6-[Bis(2-chloroethyl)amino]-6-deoxygalactopyranose Hydrochloride (C6-Galactose Mustard), a New Alkylating Agent with Reduced Bone Marrow Toxicity

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ABSTRACT

We have previously reported that chloroethyl nitrosoureas and nitrogen mustard bone marrow toxicity can be selectively reduced by placement of the cytotoxic group on specific positions of a glucose molecule. We have now synthesized and evaluated a new drug in which the mustard cytotoxic group is attached to the carbon-6 position of galactose (C6-GLM). C6-GLM, administered i.p. as a single 10% lethal dose of 15.5 mg/kg, produced a 121% increase in life span (ILS) in mice bearing the ascitic P388 leukemia, compared to a 60% ILS with a 10% lethal dose of nitrogen mustard (P < 0.01). A single p.o. dose of C6-GLM, 16 mg/kg, produced an ILS of 58%. Against i.p.-implanted B-16 melanoma, i.p. C6-GLM produced a 56% ILS compared to 30% with an equitoxic dose of nitrogen mustard (P < 0.01). The activity of the two drugs for Ehrlich ascites was comparable, with 60% survivors with the galactose mustard. A single 10% lethal dose of C6-GLM reduced the white blood cells to 74% of control; circulating granulocytes remained at 91% of initial values. With nitrogen mustard, the nadir white blood cell count was 57% of control with an absolute granulocyte count of 70% of initial values (P < 0.01). The toxicity of melphalan was considerably greater, with a lower and more protracted white blood cell nadir and an absolute neutrophil count nadir of 49% of control. These findings paralleled the relative decrements in bone marrow DNA synthesis produced by the three drugs. Measurement of human bone marrow granulocyte-macrophage colony-forming units, following in vitro exposure to graded concentrations of the three mustards, confirmed the bone marrow sparing properties of C6-GLM. At the highest concentration, 1 x 10−2 m, the latter drug produced only a 33% reduction in colonies compared to a 75% reduction with nitrogen mustard and a virtual elimination of activity of colony-forming units with melphalan. The demonstration of antitumor activity, at least equivalent to nitrogen mustard, without the necessity of significant bone marrow toxicity supports the development of C6-GLM for clinical trials in humans.

INTRODUCTION

Despite the development of many new classes of anticancer drugs during the past three decades, alkylating agents retain a strategic role in the treatment of most forms of human cancer. Their clinical utility is, however, limited by the almost universal problem of bone marrow toxicity. Structure-activity analyses with alkyl nitrosoureas have demonstrated that placement of the cytotoxic moiety on the carbon-2 position of glucose, as in the case of streptozotocin and chlorozotocin, results in a significant decrease in the alkylation of transcriptionally active chromatin of bone marrow, whereas covalent binding to the template-active region in tumor is equivalent to that observed with the more myelotoxic analogues (7, 8).

These observations have been extended to the bifunctional nitrogen mustard class of alkylating agents. Analogues in which this cytotoxic group was conjugated to the carbon-2 or -6 positions of glucose were more active than nitrogen mustard for the P388 murine ascitic leukemia and had reduced toxicity for bone marrow. In contrast, placement of the mustard function on carbon 1 (o or f position) produced compounds with significant myelosuppressive activity (9).

To determine the specificity of the sugar moiety, we have now synthesized a new galactose analogue in which the cytotoxic group is attached to the carbon-6 position of galactose. The purpose of this paper is to describe the biological properties of this C6-GLM in comparison with HN2 and L-PAM.

MATERIALS AND METHODS

6-[Bis(2-chloroethyl)amino]-6-deoxy-d-galactopyranose hydrochloride (C6-GLM; Fig. 1) was prepared in our laboratory. The synthesis and chemical characterizations are reported elsewhere. HN2 (NSC-762) and L-alanine, 3-p-bis(2-chloroethyl)aminophenyl (L-PAM, melphalan, NSC-8806) were kindly provided by the Drug Development Branch of the National Cancer Institute. For in vivo studies, C6-GLM and HN2 were dissolved in 0.9% sodium chloride at 4°C. L-PAM was dissolved in ethyl alcohol containing less than 1% concentrated hydrochloric acid, and this solution was added to hydroxypropyl cellulose (0.3% in saline) to give a final concentration of 4% ethyl alcohol and 96% hydroxypropyl cellulose. Control mice received either saline or the ethyl alcohol-hydroxypropyl cellulose diluent. For all animal studies, the compounds were prepared immediately prior to use and administered i.p. in a volume of 0.1 ml/10 g body weight. The LD10 was determined for each drug using groups of 10 normal male BALB/c x DBA/2 F1 (hereafter called CD2F1) mice which were administered graded i.p. doses of each drug and observed for a period of 45 days.

