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Utilization of the HTSCA and CFU-C assay to identify two new 2-chloroethylnitrosourea congeners of amino acid amides with increased in vitro activity against human glioma compared with BCNU*

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Summary. *AspCNU and SarCNU are two amino acid amide congeners (L-asparaginamide and sarcosinamide congeners) of chloroethylnitrosoureas. The in vitro myelotoxicity of these agents compared with BCNU at 1–8 μg/ml was determined in bone marrow cells from normal volunteers in the CFU-C assay. AspCNU and SarCNU were significantly ($P < 0.05$) less myelotoxic than BCNU at equivalent microgram concentrations. SarCNU or AspCNU at 3 μg/ml demonstrate equivalent in vitro myelotoxicity to BCNU 1 μg/ml.*

We used the human tumor stem cell assay (HTSCA) to investigate in vitro antitumor activity. We obtained four specimens of malignant glioma and one specimen of meningioma from patients not previously treated with chemotherapy. AspCNU and SarCNU were significantly ($P < 0.05$) more active than BCNU at 1–3 μg/ml concentrations in the HTSCA in all four malignant glioma specimens. In the one meningioma specimen, BCNU was significantly ($P < 0.05$) more active than either AspCNU or SarCNU at all concentrations studied. These results suggest that AspCNU or SarCNU at doses that should produce less myelotoxicity than BCNU may be more active than BCNU against gliomas.

Introduction

Current anticancer agents have a limited spectrum of clinical activity. The tumors that are generally the most responsive to anticancer chemotherapy are those that have a rapid doubling time [18]. This may be related to the screening system used to develop new anticancer agents. Novel agents must first demonstrate activity against L1210 leukemia and/or P388 leukemia in mice. These tumors have a rapid doubling time (12–24 h), which would favor the selection of agents active against rapidly growing tumors [11].

To identify anticancer agents active against slow-growing tumors such as colon cancer, non-small cell lung cancer, melanoma, and glioma, it may be necessary to alter the initial screening system. An alternative screening system may be the in vitro human tumor stem cell assay (HTSCA) [4, 13, 21]. While there are many problems with the assay, which might render it inapplicable for individual patients, it may be useful to screen new potential anticancer agents [16]. The HTSCA has already been used for in vitro phase II trials in an attempt to identify more active agents against specific tumors [14]. In vitro phase-II trials in the HTSCA with new anticancer agents are limited by a lack of knowledge of in vivo pharmacokinetics. Therefore, it is necessary to use 'very high' concentrations in the HTSCA of previously untested agents to avoid underestimating their anticancer activity. This could result in overprediction of antitumor activity.

The majority of anticancer agents have myelosuppression as their dose-limiting toxicity [2]. The CFU-C assay is an in vitro agar system that allows the development of myeloid precursors found in the normal human bone marrow. Colonies of white blood cells (granulocytes and monocytes) are formed from the myeloid precursors. Most anticancer agents injure myeloid precursor cells and in so doing produce dose-limiting toxicity [9]. Thus, it may be possible to use the CFU-C assay to determine the in vitro toxicity of new anticancer agents and then use the 'toxic' concentrations found in the CFU-C assay for testing in the HTSCA. Salmon et al. have reported a similar in vitro trial comparing two anthracyclines [15].

Recently Dr Suami has synthesized several amide congeners of chloroethylnitrosoureas including an L-asparaginamide congener (AspCNU) and a sarcosinamide congener (SarCNU) (Fig. 1) [19]. We have used the HTSCA to investigate the activity of AspCNU and SarCNU as compared with BCNU (bis-chloroethylnitrosourea), a clinically active chloroethylnitrosourea, in four malignant glioma specimens. The toxicity of these new agents compared with BCNU was determined in the CFU-C assay performed in bone marrow

