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In vitro comparative studies of the myelotoxicity and antitumor activity of 6-[bis-(2-chloroethyl)-amino]-6-deoxy-D-glucose versus melphalan utilizing the CFU-C and HTSCA assays*

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Summary. 6-[Bis-(2-chloroethyl)-amino]-6-deoxy-D-glucose (C-6) is a new glucose-containing nitrogen mustard that has significant activity for murine P388 leukemia with relative sparing of bone marrow in mice. The in vitro myelotoxicity of C-6 compared with that of melphalan, a clinically active, myelosuppressive nitrogen mustard, was determined in the CFU-C assay in human bone marrow samples obtained from normal volunteers. There was no significant difference between the myelosuppressive actions of C-6 and melphalan at any of the concentrations used except for 4.0 μM, at which C-6 was significantly ($P < 0.05$) more toxic than melphalan. Both agents decreased the number of bone marrow cell colonies to approximately 12% of control at 6.6 μM (1 h incubation), which is a good approximation of melphalan's CxT (concentration by time) in man.

We used the human tumor stem cell assay (HTSCA) to investigate in vitro antitumor activity. We obtained two specimens of malignant melanoma and two of malignant ovarian carcinoma from patients not previously treated with chemotherapy. The antitumor activity of melphalan was either similar to or greater than that of C-6 at all concentrations utilized against any of the four tumor specimens, except at 1.3 μM for tumor I. In particular, there was no significant difference in the antitumor activities of the two agents at 6.6 μM. These results suggest that C-6 will not be less myelosuppressive than melphalan at doses that produce equivalent antitumor activity in man.

In addition, C-6 did not demonstrate increased myelotoxicity for normal human bone marrow cells incubated in glucose-deficient medium as against medium containing 300 mg% glucose at any of the concentrations used. This suggests that C-6 is not transported into normal human bone marrow cells via the glucose transport system, despite the presence of a glucose moiety within the molecule.

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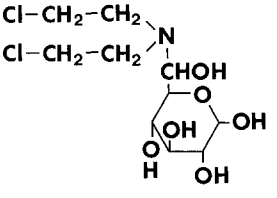
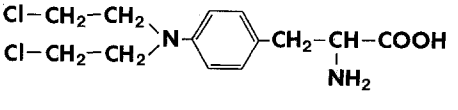
Introduction

6-[Bis-(2-chloroethyl)-amino]-6-deoxy-D-glucose (C-6) is a new glucose-containing nitrogen mustard that has significant activity for murine P388 leukemia with relative sparing of bone marrow in mice (see Fig. 1) [3]. It is the latest in a line of glucose-containing alkylating agents developed in an attempt to find one with high antitumor activity but with relatively low myelotoxicity in man. Structure-activity studies with the nonmyelosuppressive methylnitrosourea, streptozotocin, and its cytotoxic group, 1-methyl-1-nitrosourea, suggested that this bone marrow toxicity could be reduced by the attachment of the cytotoxic group to a glucose moiety [15]. To evaluate the influence of the glucose carrier for the more active chloroethylnitrosourea class of alkylating agents, chlorozotocin (2-[3-(2-chloroethyl)-3-nitrosoureido]-D-glucopyranose) was synthesized [10]. Chlorozotocin has shown curative activity for murine L1210 leukemia with relative sparing of bone marrow in mice [2, 20]. Structure-activity analysis of both methyl and chloroethylnitrosoureas have demonstrated that all nitrosoureas with hexose carriers have lower myelotoxicity in mice than do nitrosoureas without hexose carriers [14, 15]. This is unlike the significant leukopenia seen in mice at optimal anti-L1210 leukemia doses of the clinically active chloroethylnitrosourea, CCNU. This has been correlated with a preferential ratio of alkylation of L1210 DNA to



6- bis(2-chloroethyl) amino-
6-deoxy-D-Glucose, (C-6)



L-Phenylalanine Nitrogen Mustard,
(Melphalan)

