2 min to remove cellular debris. The protein and DNA contents were measured, and then fixed amounts of protein from each sample were incubated with substrate DNA, which was prepared by incubating calf thymus DNA with $^{[3H]}$methylnitrosourea. The mixture was incubated for 60 min at 37°C, then precipitated with 14% trichloroacetic acid at 4°C. AGAT activity in cell extracts was determined by removal of the $^{[3H]}$methyl group from $^{[3H]}$methylguanine presented as $^{[3H]}$methylguanine-DNA. The alkylated $^{[3H]}$methylguanine and $^{[3H]}$methylguanine bases were liberated by acid hydrolysis, separated by high-performance liquid chromatography, and quantified by liquid scintillation. AGAT activity was expressed as fmol $^{[3H]}$methyl removed/μg DNA.

Statistical Analysis. This study was designed as a two-stage trial as described by Simon (18). To reduce the risk of treating patients on an unacceptable treatment regimen, an early stopping rule was specified, which approximated our expectations regarding future clinical judgments in this situation. Fifteen evaluable patients were to be entered in the first stage of this trial. If at least three of those patients had evidence of response, an additional 13 patients were to be accrued for a total of 28 patients. This study was designed to detect a response rate of 35% with a power of 80% and an α of 0.05, for a response rate less than 15%.

Response Criteria. Standard ECOG response criteria were used, and all patients who completed one cycle of chemotherapy (6 weeks) were assessed for response.

RESULTS

Seventeen patients were enrolled in this trial. Of these, 15 were evaluable. One patient developed rapidly progressive disease, resulting in his death 3 weeks after his first cycle of treatment. A second patient refused further follow-up after the administration of chemotherapy. The patient characteristics are summarized in Table 1. All patients had received prior chemotherapy with DTIC-based regimens. One had previously received therapy with a regimen containing both DTIC and BCNU. The majority had received prior immunotherapy.

A total of 29 (range, 1–5; median 1) cycles of therapy were administered. Only four patients received more than two cycles of therapy, and the majority received only one. The toxicities associated with this therapy are summarized in Table 2. There were five episodes of grade 3 thrombocytopenia in three patients
and three episodes of grade 4 thrombocytopenia. One of these episodes of grade 4 thrombocytopenia was associated with grade 4 neutropenia. No patients were hospitalized for fever or episodes of grade 4 thrombocytopenia was associated with the prior cycles but developed progressive pulmonary metastases. The second patient had no significant toxicities associated with her known pulmonary metastases. After his third cycle, however, he developed persistently elevated liver function tests following his third cycle of therapy, despite holding further therapy. One patient who had grade 4 thrombocytopenia with her first cycle of therapy had persistent grade 2 thrombocytopenia, which precluded administration of further cytotoxic therapy. She subsequently developed moderate pancytopenia. A bone marrow biopsy performed 12 months after study entry revealed dysplastic changes in all three cell lineages, consistent with a diagnosis of myelodysplasia. No cytogenetic analysis was performed. She died of progressive melanoma 7 months after this diagnosis. There were two treatment-related deaths due to pulmonary toxicity. In both cases, the pulmonary toxicity occurred following the third cycle of chemotherapy and manifested as rapidly progressive respiratory insufficiency. The first patient had developed grade 3 thrombocytopenia with her first two treatment cycles. At autopsy, she had pulmonary fibrosis and hyaline membrane disease without evidence of lymphangitic spread of her known pulmonary metastases. The second patient had no significant toxicities associated with the prior cycles but developed progressive pulmonary insufficiency, resulting in his death on day 41 of his third treatment cycle.