Antitumor Activity. The murine P388 leukemia, maintained in female DBA/2 F1 mice, was used to evaluate antitumor activity in three replicate experiments. This tumor was selected because of its known sensitivity to nitrogen mustard (10). Male CD2F1, mice, weighing 18 to 24 g and maintained on Lab-Blox laboratory chow pellets and water, ad libitum, were used. Each drug was administered i.p. (0.1 ml/10 g body weight) to groups of 10 male CD2F1 mice on Day 1 after implantation of 1 x 105 P388 leukemia cells i.p. The LD10 doses used were determined in normal CD2F1 mice following a single i.p. injection. Mean survival of drug-treated animals was compared to the survival of control tumor-bearing animals that received appropriate volumes of vehicle. In addition to measurement of mean survival days, the percentage of ILS was calculated as follows.

\[
\text{% of ILS} = \left(\frac{T - C}{C}\right) \times 100
\]

where T is the mean survival days of the treated mice, and C is the mean survival days of the untreated mice.

The Ehrlich ascites was used as an additional murine tumor system...
for confirmation of therapeutic activity. Ehrlich ascites was maintained i.p. in female BALB/c mice. Treatment as described in the previous section was administered as a single i.p. dose 1 day after implantation of $2 \times 10^6$ cells/0.10 ml in groups of 10 male C57BL × DBA/2 F1, (hereafter called BD2F1) mice (6 to 9 wk old). Two replicate experiments were performed.

The third tumor in which therapeutic activity was assessed was the i.p.-implanted B16 melanoma. Male BD2F1 mice were inoculated with 0.5 ml of a 10% (wt/vol) tumor brei. Treatment was administered as a single i.p. LD10 dose 1 day after tumor implantation. Three replicate experiments were performed. Statistical analysis was performed using the Student t test of significance.

Toxicity for the Hematopoietic System of Mice. Groups of 10 normal male CD2F1 mice were treated i.p. with drug as described previously. Measurement of peripheral leukocyte (WBC) count was performed using a 20-μl sample of reticulobitual blood obtained on Day 3, 4, 5, or 10 following i.p. administration of LD10 doses. Blood samples obtained were diluted in 9.98 ml of Isoton, a physiological diluent, and counted in a Coulter Counter after lysis with Zapoglobin (Coulter Diagnostics, Hialeah, FL). Absolute neutrophil counts were performed on Wright-stained smears prepared from blood obtained on the day of WBC nadir. WBC and absolute neutrophil counts are expressed as a percentage of values from control mice receiving drug vehicle only. Statistical analysis was performed using the Student t of significance.

Normal male BD2F1 mice were used to investigate the comparative effects of the three drugs on in vivo murine bone marrow DNA synthesis. For each compound, animals received a single i.p. injection of the LD10 dose. Control mice received either the saline or ethyl alcohol-hydroxypropyl cellulose diluent. One h prior to sacrifice, 30 μCi of [methyl-3H]thymidine (New England Nuclear, Boston, MA) were given i.p. to each animal. Four mice from each treatment group were sacrificed by cervical dislocation at 8, 24, 48, 72, and 96 h posttreatment. Both femurs and tibias were removed, and the marrow cells were expressed with phosphate-buffered saline (0.15 N NaCl-0.01 M phosphate buffer, pH 7.4). All marrow cells for each experimental point were pooled, and the DNA content of each pooled sample was extracted using a modification of the method of Schneider (11). A 0.5-ml aliquot of each final supernatant was added to 10 ml of liquid scintillation fluid (Aqueous Counting Scintillant; Amersham Corporation, Arlington Heights, IL) and counted in a Coulter Counter after lysis with Zapoglobin (Coulter Diagnostics, Hialeah, FL). Absolute neutrophil counts were performed on Wright-stained smears prepared from blood obtained on the day of WBC nadir. WBC and absolute neutrophil counts are expressed as a percentage of values from control mice receiving drug vehicle only. Statistical analysis was performed using the Student t of significance.

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Toxicity for Human Bone Marrow CFU-GM. Bone marrow was aspirated from the iliac crests of normal volunteers. Cells were layered on Ficoll and centrifuged at 1200 rpm at 25°C for 30 min. The interface layer containing nucleated cells was removed and washed 3 times with RPMI medium. The cells were resuspended in Pike-Robinson-McCoy's medium at a cell concentration of 5 x 10^6/ml. Test drug, at concentrations of 0.01 to 0.001 μM, was added for an incubation period of 90 min at 37°C. The conditions of the colony-forming assay, a modification of published methods (13), were as follows: Pike-Robinson-McCoy's medium, 1.3 ml; bone marrow cells (5 x 10^6/ml), 0.1 ml; giant cell tumor-conditioned medium (GIBCO, Grand Island, NY), 0.5 ml as colony-stimulating factor; agar (0.3%), 0.2 ml. The suspension was plated in triplicate, 0.4 ml per well, and incubated in 5% CO2 at 37°C. Colonies (50 cells or more) and clusters (30 to 50 cells) were counted on Day 10 and compared with vehicle control samples.

