# 6-Bis-(2-chloroethyl)amino-6-deoxy-D-galactopyranose Hydrochloride: Synthesis, Chemical Characterization, Murine P388 Antitumor Activity, and Bone Marrow Toxicity

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**Abstract** □ 6-Bis-(2-chloroethyl)amino-6-deoxy-D-galactopyranose hydrochloride has been synthesized, characterized, and evaluated for antitumor activity and bone marrow toxicity in mice. The 1D- and 2D-NMR studies show the compound to exist as a beta-anomer chair conformation (23%), alpha-anomer chair conformation (22%), and several equilibrating boat conformations or furanose forms (55%). A single ip $LD_{10}$ dose of 15.0 mg/kg produced antitumor activity against the murine P388 leukemia superior to that achieved with an equitoxic dose of nitrogen mustard. In normal mice, this 15.0-mg/kg dose produced minimal depression of peripheral white blood cells and no significant decrease in absolute neutrophil counts. A reduction in toxicity was also demonstrated for human bone marrow CFU-GM, as compared with nitrogen mustard and L-PAM. This and other sugar-containing mustard compounds may represent a class of antineoplastic alkylating agents with reduced bone marrow toxicity.

Despite the development of many new classes of antineoplastic agents, the alkylating agents maintain a critical role in the management of a number of human cancers. Toxicity to bone marrow is the primary limitation to their effective use. In previously published studies, our laboratory has identified the attachment of the nitrosourea cytotoxic group to carbon-2 of glucose as a specific structural modification that can be correlated with reduced bone marrow toxicity.[1-3] Additional studies in Japan with 1-(2-chloroethyl)-3-(β-D-glucopyranosyl)-1-nitrosourea (GANU) confirmed the decreased myelotoxicity of glucose-containing nitrosoureas.[4] To further evaluate this marrow-sparing property conferred by the glucose moiety, we replaced the nitrosourea cytotoxic moiety with another class of alkylating agent, a bifunctional nitrogen mustard. New analogues were synthesized with the mustard cytotoxic group positioned at carbon-1 (D- and L-isomers), carbon-2, or carbon-6 of the aminoglucose molecule.[5,6] Optimal antitumor activity for the murine P388 leukemia (single ip dose) was not statistically different among the glucose analogues, and all demonstrated activity superior to that achieved with an equitoxic single dose of nitrogen mustard. The glucose analogues were also evaluated in normal mice for their effects on the hematopoietic system.[6] The carbon-2 and carbon-6 analogues produced significantly less depression of peripheral white blood cell (WBC) and absolute neutrophil counts than did an equitoxic dose of nitrogen mustard or L-PAM (4-[bis(2-chloroethyl)amino]-L-phenylalanine), two mustard alkylating agents in clinical use.

To further evaluate the specificity of the sugar attached to the mustard cytotoxic group, we have synthesized 6-bis-(2-chloroethyl)amino-6-deoxy-D-galactopyranose hydrochloride, and have evaluated its murine antitumor activity (P388 leukemia) and bone marrow toxicity.

## Experimental Section

**Synthesis of 6-Bis-(2-chloroethyl)amino-6-deoxy-D-galactopyranose Hydrochloride**—The following instruments were used to determine physical properties: Gallenkamp melting point apparatus; Bruker AM-300 WB, for measurement of $^1H$ NMR spectra (300.133 MHz) and $^{13}C$ NMR spectra (75.469 MHz). Chemical shifts are reported in parts per millions (δ values), using tetramethylsilane as an internal standard, except for 4 for which $D_2O$ with TSP [3-(trimethylsilyl)propionic-2,2,3,3-$d_4$ acid, sodium salt] was used. Abbreviations used are: s, singlet; d, doublet; t, triplet; m, complex multiplet. Coupling constants (J values) are in Hz.

1,2:3,4-Di-O-isopropylidene-D-galactopyranose (purity, 99%) was purchased from Aldrich Chemical, Milwaukee, WI. All solvents used were reagent grade. Evaporations were performed under reduced pressure with a rotary evaporator. Reported melting points are uncorrected. Elemental analysis of the final product was performed by Galbraith Laboratories, Knoxville, TN. The synthesis is outlined in Scheme I.