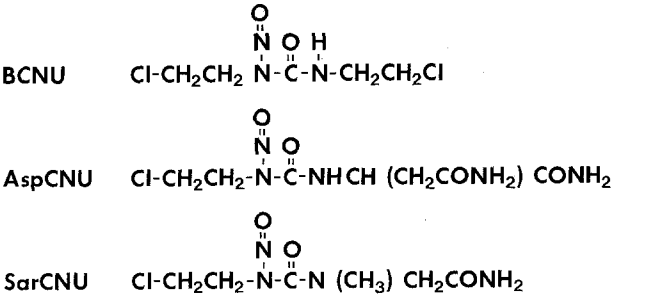

Fig. 1. Chemical formulas of the three chloroethylnitrosoureas utilized in this study. The molecular weights of BCNU, AspCNU, and SarCNU are 214, 266, and 223, respectively. The LD_{50} dose in BDF_1 mice is approximately 164 μmol/kg for BCNU and 1,758 μmol/kg for SarCNU [19]

* *Abbreviations* are: AspCNU, a chloroethylnitrosourea congener of L-asparaginamide; SarCNU, a chloroethylnitrosourea congener of sarcosinamide and BCNU, bis-chloroethylnitrosourea

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samples obtained from normal volunteers. The results reported in this article suggest that AspCNU and SarCNU may be more active than BCNU against malignant gliomas, while being less myelotoxic.

Materials and methods

Collection of cells. Bone marrow cells were obtained from three normal volunteers following informed consent. A bone marrow aspiration was performed from the posterior iliac crest. The aspirate was collected in a syringe containing approximately 100 U of preservative-free heparin (Sigma Chemical Co., St. Louis, Mo) per milliliter bone marrow. The RBCs were separated from mononuclear cells by centrifugation on Ficoll-Hypaque reagent (Pharmacia Fine Chemicals, Pharmacia Inc., Piscataway, NJ) at 600 *g* for 45 min at room temperature. The interfaces containing the mononuclear cells were then treated in the same fashion as the tumor cells (described below).

Tumor specimens were obtained from patients undergoing surgery for removal of suspected malignant glioma lesions. These patients had not received prior chemotherapy. In addition we obtained six specimens of 'normal' brain. These specimens came from the frontal lobe of autopsied patients in whom the brain was free of tumor. All autopsies were performed within 24 h of the patients' death. The tumor or normal brain specimens were placed in McCoy's 5A medium plus 10% heat-inactivated fetal calf serum, 1% penicillin and 1% streptomycin (Grand Island Biological Co., Grand Island, NY) under aseptic conditions. The specimens were mechanically dissociated with scissors and treated with an enzyme cocktail consisting of collagenase type 1 (1.2% of 150 U/mg) and 0.001% DNAase I (Sigma) but without hyaluronidase [5]. The mixture was then forced successively through 20–200× mesh. RBCs were separated from tumor cells by centrifugation on Ficoll–Hypaque at 150 *g* for 20 min at room temperature. The cell suspension was then forced through a 400× mesh and washed with the enriched McCoy's medium. Viability of cells derived from solid tumors was estimated by trypan blue to be ≧ 90% in all five tumor specimens and the six normal brain specimens. Control samples from the normal brain specimens were processed in an identical fashion to control tumor samples as outlined below.

In vitro exposure of tumor cells and normal bone marrow cells to drugs. BCNU was kindly supplied by the Drug Development Branch, National Cancer Institute, Bethesda, Md. AspCNU and SarCNU were kindly supplied by Dr Suami, Keio University, Yokohama, Japan. Each drug under investigation was prepared in sterile sodium citrate buffer, 0.001 *M*, pH 4.0. Samples (1 ml) of bone marrow or tumor cells at 2×10^6 cells/ml were preincubated at 37° C for 15 min in enriched McCoy's 5A medium with 0.02 *M* HEPES buffer without serum. After the addition of appropriate concentrations of BCNU, SarCNU, AspCNU, or citrate buffer, the samples were incubated for 1 h at 37° C. They were then centrifuged at 150 *g* for 10 min and washed twice with the enriched McCoy's medium.