Fig. 1. Chemical structures of C-6 *(top)* and melphalan *(botton)*

murine bone marrow DNA by 0.1 m*M* chlorozotocin compared with equimolar CCNU in vitro. (The L1210: bone marrow DNA ratio was 1.3 for chlorozotocin and 0.6 for CCNU [16]). This favorable ratio was shown not to be secondary to differences in uptake of the two agents by L1210 leukemia and murine bone marrow cells. In addition, chlorozotocin uptake in murine L1210 leukemia cells appears to be via a passive mechanism and not via the glucose transport system, despite the presence of a glucose moiety within the chlorozotocin molecule [11, 12]. It has recently been reported that chlorozotocin and CCNU alkylate preferentially at different sites within murine bone marrow cell chromatin, but at the same site in L1210 leukemia cell chromatin [6]. This may contribute to both the favorable ratio of alkylation of DNA in the two cells and the reduced myelotoxicity of chlorozotocin compared with CCNU. Unfortunately, chlorozotocin's dose-limiting toxicity in man is myelosuppression [9, 17].

C-6 was evaluated to determine whether a glucose carrier attached to a nitrogen mustard would decrease myelotoxicity in a similar fashion. C-6 has demonstrated equivalent lethal toxicity and significant anti-P388 leukemia activity in mice compared with melphalan, a clinically active, myelosuppressive nitrogen mustard [3, 5]. At its optimal anti-P388 leukemia dose, C-6 is significantly less myelotoxic than melphalan in mice. We have investigated the in vitro antitumor activity of C-6 compared with melphalan in four human tumor specimens with the aid of the human tumor stem cell assay (HTSCA). The in vitro myelotoxicity of C-6 compared with that of melphalan was determined in the CFU-C assay in human bone marrow samples obtained from normal volunteers. In addition, we have investigated the effect of glucose on the cytotoxicity of C-6 with normal human bone marrow cells in the CFU-C assay.

Materials and methods

Collection of cells. Bone marrow cells were obtained from six 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 units preservative-free heparin (Sigma Chemical Co., St. Louis, Mo) per ml 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 melanoma or ovarian carcinoma lesions. These patients had not received prior chemotherapy. Under aseptic conditions, the tumors were placed in McCoy's 5A medium with 10% heat-inactivated fetal calf serum, 1% penicillin and 1% streptomycin (Grand Island Biological Co., Grand Island, NY). The solid tumor specimens were mechanically dissociated with scissors and, when necessary, treated with an enzyme cocktail consisting of collagenase type 1 (1.2% of 150 units/mg) and 0.001% DNAase I (Sigma) but without hyaluronidase [8]. The mixture was then forced successively through 20–200× meshes. 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 all tumor cells was estimated by trypan blue to be ≧90% in all specimens.

In vitro exposure of tumor cells and normal bone marrow cells to drugs. Melphalan and C-6 were kindly supplied by the Drug Development Branch, National Cancer Institute, Bethesda, Md. Melphalan was dissolved in an ethanol-water-hydrochloric acid solution while C-6 was dissolved in DMSO. Samples (1 ml) of bone marrow or tumor cells at 2×10^6 cells/ml were preincubated at 37 °C for 15 min in McCoy's 5A medium with 0.02 *M* HEPES buffer without serum. After the addition of appropriate concentrations of melphalan, C-6 or vehicle, the samples were incubated for 1 h at 37 °C. The composition of the vehicles added to the bone marrow or tumor cells was kept constant in all tubes, including controls (i.e., 3.00 µl DMSO and 2.25 µl ethanol added per ml cells). They were then centrifuged at 150 *g* for 10 min and washed twice with the enriched McCoy's medium. In addition, experiments with normal human bone marrow cells were performed in McCoy's 5A medium both with and without glucose. The glucose concentration in McCoy's 5A medium is 300 mg%.

HTSCA. The culture system used in this study has been extensively described elsewhere [7, 19, 23]. 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 units/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). Then 1-ml volumes of the resultant mixture were 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 and a variety of nutrients as previously described [7, 19, 23]. Immediately before use, 10 ml 3% trypticase soy broth (Grand Island Biological Co.) and 0.3 ml 88 m*M* asparagine and 5% DEAE-dextran were added to 40 ml enriched underlayer medium. Agar was added to this enriched medium to give a final concentration of 0.5%, and underlayers were poured into 35-mm petri dishes.