**Preparation of 1,2:3,4-Diisopropylidene-6-O-p-tolylsulfonyl-α-D-Galactopyranose (1)**—To an ice-cold solution of 1,2:3,4-di-O-isopropylidene-α-D-galactopyranose (30 g, 0.115 mol) in dry pyridine (distilled from KOH) was slowly added p-toluenesulfonyl chloride (33 g, 0.173 mol). The mixture was stirred at 0–5 °C for 1 h, and then at room temperature overnight. The resulting precipitate was filtered and washed with acetone. This filtrate was then treated with decolorizing carbon, which was subsequently removed by filtration. The solvents were removed under reduced pressure, giving the crude product as a solid residue which was washed with $H_2O$ and dried (41.5 g, 92% yield). Recrystallization from 95% ethanol yielded fine needles (35 g, 84% yield), with a melting point of 89–90 °C (Lit.[7] 87–89 °C); NMR ($CDCl_3$): 1.281 (s, 3H, $CH_3$), 1.314 (s, 3H, $CH_3$), 1.344 (s, 3H, $CH_3$), 1.502 (s, 3H, $CH_3$), 2.44 (s, 3H, aromatic $CH_3$), 4.0, 4.1 (d of d of d, $J$ = 6.6, 11.9, 1.4 Hz, 2H, $H_6\alpha,\beta$), 4.188 (d of d of d, $J$ = 2.7, 6.6, 1.4 Hz, 1H, $C_5H$), 4.218 (d of d, $J$ = 2.7, 2.4 Hz, 1H, $C_4H$), 4.292 (d of d, $J$ = 5.0, 2.5 Hz, 1H, $C_2H$), 4.585 (d of d, $J$ = 7.9, 2.4 Hz, 1H, $C_3H$), 5.452 (d, $J$ = 5.0 Hz, 1H, $C_1H$, 7.328 (d, $J$ = 8.2 Hz, 2H, aromatic 3,5 protons), and 7.806 ppm (d, $J$ = 8.4 Hz, 2H, aromatic 2,6 protons). All hydrogens were assigned by a 2DFT COSY experiment.

**Preparation of 1,2:3,4-Diisopropylidene-6-bis-(2-hydroxyethyl)amino-6-Deoxy-α-D-Galactopyranose (2)**—Compound 1 (21 g, 0.052 mol) was suspended in 225 mL of freshly distilled diethanolamine and heated at 150–160 °C for 4–4.5 h under a dry nitrogen atmosphere with vigorous stirring. The viscous mixture was cooled to room temperature and added to 800 mL of methylene chloride. The methylene chloride solution was washed twice with 250-mL portions of water, and then dried over anhydrous sodium sulfate. Concentration under reduced pressure and subsequent distillation at 0.5 mmHg and 190 °C gave 13.8 g of a clear viscous syrup (76% yield); NMR ($CDCl_3$): 1.327 (s, 3H, $CH_3$), 1.349 (s, 3H, $CH_3$), 1.460 (s, 3H, $CH_3$), 1.547 (s, 3H, $CH_3$), 2.64 (m, 2H, $H_6\alpha,\beta$), 2.68–2.76 (m,4H,N—$CH_2$—), 2.96 (broad s,2H,OH), 3.613 (m, 4H, N—C—$CH_2$), and 3.892 ppm (d of t, $J$ = 6.7,1.9 Hz, 1H, $C_5H$).

**Preparation of 1,2:3,4-Diisopropylidene-6-Bis-(2-Chloroethyl)amino-6-Deoxy-α-D-Galactopyranose Hydrochloride (3)**—Compound 2 (2 g, 0.0057 mol) was dissolved in 25 mL of dry methylene chloride and 6 mL of thionyl chloride, refluxed for 15 min, and then immediately evaporated to dryness under reduced pressure. Repeated addition of fresh dry methylene chloride followed by evaporation to dryness under reduced pressure yielded an off-white