HTSCA. The culture system used in this study has been extensively described elsewhere [4, 13, 21]. The overlayer in which tumor cells were suspended consisted of 0.3% agarose (Bethesda Research Lab., Rockville, Md) in enriched Connaught Medical Research Laboratories Medium 1066 supplemented with 15% horse serum (Grand Island Biological Co.), gentamicin (8 μg/ml, Schering, Pointe Claire, Quebec), glutamine (2 m*M*, Sigma), $CaCl_2$ (4 m*M*, Fisher Scientific Co., NJ) and insulin (2 U/ml, Connaught). Just before use, several substances were added to this enriched medium (0.66 m*M* asparagine, DEAE-dextran 0.4 mg/ml and 0.01 m*M* dithiothreitol, Sigma). Aliquots of 1 ml of the resultant mixture was pipetted onto 2-ml feeder layers in 35-mm plastic petri dishes (Falcon Plastics). The final concentration of cells in each culture was 6.66×10^5 cells in 1 ml agarose medium.

The feeder layers used in this study consisted of McCoy's 5A medium plus 15% heat-inactivated fetal calf serum plus epidermal growth factor (30 ng/ml enriched medium, Sigma) and a variety of nutrients as previously described [4, 13, 21]. Immediately before use, 10 ml 3% trypticase soy broth (Grand Island Biological Co.) and 0.3 ml 88 m*M* asparagine–5% DEAE-dextran were added to 40 ml enriched underlayer medium. Agar was added to this enriched medium, resulting in a final concentration of 0.5% and underlayers were poured into 35-mm petri dishes.

All the drug-treated cells were plated onto feeder layers containing 30 ng/ml epidermal growth factor, whereas cells for control were cultivated with and without epidermal growth factor. After preparation of both bottom and top layers, the control plates were examined under an inverted microscope (Ernst Leitz, Wetzlar, Germany) to ensure the presence of a good single-cell suspension. All drug concentrations were done in duplicate (3–4 plates per concentration). The plates were then incubated at 37° C in a 7.5% CO_2 humidified incubator for 3–6 weeks.

The number of colonies (≧ 40 cells) on control and drug-treated plates was determined by counting the colonies on an inverted microscope at 40× magnification. An 'evaluable' colony was determined according to the equation of Moon [8].

CFU-C assay. Bone marrow cells were cultured in the CFU-C assay with modifications [10]. A single-layer agar system was used. Cells were suspended in 4 ml enriched Iscove's DMEM (Flow Laboratories, Mississauga, Ontario) with 20% fetal calf serum, 0.5% placental conditioning medium, and gentamicin 8 μg/ml, plus 1 ml 3% agar. Aliquots of 1 ml were cultured in quadruplicate at 37° C in a 7.5% CO_2 humidified incubator for 10–14 days. Control plates had 124 ± 40 colonies.

Statistical analysis. An analysis of variance with intergroup comparison by least significant difference was used to evaluate the data [17].

Results

The CFU-C assay, which allows for the development of white blood cell (granulocytic and monocytic) colonies from myeloid precursors of normal human bone marrow, was used as a system for determining the toxicity of each of the three chloroethylnitrosoureas. The results of three bone marrow specimens obtained from normal volunteers were pooled because they were similar (Fig. 2). BCNU at concentrations that are clinically obtainable (1–3 μg/ml) produced significant myelotoxicity [12]. AspCNU and SarCNU were significantly ($P < 0.05$) less myelotoxic than BCNU at all drug concentrations (Fig. 2). There was no significant difference between the myelosuppressive action of AspCNU and SarCNU at any of the concentrations utilized.