After preparation of both the overlayers and the feeder layers, the 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 used 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 [13]. Control samples had 108 ± 32 colonies/plate (mean ± SE).

CFU-C assay. Bone marrow cells were cultured in the CFU-C assay with modifications [18]. A single-layer agar system was utilized. 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 for cells incubated in McCoy's 5A medium with glucose had 116 ± 35 colonies, while control plates for cells incubated in medium without glucose had 90 ± 9 colonies (means ± SE).

Statistical analysis. An analysis of variance with intergroup comparison by least significant difference was utilized to evaluate the data [22].

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 both melphalan and C-6 [18]. The results of bone marrow specimens obtained from normal volunteers were pooled because they were similar. For bone marrow cells incubated in medium containing glucose, melphalan at clinically achievable (CxT) concentrations (4.0–6.6 μ*M*) produced significant in vitro myelotoxicity (Fig. 2A) [1]. There was no significant difference between the myelosuppressive action of C-6 and melphalan at any of the concentrations used except at 4.0 μM, where C-6 was significantly ($P<0.05$) more toxic than melphalan. Both agents decreased the number of bone marrow cell colonies to approximately 12% of that in control dishes at 6.6 μ*M*. Both C-6 and melphalan demonstrated less myelotoxicity in glucose-deficient media at all concentrations used (Fig. 2B).

The HTSCA was utilized as an in vitro assay for antitumor activity. Two solid malignant melanoma specimens (tumors I and II) and two malignant ovarian carcinoma specimens (tumors III and IV) were obtained from patients who had not received previous chemotherapy. The results of each tumor assay with C-6 and melphalan are presented in Fig. 3. The activity of C-6 against tumor I was not significantly different than that of melphalan at any of the concentrations used except 1.3 μ*M*, where C-6 was significantly ($P<0.001$) more active. Melphalan was more effective than C-6 at 1.3 ($P<0.025$) and 2.6 μ*M* ($P<0.05$) against tumor II. There was no significant difference in activity between these two drugs at the other concentrations used against this tumor. Melphalan significantly ($P<0.05$) reduced the colony count compared with control at 1.3 μ*M* against tumor II. There was no significant difference in activity between C-6 and melphalan at any concentration against tumor III. Both drugs significantly ($P<0.05$ for

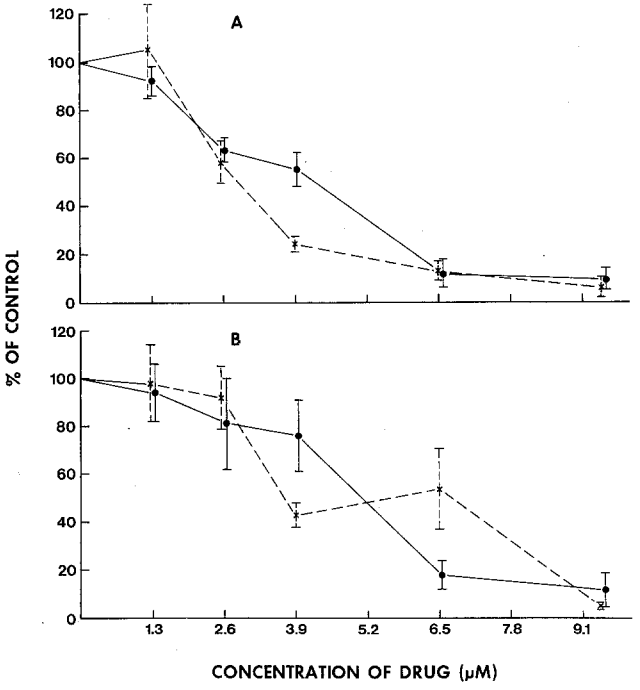


Fig. 2. A, B. The results of the in vitro myelotoxicity of C-6 and melphalan incubated in McCoy's 5A medium with and without 300 mg% glucose in the CFU-C assay, presented as percentages of control colony values, where control samples were incubated with vehicle only. Each drug concentration or vehicle was incubated in McCoy's 5A medium with 300 mg% glucose **(A)** or McCoy's 5A medium without 300 mg% glucose **(B)** with 2×10^6 normal human bone marrow cells per ml for 1 h at 37 °C. The results shown are means ±SE of six experiments. All drug concentrations were used in duplicate. ___, L-PAM; –, C-6

