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Sequential therapy with dacarbazine and carmustine: a phase I study

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Abstract. Depletion of the DNA-repair protein O^6-alkylguanine-DNA alkyltransferase (AGT) increases the sensitivity of cells in culture and of human tumor xenografts to chloroethylnitrosoureas such as carmustine (BCNU). We have previously demonstrated that dacarbazine (DTIC) can deplete AGT activity in cells in culture and in human tumor xenografts. A phase I trial of DTIC followed immediately by BCNU was conducted to determine the DTIC dose resulting in maximal depletion of AGT in the peripheral blood mononuclear cells (PBMC) of cancer patients and to determine the maximally tolerated dose of DTIC given as a 4-h infusion immediately prior to a fixed dose of BCNU. A 4-h infusion of DTIC followed by a 2-h infusion of BCNU was given to 42 patients with refractory solid tumors. Complete depletion of AGT activity was not achieved at DTIC doses of up to 750 mg/m^2. The dose-limiting toxicity was hematologic, although at higher doses of BCNU ($\geq$100 mg/m^2) we observed significant nonhematologic toxicity. Our recommended phase II doses are 1,000 mg/m^2 DTIC followed by 75 mg/m^2 BCNU. AGT activity in PBMC of the 28 patients studied decreased to a mean of 62% $\pm$ 11% (SE) of the baseline value at 4 h after initiation of the DTIC infusion. At 24 h after initiation of the DTIC infusion, AGT activity in PBMC was depleted to a mean of 65% $\pm$ 14% of the baseline value. There was no direct correlation between the DTIC dose and the extent of AGT depletion. Baseline PBMC AGT levels varied widely among patients.

Key words: Alkyltransferase – Nitrosourea – Dacarbazine – Carmustine

Introduction

The chloroethylnitrosoureas, including carmustine [1,3-bis(2-chloroethyl)-1-nitrosourea, BCNU], have activity against many tumors. They have limited usefulness in human malignant disorders, in part because of cumulative toxicity [26]. Cellular resistance to this class of alkylating agents may be related to the level of the DNA-repair protein, O^6-alkylguanine-DNA alkyltransferase (AGT). AGT selectively removes adducts from the O^6 position of guanine in DNA by a stoichiometric transfer of the alkyl group to a cysteine moiety within the active site of the protein [30]. Removal of an alkyl group from DNA inactivates the AGT molecule, and restoration of activity requires new protein synthesis [31]. The sensitivity of cell lines to the chloroethylnitrosoureas is inversely correlated with cellular expression of the AGT protein and is proportional to the number of DNA interstrand cross-links formed in cells exposed to chloroethylnitrosoureas. There is considerable evidence suggesting that the AGT protein removes or reacts with alkyl groups at the O^6 position of guanine prior to the formation of lethal interstrand cross-links [2, 10, 18, 20, 33].

Depletion of AGT activity in tumor cells grown in culture results in an increase in the sensitivity of cells to the cytotoxic effects of O^6-alkylating agents. Cellular AGT levels can be lowered indirectly by exposure to methylating agents [13, 15, 24, 25, 32] or directly by exposure to O^6-methylguanine [3, 16, 19, 35] or O^6-benzylguanine [4–6, 28]. The mechanism for AGT depletion by methylating agents involves autoinactivation of the AGT protein following rapid repair of methylated DNA.

Dacarbazine (DTIC) methylates DNA at 12 sites, including the O^6 position of guanine. In animal studies, DTIC has been found to be one of the most effective agents in

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causing depletion of AGT in comparisons of a series of alkylating agents [24]. DTIC has been shown to deplete both human lymphocytes [21] and human tumor xenografts of AGT activity [27]. Thus, DTIC may be useful as a modulator of BCNU activity. Although DTIC and BCNU have been used together for many years in the treatment of melanoma, the combination has not been proven superior to therapy with DTIC alone. BCNU is usually given before or with the first of several daily doses of DTIC [23]. Such schedules are unlikely to take advantage of the potential for a synergistic effect due to AGT depletion by DTIC, since depletion must occur prior to BCNU exposure. Our recent work with O^6-benzylguanine demonstrates the requirement for AGT depletion prior to BCNU exposure [28].