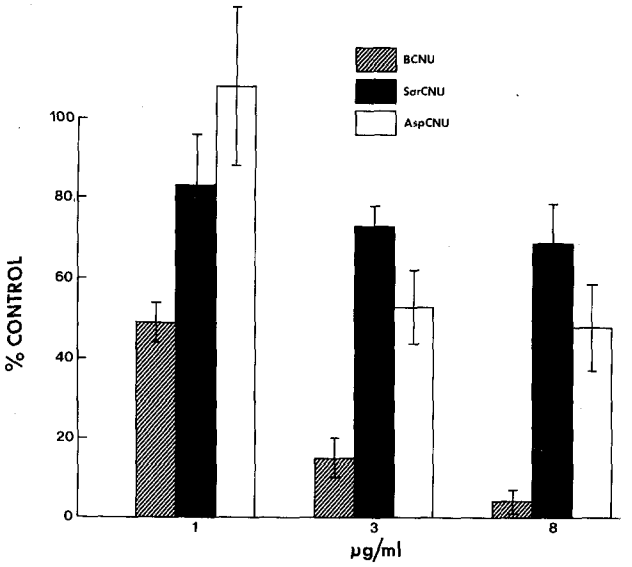

Fig. 2. Results of the in vitro myelotoxicity tests of each chloroethylnitrosourea in the CFU-C assay as percentages of myelotoxicity in control colonies where control samples were incubated with vehicle (0.001 *M* citrate, pH 4) only. Each drug concentration or vehicle was incubated in McCoy's 5A medium with 2×10^6 normal human bone marrow cells/ml for 1 h at 37° C. The results shown are means ± SE from three experiments. All drug concentrations were used in duplicate

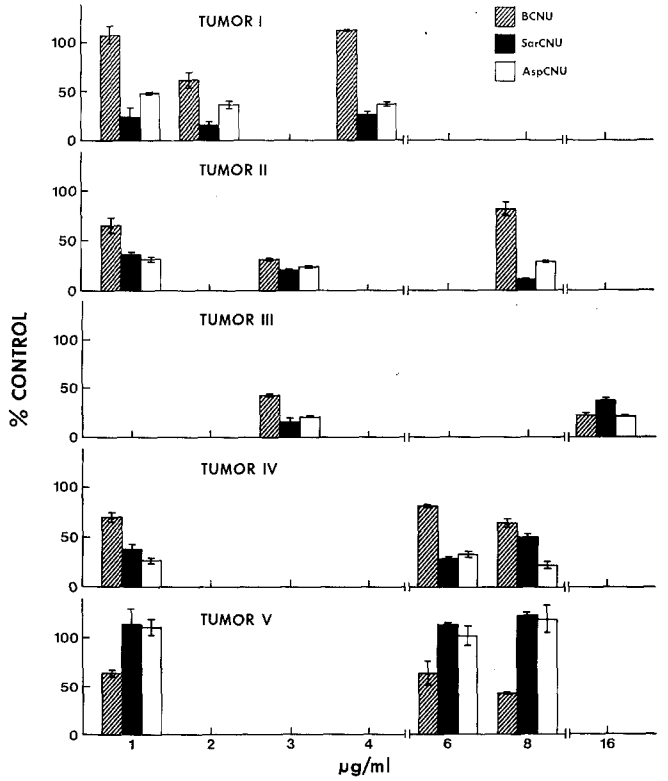

Fig. 3. In vitro antitumor activity of each chloroethylnitrosourea in the HTSCA with four malignant glioma specimens plus one meningioma specimen (tumor V). Results are expressed as a percentage of the activity in control colonies where control samples were incubated with vehicle (0.001 *M* citrate, pH 4) only. Each drug concentration or vehicle was incubated in McCoy's 5A medium with 2×10^6 cells per ml for 1 h at 37° C. The results are expressed as the mean ± SE from all petri dishes at that drug concentration. All drug concentrations were done in duplicate