Organ Toxicity Studies. Groups of five male BD2F1 mice were given injections of C6-GLM i.p. at doses of 15.5 mg/kg (LD10) or 27 mg/kg (LD50). On Day 3 after drug administration, blood was obtained from the retroorbital sinus for measurement of serum creatinine levels (14). These same experimental mice were then sacrificed by cervical dislocation, and kidneys and liver were removed, rinsed in cold phosphate-buffered saline, and fixed in formalin. Tissue sections were cut at a thickness of 5 μm, and the stain used was hematoxylin-eosin. Slides of sections from drug-treated mice were read blindly versus control slides.

RESULTS

The LD10 dose of C6-GLM in normal mice is 15.5 mg/kg or 37 μmol/kg (Table 1). The corresponding LD10 dose for HN2 was 2.9 mg/kg (15.1 μmol/kg), while for L-PAM it was 12 mg/kg (39 μmol/kg). The LD10 proved to be the maximally effective single dose, as determined in dose-ranging studies, for each of the three murine tumors in which the three nitrogen mustards were compared.

Antitumor Activity. In P388 leukemia, a single i.p. LD10 dose of C6-GLM resulted in a mean survival time of 21.4 days compared to 9.7 days for vehicle-treated tumor-bearing control mice. The corresponding percentage of ILS was 121% (Table 2). Nitrogen mustard, administered as a single LD10 dose, resulted in a 15.5-day mean survival time and an ILS of 60%. The survivals achieved with C6-GLM were significantly longer than those achieved with HN2 ($P < 0.01$). With L-PAM, the mean survival time was in excess of 21.9 days, with 4 of 30 mice living more than 45 days.

C6-GLM also demonstrated activity against i.p. P388 leukemia when the drug was administered as a single p.o. dose. At a drug dose of 14 mg/kg, the ILS was 48%. A p.o. dose of 16 mg/kg produced a 58% ILS, with 2 of 10 deaths attributed to drug toxicity. At 18 mg/kg, the ILS was 34%, and the number of deaths due to drug toxicity was 4 of 10.

All three nitrogen mustards demonstrated excellent activity against the Ehrlich ascites tumor. The mean survival time of
vehicle-treated tumbled mice was 15 days. With C6-GLM, 12 of 20 mice treated with a single i.p. LD_{10} dose were alive on Day 40. This was equivalent to 13 of 20 and 12 of 20 survivors observed with HN2 and L-PAM, respectively.

In the i.p.-implanted B-16 melanoma model, all vehicle-treatment control mice were dead by Day 18 with a mean survival time of 17.5 days. C6-GLM, administered as a single LD_{10} dose, produced a 56% ILS, compared to a 30% ILS produced by an equitoxic dose of HN2 (P < 0.01). With L-PAM, the ILS was 65%.

Relative Toxicity for Murine Bone Marrow. With the administration of a single i.p. LD_{10} dose, the nadir WBC count with C6-GLM appeared on Day 3 and was 74% of the control count of 8597/mm^3 (Fig. 2), and granulocytes remained at 91% of control values. With HN2, the nadir WBC count was reduced to 57% of control, with an absolute neutrophil count 70% of base line (P < 0.01). L-PAM was significantly more toxic, producing a lower and more protracted WBC nadir with a reduction to 40 to 45% of control during Days 3 to 5. By Day 10 after L-PAM administration, the WBC count had recovered to only 77% of control, whereas complete reversal was observed with both C6-GLM and HN2. The nadir absolute neutrophil count with L-PAM was 49% of control.

These studies were complemented by measurements of bone marrow DNA synthesis at serial time points after i.p. administration of the three mustards at their respective LD_{10} doses (Fig. 3). With C6-GLM, DNA synthesis was reduced from a pretreatment value of 2123 ± 201 (SD) dpm/μg DNA to 1338 ± 191 dpm/μg DNA by 8 h, a 37% decrement; thymidine incorporation returned to control activity by 24 h. HN2 decreased DNA synthesis by 59% (P = 0.01) at 8 h, to 869 ± 89 dpm/μg DNA. L-PAM produced a 67% reduction in bone marrow DNA synthesis at 8 h which further decreased to an 81% reduction at 24 h; DNA synthesis did not return to baseline rate until 72 h after drug administration.