No complete responses were seen among the evaluable patients on this trial; however, 2 (13%) of the 15 patients had partial responses. A partial response following the second cycle of therapy was seen in a patient with multiple pulmonary nodules. After his third cycle, however, he developed persistently elevated liver function tests of uncertain cause and 2 months later was noted to have progression of his disease. The other responder was a patient with metastatic melanoma to his gastric wall, who was shown to have a marked reduction in the size of his mass by endoscopy. This patient received a total of five cycles of therapy, with his fourth and fifth cycles resulting in the development of transient grade 3 hepatic toxicity. Following his fifth cycle, his therapy was discontinued, and he remained in a stable partial response for eight months before developing recurrent disease. Two patients had stable disease following their second course of therapy and received a third cycle. Both developed fatal pulmonary toxicity as described above.

Specimens for assay of AGAT activity in peripheral blood lymphocytes were obtained from all patients. Paired specimens with sufficient lymphocytes for assay were obtained in 14 patients (Fig. 1). Nine of 14 patients had statistically significant (day 1 mean, 2.89 fmol/μg DNA; day 3 mean, 1.23 fmol/μg DNA; P = 0.002) decreases in AGAT activity, with a mean decrease of 53% (range, 22–81%). Two patients had no change, and 3 had increases in enzyme activity. Mean data for all groups are summarized in Table 3. One of the patients who responded and both patients with stable disease and subsequent pulmonary toxicity are among the 9 patients who had significant declines in their AGAT activity. The patient who developed myelodysplasia (1.54 fmol/μg DNA) and the patients with pulmonary toxicity (1.03 and 1.07 fmol/μg DNA, respectively) had among the lowest pretreatment levels of AGAT activity. On day 3, the patients who developed pulmonary toxicity had very low levels (0.72 and 0.95 fmol/μg DNA, respectively), whereas the patient with myelodysplasia had an increase in her level (3.12 fmol/μg DNA). These numbers are too small to postulate any association between these levels. When all the paired data are considered, there is no statistically significant change in AGAT activity following the administration of three doses of STZ. This lack of significance is largely due to the single patient whose posttreatment value rose markedly, reaching a level more than 2 SD above that of any other patient in the study. There was no significant correlation between a decline in AGAT activity and hematological, hepatic, or pulmonary toxicity.

**DISCUSSION**

DTIC and the BCNU are among the most active single agents in the treatment of melanoma, with documented response rates ranging from 10–25% (19–22). Based on this activity, they have been incorporated into combination chemotherapy regimens, which in Phase II trials have shown some improvement in response rate. Although the response to these regimens...
indicates activity in the treatment of metastatic melanoma, in randomized comparisons, they have not been proven superior to DTIC alone (16). DTIC remains the cornerstone of chemotherapy for advanced melanoma.

DTIC and the nitrosoureas share common sites of activity on the DNA strand, one of which is the O^6 position of guanine (6, 23). DTIC is known to methylate this site, and this is a preferred site of activity for BCNU. AGAT protects this site from alkylation and limits the cytotoxicity of these agents. AGAT is present in highly variable amounts in melanoma cell lines and tumor specimens; lymph node and skin metastases demonstrate somewhat less variability (24–26). Although these reports have small numbers of specimens, at least one suggests that AGAT may be inducible in tumor specimens, and that the level of AGAT may correlate with survival (25). The demonstration of AGAT activity in melanoma and the development of strategies to modulate its activity served as the bases for this trial. The combination of DTIC and fotemustine has been shown to deplete AGAT activity in peripheral blood lymphocytes, and trials using the combination have shown considerable activity in patients with melanoma (27–29). One of these trials used widely separated doses in a regimen that would not be expected to deplete AGAT activity and to potentiate cytotoxicity via this mechanism (27). Despite this, the response rate to this regimen (33%) was comparable to one using a strategy designed to modulate AGAT-mediated resistance (28).