Previous studies by Zeller et al. [36] and Skipper [34] have demonstrated synergistic toxicity in animals for combinations of 1-methyl-1-nitrosourea plus BCNU and of DTIC plus 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU), but the therapeutic advantage of such combinations has not been fully explored. The objective of our phase I study was to determine the maximally tolerated dose of DTIC given prior to a fixed dose of BCNU. Concomitantly, we evaluated the depletion of AGT activity in peripheral blood mononuclear cells (PBMC) of cancer patients treated with DTIC and BCNU.

Patients and methods

Patient selection. Patients with any histologically confirmed malignancy except leukemia for which there was no effective therapy or which was refractory to standard therapy were eligible for this trial. Other eligibility criteria included a life expectancy of >8 weeks; a Karnofsky performance status of $\geq 60\%$; a WBC of $>4.0\times10^9$/l; a hematocrit value of $>30\%$; a platelet count of $>100\times10^9$/l; a serum creatinine level of ≤ 1.8 mg/dl; total bilirubin, alkaline phosphatase, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) values of ≤ 1.5 times normal; no radiotherapy or chemotherapy within 4 weeks of entry to the study (6 weeks for mitomycin C or nitrosoureas); no immunotherapy within 2 weeks; and no serious concomitant medical or psychiatric illness. After two patients treated at the first dose level experienced excessive toxicity, the following eligibility criteria were added: forced expiratory volume in 1 s (FEV_1), $\geq 60\%$ of the predicted value; carbon monoxide-diffusing capacity ($D_L CO$), $\geq 60\%$ of the predicted value; room-air oxygen pressure (PO_2), ≥ 60 mmHg; and no history of hepatic irradiation. All patients signed an informed consent document approved by the Institutional Review Board.

Treatment plan. DTIC was mixed in 250 cc normal saline, shielded from light, and infused over 4 h. BCNU was mixed in 250 cc normal saline and infused over 2 h immediately following completion of the DTIC infusion. The antiemetic regimen consisted of three doses of ondansetron (8 or 12 mg) or three doses of metoclopramide (2 mg/kg) plus diphenhydramine. Blood for determination of PBMC AGT activity was obtained at baseline (10 cc), immediately before the start of the BCNU infusion (30 cc), and 24 h after the beginning of the DTIC infusion (30 cc).

Patient evaluation and follow-up. Patients were evaluated for toxicity by history and physical examination every 2 weeks. A complete blood count was performed weekly, and serum chemistries to measure liver and kidney function were obtained every other week. Treatment was repeated every 6 weeks. Treatment could be delayed for up to 2 weeks if the WBC was $<4.0\times10^9$/l or the platelet count was $<100\times10^9$/l. Patients who developed grade 3 nonhematologic toxicity or grade 4 hematologic toxicity could be retreated with a 33%-reduced BCNU dose if the toxicity resolved by week 8. Protocol treatment was discontinued if there was evidence of disease progression.

Dose-limiting toxicity. Dose-limiting toxicity was defined as grade 4 hematologic toxicity occurring during the first chemotherapy cycle or grade 3 nonhematologic toxicity occurring during any treatment cycle. If one patient experienced dose-limiting toxicity, up to three additional patients were entered at that dose level. Dose escalation was not permitted in individual patients. The maximally tolerated dose was considered to be that level at which three of six patients experienced dose-limiting toxicity.

Drugs and reagents. [^{3}H]-Methylnitrosourea was prepared by Amersham Corp. (Arlington Heights, Ill.) and the dye for protein determination was purchased from BioRad Corp. (Hercules, Calif.). All other biochemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Sample preparation. Whole blood (10 cc for baseline samples and 30 cc for 4- and 24-h samples) was drawn into sterile vacuum tubes containing ethylenediaminetetraacetic acid (EDTA). The blood was diluted 1 : 1 with phosphate-buffered saline and centrifuged over a Ficoll gradient. The mononuclear cell layer was removed and cells were washed twice in ice-cold saline. Red blood cells were lysed by briefly (20 s) suspending the pellet in deionized water. Cell pellets were frozen at –20° C and stored at –80° C until the AGT assay was performed.