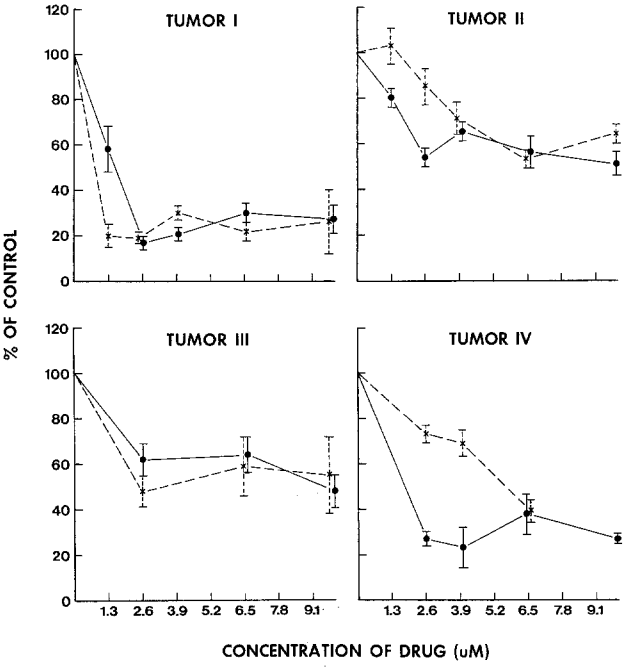


Fig. 3. Results of the in vitro antitumor activity of C-6 and melphalan in the HTSCA with two malignant melanoma specimens (tumors I and II) and two malignant ovarian carcinoma specimens (tumors III and IV) as percentages of control colony values where control samples were incubated with vehicle only. Each drug concentration or vehicle was incubated in McCoy's 5A medium containing 300 mg% glucose with 2×10^6 tumor cells per ml for 1 h at 37 °C. The results are expressed as means ±SE of all petri dishes at that drug concentration. All drugconcentrations were done in duplicate. ___, L-PAM; –, C-6

melphalan; $P<0.01$ for C-6) reduced the colony count compared with control at 2.6 μM against this tumor. Melphalan was significantly more active than C-6 at 2.6 ($P<0.025$) and 4.0 μM ($P<0.005$) against tumor IV. No significant difference in activity between the two drugs was observed for 6.6 μM against this tumor. For all four tumors there is essentially no clear correlation between dose and survival for either C-6 or melphalan. Partial dose-response curves are seen for melphalan in tumor I, C-6 and melphalan in tumor II, and C-6 in tumor IV.

Discussion

Many anticancer agents are characterized by a narrow therapeutic index, reflecting their failure to discriminate effectively between target and normal tissues. In man, bone marrow suppression is generally the most important dose-limiting toxicity, particularly for alkylating agents [4]. Structure-activity studies with the nonmyelosuppressive, glucose-containing methylnitrosourea, streptozotocin, and its cytotoxic group, l-methyl-l-nitrosourea, suggested that this bone marrow toxicity could be reduced by the attachment of the cytotoxic group to a glucose moiety [15]. Chlorozotocin, a glucose-containing chloroethylnitrosourea, was shown to have curative activity for murine L1210 leukemia with relative sparing of bone marrow in mice, but nevertheless it has delayed and cumulative myelosuppression as its dose-limiting toxicity in man [2, 9, 17, 20].

Melphalan is a clinically active, myelosuppressive nitrogen mustard, which has demonstrated significant murine P388 leukemia antitumor activity [5]. C-6, a glucose-containing nitrogen mustard, has an equimolar LD_{10} and significant murine P388 leukemia antitumor activity compared with melphalan in $CD2F_1$ mice [3, 5]. However, C-6 lacks substantial myelotoxicity at its LD_{10} in mice. Therefore, C-6 may have the same antitumor activity as melphalan but less myelotoxicity in man. Conversely, it is possible that at similar doses C-6 may cause similar myelotoxicity to melphalan in man, but with a higher antitumor activity. In either case, C-6 would have a higher therapeutic index than melphalan. We have investigated the activities of C-6 and melphalan against human bone marrow cells and human tumor cells in vitro by applying the CFU-C and HTSCA assays in combination in an attempt to test this hypothesis.