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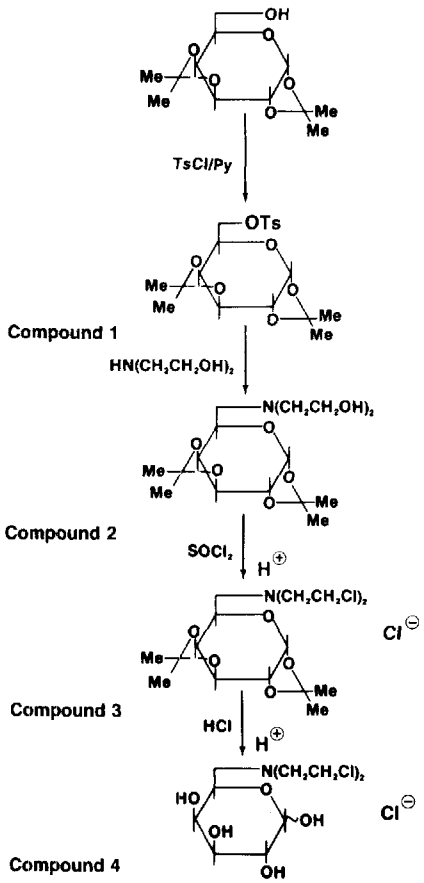



**Scheme I**—Synthetic scheme for 6-bis-(2-chloroethyl)amino-6-deoxy-D-galactopyranose hydrochloride.

hygroscopic solid (1.9 g, 78% yield); NMR ($CDCl_3$): 1.315 (s, 3H, $CH_3$), 1.324 (s, 3H, $CH_3$), 1.428 (s, 3H, $CH_3$), 1.659 (s, 3H, $CH_3$), 3.26 (d of d, $J$ = 15.3,7.9 Hz, 1H, $H_6\ \beta_?$), 3.485 (d, $J$ = 15.3 Hz, 1H, $H_6\ \alpha_?$), 3.58 (broad s, 4H, N—C—$CH_2$—), 4.038 (broad s, 4H, N—C—$CH_2$—), and 4.483 ppm (d, $J$ = 8.4 Hz, 1H, $C_5H$).

**Preparation of 6-Bis-(2-Chloroethyl)amino-6-Deoxy-D-Galactopyranose Hydrochloride (4)**—Compound 3 (1 g, 0.0023 mol) was added to 10 mL of 6 M HCl in a flask fitted with a reflux condenser and refluxed for 10 min. The solution was cooled to room temperature and extracted twice with methylene chloride, and the aqueous layer was then treated with decolorizing carbon. After removing the carbon by filtration, the liquid was concentrated to 5 mL. Addition of acetone to this solution precipitated the final product, a hygroscopic white solid, in 82% yield; NMR ($D_2O$: 3.4–4.6 $\delta$, galactose and N-($CH_2CH_2Cl)_2$ protons; HDO, 4.8 $\delta$; 5.3 anomeric proton; disappearance of isopropylidene protons (methyl groups) at 1.3-1.7 $\delta$.

*Anal.*—Calc. for $C_{10}H_{20}NO_5Cl_3 \cdot 0.5\ H_2O$: C, 34.35, H, 6.05, N, 4.01. Found: C, 34.26, H, 5.75, N, 3.95.

**Chemical Alkylating Activity**—Comparative in vitro chemical alkylating activities for 4 or 6-bis-(2-chloroethyl)amino-6-deoxy-D-galactopyranose hydrochloride, nitrogen mustard, and L-PAM were estimated by reaction with 4-(*p*-nitrobenzyl) pyridine (NBP). Nitrogen mustard and L-PAM were kindly provided by the Drug Development Branch, National Cancer Institute, Bethesda, MD. An aliquot with 0.02–3.0 $\mu$mol of each compound dissolved in acetone was added to 1.5 mL of 5% (w/v) NBP in acetone. Four milliliters of 0.025 M acetate buffer, pH 6, were then added and the mixture was incubated at 37 °C for 2 h. Next, 2 mL of acetone and 3 mL of ethyl acetate were added to each tube (on ice). The mixture was made alkaline with 1.5 mL of 0.25 M NaOH, vortexed, and then centrifuged at 3000 rpm for 15 s. Absorbance of the ethyl acetate layer at 540 nm was determined, and alkylating activities were compared relative to nitrogen mustard.