AGT assay. The PBMC pellets were thawed on ice and disrupted ultrasonically (2×20 s), and total cellular protein was isolated by centrifugation (22,000 *g* for 30 min at 4° C). The protein extract was allowed to react wtith [^{3}H]-methyl DNA, which was prepared by allowing [^{3}H]-methylnitrosourea to react with calf-thymus DNA [4, 9]. After incubation at 37° C for 30 min, protein and DNA were precipitated by adding ice-cold perchloric acid. The DNA was hydrolyzed in 0.1 *N* HCl, and the ratio of O^6-[^{3}H]-methylguanine/*N*7-[^{3}H]-methylguanine was compared with the control value as measured by reverse-phase high-performance liquid chromatography (HPLC) [8]. Protein was determined by the method of Bradford [1]. AGT levels were expressed as the amount (in picomoles) of O^6-methylguanine removed per milligram of protein.

Results

A total of 42 patients received 70 treatment cycles. The patients' demographics are shown in Table 1. Patients had received a median of 2 prior chemotherapy regimens, and 14 patients had received radiotherapy. Seven dose levels were explored (Table 2). Dose level 1 (300 mg/m^2 DTIC/150 mg/m^2 BCNU) was abandoned because of excessive toxicity (five of eight patients experienced grade 3 or 4 nonhematologic toxicity). Thereafter, the eligibility criteria were amended to exclude patients with poor pulmonary function and prior hepatic irradiation (see Patients and methods), and the BCNU dose was reduced by 50%.

At dose level 5 (1,000 mg/m^2 DTIC/75 mg/m^2 BCNU), three of five patients experienced grade 4 neutropenia during the first cycle of chemotherapy (Table 3). At dose level 6 (750 mg/m^2 DTIC/100 mg/m^2 BCNU), two of six patients developed grade 4 neutropenia and two of six patients developed grade 4 thrombocytopenia during the first cycle. At dose level 7 (750 mg/m^2 DTIC/125 mg/m^2 BCNU), only one of six patients developed grade 4 neu-

Table 1. Patients' characteristics (n = 42)

M/F	21/21
Median age (range)	60 (38–81) years
Diagnosis:	
GI:	
Colon cancer	23
Gastric cancer	2
Rectal cancer	4
Small bowel cancer	1
Sarcoma	3
Renal cancer	4
Lung cancer	2
Melanoma	1
Ovarian cancer	2
Prior chemotherapy regimens:	
0	0
1	17
2	10
≥3	15
Prior radiotherapy	14
Median performance status (range)	80 (60–100)

Table 2. Dose levels

Dose level	DTIC dose (mg/m²)	BCNU dose (mg/m²)	Patients entered (n)	Treatment cycles (n)
1	300	150[a]	8	11
2	300	75	3	3
3	500	75	8	14
4	750	75	6	12
5	1,000	75	5	8
6	750	100	6	11
7	750	125	6	11
Totals			42	70

[a] Dose of BCNU reduced in subsequent cycles due to toxicity

tropenia, but three of six patients developed grade 4 thrombocytopenia during the first cycle.

In all, 25 patients received >1 treatment cycle. Nine patients required a 33% reduction in the BCNU dose for grade 4 myelosuppression. Treatment delays of 1–2 weeks for prolonged neutropenia ($<4\times10^9$/l) or thrombocytopenia ($<100\times10^9$/l) were necessary in five patients. For patients who received two cycles, the median nadir WBC during cycle 2 was 73% of the median nadir during cycle 1, despite a 33% dose reduction in four of nine patients. For patients who developed leukopenia or thrombocytopenia, the median time to nadir was 21 days.

Severe nonhematologic toxicity occurred in nine patients, including five treated at dose level 1, two treated at dose level 6, and two treated at dose level 7 (Table 4). Three episodes involved infection. Patient 36 developed a fever on day 9 of cycle 2 and died on day 15 with a WBC of 0.1×10^9/l. Patient 8 developed apparent pneumonia during cycle 2. His WBC nadir was 3.2×10^9/l. Intravenous antibiotics were given and the patient improved transiently, although he died on day 25. Blood and sputim cultures were negative, raising the possibility that this might have represented severe pulmonary toxicity. Patient 37 died of respiratory failure on day 42 of cycle 3. Autopsy revealed pulmonary aspergillosis and interstitial pneumonitis with fibrosis and cytologic atypia of type II pneumonocytes consistent with BCNU toxicity. Patient 1 and 32 died of pulmonary failure. Both patients had significant pulmonary metastases.