Relative Toxicity for Human Bone Marrow (CFU-GM). Each drug produced a concentration-dependent decrease in human CFU-GM. C6-GLM proved to be the least toxic of the three mustards in this system (Fig. 4). At the highest concentration, 0.01 mM, the galactose analogue decreased stem cells by only 33% compared to control values (44 ± 5.9 colonies for C6-GLM versus 66 ± 6.4 colonies for vehicle controls). HN2 at the same concentration reduced colonies to 25% of control (16 ± 2.1, P < 0.01), whereas L-PAM produced a virtual elimination of CFU-GM activity.

Organ Toxicity Studies. Liver and renal samples were examined from mice 3 days after they had been administered an LD_{10} (15.5 mg/kg) or an LD_{100} (27 mg/kg) dose of C6-GLM and compared blindly to control histology. There was no significant histopathological change in the liver. The kidneys from mice treated with the low and high toxic doses of C6-GLM demonstrated focal tubular degenerative changes, including cytoplasmic vacuolation and exfoliations of cells into the lumen. Serum creatinine levels of these mice, measured concurrently, demonstrated no significant change when compared to vehicle-treated controls: 0.4 ± 0.04 mg/dl for controls versus 0.45 ± 0.07 mg/dl after 15.5 mg/kg and 0.6 ± 0.1 mg/dl after 27 mg/kg.

**DISCUSSION**

Alkylation agents remain one of the most important classes of chemotherapeutic agents for the treatment of human cancer,
whether used singly or as a component of regimens of combination chemotherapy. The relative inability of most available drugs to adequately distinguish between normal bone marrow and tumor requires that significant, and at times life-threatening, myelosuppression be produced in order to ensure that therapeutically effective doses have been administered. The myelosuppression observed with the nitrogen mustard class is generally acute, and it can be severe. This is particularly the case with L-PAM, which is well recognized for its ability to produce profound and sustained granulocytopenia (15).

We have previously demonstrated that the bone marrow toxicity of the nitrosoureas can be reduced by conjugation of the cytotoxic moiety to glucose. In the case of streptozotocin, chlorozotocin, and 1-(2-chloroethyl)-3-(β-D-glucopyranosyl)-1-nitrosourea, maximum antitumor activity was achieved in murine tumors at LD10 doses which produced little or no reduction in circulating granulocytes (2, 3). This phenomenon has been correlated with a selective reduction in alkylation of total bone marrow DNA and of the transcriptionally active fraction of murine and human bone marrow chromatin, in contrast to the binding of the myelosuppressive CCNU (7, 8). In murine marrow, the glucose analogues were further shown to preferentially alkylate DNA of the linker regions of chromatin substructure, whereas the myelotoxic CCNU bound more actively to DNA of nucleosomes (7). In contrast to the differing pattern of chromatin alkylation of bone marrow, all analogues were found to preferentially bind to template-active regions of L-1210 leukemia and HeLa cell chromatin.

We have subsequently extended this concept to the bifunctional nitrogen mustard class of alkylating agents. Four glucose mustard analogues were compared (9). Attachment of the cytotoxic moiety to carbon 2 produced a compound that, based upon molar dose required for lethality (LD10), was the most toxic: 12-fold greater than a carbon-6 analogue and 19-fold greater than the D- and L-isomers of the carbon-1-substituted analogue. Antitumor activity for the murine P388 ascitic leukemia was not different for the four glucose mustards, but superior to that of nitrogen mustard. Distinct differences were, however, demonstrated in regard to bone marrow toxicity. Placement of the bifunctional mustard in the D- or L-position on carbon 1 of glucose produced compounds whose myelosuppressive properties were equivalent to nitrogen mustard. Carbon-6 or -2 analogues, in contrast, possessed good relative bone marrow sparing properties, with nadir absolute neutrophil counts 80 to 86% of control values after an LD10 dose.

In the present studies, we have demonstrated that the glucose sugar is not required. C-6 galactose mustard has antitumor activity at least equivalent, and probably slightly superior, to the comparable C-6 glucose mustard and with good bone marrow sparing properties. Efficacy for P388 leukemia and i.p. B16 melanoma was significantly greater than nitrogen mustard at comparable LD10 doses, whereas both drugs were highly and equally effective for Ehrlich ascites tumor. The toxicity of C6-GLM for murine bone marrow was significantly less than that of HN2 or L-PAM. The latter drug, consistent with its well-recognized myelosuppressive properties in humans, produced a major reduction in circulating neutrophils and a profound reduction in bone marrow DNA synthesis. Both parameters of hematological toxicity were prolonged relative to C6-GLM, requiring an extended period of observation for eventual recovery. The relative toxicity of each drug for human marrow CFU-GM was consistent with the murine data. C6-GLM was significantly less toxic than HN2 and L-PAM at a comparable molar concentration.

Based upon the demonstration of antitumor activity at least equivalent to HN2, but without the necessity for significant bone marrow toxicity, C6-GLM is a candidate for further preclinical development and subsequent Phase I and II clinical trials that are specifically designed to test this concept in humans.

REFERENCES

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