Our trial demonstrated a low level of response in patients with tumors known to be refractory to DTIC. Several factors could account for the low response rate. The data from this trial suggest that STZ given on this schedule is not an effective modulator of AGAT activity, as indicated by the highly variable levels of enzyme activity on day 3. The mean decrease in AGAT activity following three doses of STZ was only 53% in the nine patients whose levels declined. This calculation does not take into account the patients who appeared to have stable or increased levels. This is smaller than the decrease in AGAT activity demonstrated in the Phase I trial (>89%), but that trial reported levels on only six patients. A recent report has suggested that a maximal dose of STZ given approximately 6 h prior to BCNU is likely to be more effective at depleting AGAT activity (15). Even with this schedule, however, substantial enzyme activity remains since the decrease in enzyme activity was 81% in peripheral blood lymphocytes and 78% in the target tissues. Other possible factors in the poor response are the marked interpatient variability of the AGAT levels and the inconsistent inhibition of activity by STZ. Changes in AGAT levels in peripheral blood lymphocytes are known to have significant interindividual variability and to serve only as general indicators of the degree of AGAT depletion in target tumors (15). No prior trial of this combination has reported an increase in levels following STZ administration. Three of the patients enrolled on this trial had increased levels of enzyme activity on day 3 when compared with pretreatment values. Day-to-day variations in AGAT levels are small (15); thus, interindividual variability is unlikely to account for this observation. The cause of this finding is unclear, but it is another indicator that STZ is unlikely to be effective when used alone as a modulator of AGAT activity. Finally, induction of resistance by augmentation of AGAT or other mechanisms of resistance due to the prior exposure to DTIC and the prolonged treatment cycles required to recover from the toxicity of BCNU may also have contributed to the poor response rate.

Although residual AGAT activity in target tissues may have limited response, depletion of enzyme activity in normal tissues may have exacerbated toxicity. The frequency of acute pulmonary toxicity associated with this combination appears to be increased when compared with that associated with BCNU alone (28, 30, 31). The clinical course is similar to that reported in DTIC and fotemustine trials, in which 6 of 107 patients developed rapidly progressive and fatal respiratory failure (29). One patient in the Phase I trials of STZ and BCNU died of progressive respiratory insufficiency (13). STZ is not associated with pulmonary toxicity (32). These deaths suggest that the pulmonary toxicity of the nitrosoureas may be potentiated by attempts to modulate AGAT, either through inhibition of this enzyme or through another mechanism. It has been suggested that metabolites of DTIC may deplete cellular glutathione, resulting in augmentation of the pulmonary toxicity of fotemustine (29). STZ is known to deplete erythrocyte glutathione and to induce hepatic glutathione S-transferase activity in mice (33, 34). Whether this activity contributes to the potentiation of pulmonary toxicity and whether the prior exposure to DTIC may have played a role is unknown and will require further investigation. The development of myelodysplasia in a patient treated in this trial is also of concern. Although it would appear unlikely that the myelodysplasia was due to the therapy since she received only one cycle of treatment, the early onset of significant thrombocytopenia and the interval from exposure to diagnosis are comparable to those seen in chemotherapy-induced preleukemia and leukemia (35, 36). Human myeloid precursors are known to have relatively low levels of AGAT (37), and this patient had one of the lower levels of activity in peripheral blood lymphocytes before treatment. Depletion of AGAT activity could theoretically potentiate the leukemogenic effects of nitrosoureas, raising concerns about this strategy for improving the efficacy of these agents.

The low response rate and the incidence of acute pulmonary toxicity with this combination preclude its further use in patients with refractory melanoma. Despite these concerns, depletion of AGAT remains an appropriate strategy for improving the efficacy of the alkylating agents in melanoma and other cancers. O^6-benzylguanine, which is a direct substrate for AGAT, is now entering clinical trials. This drug has shown significant inhibition of enzyme activity and potentiation of the cytotoxic activity of alkylating agents in preclinical models (38, 39). The DTIC analogue temozolomide has also been shown to deplete AGAT levels in peripheral blood lymphocytes and biopsies of melanoma tissues (40, 41) and is now entering Phase II trials. These trials should provide a good test of this therapeutic strategy. As they progress, careful monitoring will be required to assess whether more effective modulation and presumably greater efficacy results in increased toxicity.


Carmustine and streptozocin in refractory melanoma: an attempt at modulation of O-alkylguanine-DNA-alkyltransferase.

D C Smith, S L Gerson, L Liu, et al.