Three patients developed significant hepatotoxicity. Patient 3 had increasing liver-function tests on the day of chemotherapy. He died on day 19 of cycle 1. Patient 4 had previous radiation to the porta hepatis and an indwelling biliary stent. Replacement of the stent, however, did not affect the liver function. Patient 5 suffered a motor vehicle accident on day 17 of cycle 1. Levels of serum AST and lactic dehydrogenase (LDH) increased transiently after the accident, and serum bilirubin levels reached a peak 10 days later. The pattern of abnormal liver-function tests was slightly different for each patient, and each patient had significant hepatic metastases, but hepatic toxicity was seen only at dose level 1.

The percentage of baseline AGT activity present in PBMC at 4 h after initiation of the DTIC infusion and 24 h later is shown in Table 5. AGT levels were available for 42 of 76 treatment cycles. The average AGT activity at baseline was 283±47 (SE) fmol/mg protein, and there was considerable variation in AGT depletion both at 4 h and at 24 h, with no clear relationship to the DTIC dose being noted. We also found no clear relationship between the baseline AGT activity or percentage of AGT depletion and the nadir WBC or platelet count.

Table 3. Hematologic toxicity

Dose level	DTIC dose (mg/m²)	BCNU dose (mg/m²)	WBC toxicity			Platelet toxicity		
			Median nadir[b]	Nadir range[b]	Patients with grade 4[c]	Median nadir[b]	Nadir range[b]	Patients with grade 4[c]
1	300	150[a]	2.8	2.3–8.1	0/8	80	18–122	2/8
2	300	75	6.5	3.7–8.0	0/3	179	86–359	0/3
3	500	75	4.9	1.4–9.9	0/8	145	8–240	1/8
4	750	75	2.5	0.8–8.2	1/6	129	10–237	0/6
5	1,000	75	1.7	0.4–6.2	3/5	34	9–270	2/5
6	750	100	3.9	0.1–7.9	2/6	25	4–178	2/6
7	750	125	3.0	0.3–5.2	1/6	24	7–302	3/6

[a] Dose of BCNU reduced in subsequent cycles due to toxicity
[b] ($\times10^9$/l)
[c] Cycle I only

Table 4. Severe nonhematologic toxicity

Dose level	Patient	Toxicity	Grade	Notes
1	1	Pulmonary	4	Pulmonary nodules and pleural effusion; patient died on day 17
1	3	Hepatic	4	Liver functions were increasing on day of chemotherapy; patient died on day 19 of sepsis
1	4	Hepatic	3	Patient had previous radiation to porta hepatis and also had a biliary stent
1	5	Hepatic	3	Patient was in a motor vehicle accident on day 17 with ↑ LDH and SGOT; bilirubin peaked on day 28
1	8	Infection	3	Patient developed pneumonia but was not neutropenic
6	32	Pulmonary	5	Admitted on day 12, cycle 2 with thrombocytopenia, mild bleeding, and dyspnea; patient was treated for fluid overload and improved transiently but died on day 19
6	36	Infection	5	Admitted on day 9, cycle 2 with fever and neutropenia; patient died on day 15, WBC 0.1×10^9/l
7	41	Hemorrhage Cardiac	4 3	Atrial fibrillation, and GI bleeding during cycle 1; patient died on day 23, cycle 2, of progressive disease
7	37	Pulmonary	5	Admitted on day 17, cycle 3 with dyspnea; patient died on day 42 with pulmonary aspergillosis, confirmed at autopsy. Autopsy showed interstitial pneumonitis and fibrosis with cytologic atypia of type II pneumocytes consistent with BCNU toxicity

LDH, Lactic dehydrogenase

Table 5. AGT depletion

Dose level	Patients (n)	Cycles (n)	DTIC dose (mg/m^2)	BCNU dose (mg/m^2)	% Baseline AGT activity (±SE)	
					4 h	24 h
1	4	5	300	150	65± 8.5	58± 5.8
2	2	2	300	75	18±11	26± 4.9
3	3	6	500	75	44± 6.1	36±14
4	6	9	750	75	65±16	49±17
5	2	2	1,000	75	–	82±11
6	6	10	750	100	81±33	115±51
7	5	8	750	125	53±29	49±20

Discussion

This phase I trial was based on preclinical studies indicating a role for the administration of a methylating agent prior to BCNU. We elected to study DTIC because of its activity against lymphoma and melanoma and its demonstrated ability to deplete tissue AGT levels [21, 24]. In preclinical experiments we found that exposure of tumor cells in culture to temozolomide (which spontaneously hydrolyzes to form the active metabolite of DTIC) prior to BCNU treatment produces synergistic cytotoxicity. However, toxic doses of temozolomide were required to achieve significant AGT depletion. In further investigations in tumor-bearing mice, the maximally tolerated dose of DTIC was required for complete AGT depletion in HT29 xenografts [27].