There was no significant difference in the in vitro myelotoxicity of C-6 versus melphalan as measured by the CFU-C assay against human bone marrow cells incubated in medium containing glucose at any of the concentrations used except 4.0 μM, at which C-6 was significantly more toxic. In particular, both agents decreased the number of WBC colonies to approximately 12% of control at 6.6 μM after a 1-h incubation, which is a good approximation of melphalan's CxT in man [1]. This suggests that if C-6 and melphalan have similar pharmacokinetic properties in man the two agents would have similar myelotoxicities at equivalent doses. In addition, C-6 demonstrated a generally decreased meylotoxicity for bone marrow cells incubated in glucose-deficient medium compared with McCoy's 5A medium with 300 mg% glucose at all concentrations used. This suggests that C-6 is not transported into human bone marrow cells via the glucose transport system, since an increased C-6 delivery into cells would be anticipated with a corresponding increase in cytotoxicity in the absence of glucose if C-6 were transported via the glucose transport system.

The antitumor activity of melphalan was either similar to or greater than that of C-6 at all the concentrations used against any of the four tumor specimens except at 1.3 μM for tumor I as measured by the HTSCA assay. In particular, there was no significant difference in the antitumor activities of the two agents at 6.6 μM after the 1-h incubation in vitro (a good approximation of CxT for melphalan in vivo) used against the four tumor specimens. This suggests that at equivalent doses in vivo, C-6 and melphalan would have similar antitumor activities.

Only partial dose-response curves are seen for either C-6 or melphalan in the four tumors. The failure to generate clear dose-response curves in tumors II and III could be explained in part by resistance of the two tumors to the drugs or to technical artifacts of the HTSCA. (While the plates were examined for clumps at the end of the experiment, positive controls with mercuric chloride were not routinely performed at the time of these experiments.) It is conceivable that dose-response curves could be obtained in all four tumors if a much broader range of concentrations were used for C-6 and melphalan. However, the purpose of this investigation was to compare similar 'toxic' concentrations of C-6 and melphalan to those determined in the CFU-C assay. This approach appears to be justifiable, since the average clinically obtainable concentration (CxT) for melphalan of 6.6 μM for a 1-h incubation produced significant in vitro myelotoxicity (i.e., 12% of control), which correlates well with its dose-limiting myelotoxicity in man. In addition, we used other drug concentrations (i.e., 1.3–9.9 μM for 1-h incubations), which are representative of possible variations that could be seen in the clinical situation. It is highly unlikely that conventional doses of melphalan will result in a greater CxT than 9.9 μM for a 1-h incubation [1].

In summary, C-6 does not appear to be transported into normal human bone marrow cells via the glucose transport system despite the presence of a glucose moiety within the molecule. In general, C-6 does not appear to be a more effective agent than melphalan, since at equivalent concentrations the two agents have both similar in vitro myelotoxicities and antitumor activities. Further comparative in vitro antitumor studies with a greater variety of tumors will be necessary to verify whether C-6 has equivalent in vitro antitumor activity to melphalan in general. These in vitro studies could lead to incorrect conclusions if (a) the HTSCA is a poor predictor of in vivo antitumor activity or (b) the CFU-C assays fails to estimate in vivo myelotoxicity correctly. The latter possibility is likely to apply with the chloroethylnitrosoureas, which produce myelotoxicity 4–6 weeks after administration in man. Therefore, the progenitor cell which gives rise to colonies in the CFU-C assay is probably not the cell that is attacked by the chloroethylnitrosoureas. This may account for the discrepancy between the CFU-C results with human bone marrow obtained with chlorozotocin and BCNU compared with their in vivo myelotoxicity in man [17, 21]. Our studies suggest that C-6 will not be less myelosuppressive than melphalan at doses that produce equivalent antitumor activity in man. The validity of using the CFU-C assay in conjunction with the HTSCA to assess the potential therapeutic index of novel agents such as C-6 will require

careful correlation with results of clinical trials utilizing these agents.

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