The HTSCA was utilized as an in vitro assay for antitumor activity. Four malignant glioma specimens plus one meningioma specimen were obtained from patients who had not received previous chemotherapy. Control samples of tumor specimens without epidermal growth factor had 53% ± 17% of colonies, compared with control samples with epidermal growth factor. Control samples of tumor specimens with epidermal growth factor had 143 ± 29 colonies/plate. The control samples from the six normal brain specimens had 12 ± 4 colonies in the plates with or without epidermal growth factor. The results of each tumor assay with the three chloroethylnitrosoureas are presented in Fig. 3. AspCNU and SarCNU were significantly ($P < 0.001$, except $P < 0.05$ for AspCNU 2 μg/ml) more effective than BCNU at all drug concentrations used against tumor I. At a concentration of 1 μg/ml, AspCNU and SarCNU were significantly ($P < 0.001$) more active than BCNU against tumor II. All three drugs significantly ($P < 0.001$) reduced the colony count compared with control at 3 μg/ml with this same tumor. BCNU at 3 μg/ml significantly ($P < 0.001$) reduced the number of colonies compared with control with tumor III, but AspCNU and SarCNU were significantly ($P < 0.001$) more active than BCNU at this concentration. In tumor IV, AspCNU and SarCNU ($P < 0.001$ and $P < 0.01$, respectively) were more effective than BCNU at all the concentrations tested. In contrast to the other four tumors, BCNU was significantly ($P < 0.05$) more effective than AspCNU or SarCNU at all concentrations against the meningioma specimen (tumor V).

Discussion

The chloroethylnitrosoureas are a group of anticancer agents with demonstrated activity against Hodgkin's disease, small cell carcinoma of the lung, and gliomas. The dose-limiting toxicity of the chloroethylnitrosoureas is delayed cumulative myelosuppression [1, 22]. In particular, BCNU (bis-chloroethylnitrosourea) is one of the most active agents for treatment of patients with malignant gliomas. Responses are seen in approximately 40% of patients [22]. However, the combination of BCNU and radiotherapy to the brain is not significantly better than radiotherapy alone in the initial treatment of malignant gliomas [23]. A more effective anticancer agent against malignant gliomas could prove useful in combination with radiotherapy.

The two new chloroethylnitrosoureas reported here are amino acid amide congeners. These agents are as active against L1210 leukemia as other chloroethylnitrosoureas. Furthermore, amino acid carriers may alter the transport of chloroethylnitrosoureas [19]. The clinically available chloroethylnitrosoureas (BCNU, CCNU, and chlorozotocin) enter tumor cells and murine bone marrow cells by passive diffusion [6, 7]. However, chloroethylnitrosoureas with neutral amino acid carriers may be transported into cells by the neutral amino acid transport systems which are overlapping transport systems [3]. One anticancer agent, L-phenylalanine mustard, in which the nitrogen mustard group is attached to the L-phenylalanine group, is transported into cells by neutral amino acid transport systems [20]. Therefore, it is possible that AspCNU and SarCNU are transported into cells by neutral amino acid transport systems and have a different molecular pharmacology than the conventional chloroethylnitrosoureas. This was the reason they were chosen for testing in the HTSCA.

AspCNU and SarCNU demonstrate significantly less myelotoxicity than BCNU as measured by the CFU-C assay. It

appears that 3 μg/ml SarCNU or AspCNU produces CFU-C toxicity equivalent to that produced by 1 μg/ml BCNU. The results with the glioma tumor samples in the HTSCA are quite different. The lack of significant growth of normal brain specimens suggests that the colonies grown from tumor samples are colonies of tumor cell origin. There is no clear-cut dose–response curve with the limited range of concentrations used in the HTSCA. This may be secondary to a general resistance to BCNU in reference to tumors I and IV. In general, there is not a dose–response curve with SarCNU or AspCNU in any of the tumors, even though these tumors are sensitive to these drugs (reduce colony growth to 30% of control). This phenomenon of a plateau has been previously noted [15]. It is possible that the use of a larger range of concentrations would have resulted in more clear-cut dose–response curves, but there was a limited amount of tumor tissue available and we used clinically obtainable concentrations of BCNU. BCNU at concentrations that are clinically obtainable (1–3 μg/ml) was significantly less effective than either SarCNU or AspCNU at similar concentrations in all four malignant glioma samples. This suggests that SarCNU or AspCNU at doses that should produce less myelotoxicity than BCNU may be more active than BCNU against gliomas. The introduction of one of these new agents (SarCNU) into clinical trials will verify whether these in vivo predictions are correct.

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