This study was designed with the intent of escalating the dose of DTIC given prior to a fixed dose of BCNU until complete inhibition of PBMC AGT activity was achieved. However, at the doses of DTIC used in this study, complete inhibition of PBMC AGT activity could not be achieved. There was no apparent correlation between the degree of AGT depletion and the dose of DTIC given. Escalation of the DTIC dose beyond 1,000 mg/m^2 was not attempted because the BCNU dose would need to be reduced to <75 mg/m^2 to avoid severe toxicity. Using escalating doses of DTIC given prior to a single dose of fotemustine, a new nitrosourea, Lee et al. [22] also found variable and incomplete AGT depletion. There was some evidence of a dose-response effect, with 44%, 74%, and 76% AGT depletion occurring at DTIC doses of 400, 500, and 800 mg/m^2, respectively. The incomplete and variable AGT depletion seen with DTIC may relate to the need for metabolic activation in vivo. The mean baseline PBMC AGT activity detected in our patients was similar to that found in the study by Lee et al. [22]. In a phase I study of streptozotocin (four daily doses of 500 mg/m^2) and escalating doses of BCNU (single dose on day 3), Panella et al. [29] found a 90% reduction in PBMC AGT activity on day 3 in six patients. Gerson [15] found a 75% depletion of PBMC AGT after the administration of three daily doses of streptozotocin (500 mg/m^2 per day). These studies presume that PBMC AGT is a surrogate marker for tumor AGT; whether tumor AGT levels in humans fall in a parallel fashion after treatment with methylating agents is unknown.

Lee et al. [22] and Panella et al. [29] did not report significant pulmonary or hepatic toxicity in their studies, although Gerard et al. [14] described unexpected pulmonary toxicity in their experience with sequential DTIC and fotemustine. We caution against using the combination of DTIC given prior to BCNU at 150 mg/m^2. Our experience suggests that this therapy is too toxic for heavily pretreated patients, those with a poor performance status, or patients with significant pulmonary or hepatic tumor burden or dysfunction.

For phase II testing of DTIC and BCNU given according to this schedule, we recommend 1,000 mg/m^2 DTIC followed by 75 mg/m^2 BCNU (dose level 5). We also recommend monitoring of PBMC AGT levels to define further the extent of AGT depletion. At this dose level, three of five patients developed grade 4 hematologic toxicity, but there was no significant nonhematologic toxicity. At dose level 6, only two of six patients experienced grade 4 hematologic toxicity during cycle 1, although three of six patients experienced grade 4 hematologic toxicity (WBC, 3; platelet, 3) during cycle 2, and two patients developed severe nonhematologic toxicity (Table 4).

A number of preclinical studies have demonstrated the usefulness of O^6-alkylguanines as alternatives to methylating agents for modulation of the cytotoxic effects of chloroethylnitrosoureas [4, 6]. Methylating agents deplete AGT activity indirectly, whereas the O^6-alkylguanines deplete AGT directly by acting as a substrate. One of the most effective agents studied to date is O^6-benzylguanine, which requires only micromolar concentrations for a few minutes to deplete AGT activity completely in cultured cells [5, 28]. Furthermore, the therapeutic index of BCNU was increased after treatment with O^6-benzylguanine in human tumor-xenograft studies [7, 8, 11, 12, 17, 28]. In contrast, effective depletion of AGT activity using methylating agents requires toxic doses of drug. Methylating agents also introduce mutagenic lesions in DNA, increasing the possibility of secondary tumors [5, 28]. Clinical trials combining methylating agents with chloroethylnitrosoureas should be designed carefully, but the use of nontoxic modulators such as O^6-benzylguanine is likely to be more effective in enhancing the activity of nitrosoureas.

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