**Studies of Murine Antitumor Activity and Bone Marrow Toxicity**—Male BALB/c × DBA/2F (hereafter called $CD2F_1$) mice, weighing 19–24 g and maintained on Lablox laboratory chow pellets and water (ad libitum), were used. For determination of acute drug toxicity, groups of 10 normal $CD2F_1$ mice were treated intraperitoneally with single graded doses of 6-bis-(2-chloroethyl)amino-6-deoxy-D-galactopyranose hydrochloride, or C6-galactose mustard. For all studies reported, C6-galactose mustard and nitrogen mustard were dissolved in 0.85% NaCl (saline) at 4 °C immediately prior to use. The L-PAM was dissolved in ethyl alcohol containing <1% concentrated HCl, and this solution was then added to saline to give a final concentration of 4% ethyl alcohol.

The murine P388 leukemia, used to evaluate antitumor activity, was maintained by ip passage in female DBA/2F mice. Each drug was administered intraperitoneally as a single dose to groups of 10 male $CD2F_1$ mice on day 1 following implantation of $1 \times 10^6$ P388 cells, as described in a previous publication.[6]

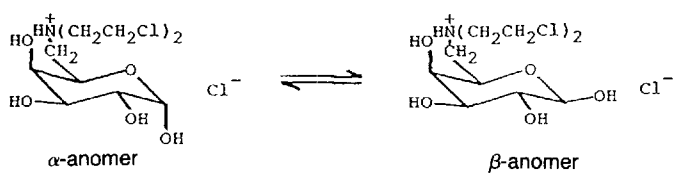
For studies of bone marrow toxicity, serial peripheral leukocyte (WBC) counts were measured using a 20-$\mu$L sample of retro-orbital sinus blood obtained from normal male $CD2F_1$ mice on day 3, 4, or 10 following ip drug administration. This 20-$\mu$L sample was added to 9.98 mL of physiological diluent (Isoton, Curtin Matheson, Washington, DC) and counted in a model ZBI Coulter counter after lysis of red blood cells with Zapoglobin (Curtin Matheson). Mean WBC counts for drug-treated animals were compared with mice that received no treatment or drug vehicle only. Absolute neutrophil counts were performed on Wright-stained smears taken on the WBC nadir day.

**Human Bone Marrow CFU-GM**—The human bone marrow CFU-GM study had the approval of the Internal Review Board of Georgetown University Medical Center. Bone marrow was aspirated from the iliac crests of normal volunteers. Cells were layered on Ficoll and centrifuged at 25 °C for 30 min. The interface layer containing nucleated cells was removed and washed three times with Pike-Robinson-McCoy (PRM) medium. The cells were then resuspended in PRM medium at a concentration of $5 \times 10^5$ per milliliter, and test drug, at concentrations of 0.01–0.001 mM, was added for an incubation period of 90 min at 37 °C. The conditions for the colony-forming assay, a modification of published methods,[8] were as follows: PRM medium, 1.3 mL; bone marrow cells ($5 \times 10^5$ mL), 0.1 mL; Giant Cell Tumor conditioned medium (GIBCO, Grand Island, NY), 0.5 mL as colony-stimulating factor; and agar (0.3%), 0.2 mL. Each sample was plated in triplicate, 0.4 mL/well, and incubated in 5% $CO_2$ at 37 °C. Colonies (50 cells or more) and clusters (30–50 cells) were counted on day 10, and compared with control samples that received no treatment or drug vehicle only.

## Results and Discussion

**Nuclear Magnetic Resonance Data for 6-Bis-(2-chloroethyl)amino-6-deoxy-D-galactopyranose Hydrochloride (4)**—The $^1H$ NMR and $^{13}C$ NMR spectra of 4 dissolved in 99.97% $D_2O$ after lyophilization from $D_2O$ are reproduced in Figures 1 and 2. The compound is present in aqueous solution as the beta-anomer chair conformation, the alpha-anomer chair conformation, along with several equilibrating boat conformations or furanose forms in a ratio of 22.7:22.1:55.2 (proton data), respectively. The alpha- and beta-anomers are shown below (see structure). Inspection of the $^{13}C$ NMR spectrum (Figure 2) shows that all carbons have two sharp peaks in a ratio of 58:42 for the beta- and alpha-chair anomers, plus a number of small broadened peaks for the various equilibrating boat conformations or furanose forms. For example, the beta-anomeric carbon is located at 99.52 ppm, the alpha-anomeric carbon is at 95.58 ppm, and the various boat or furanose anomeric carbons are at 102.7, 102.2, 99.7, and probably at 88.2, 87.7, 85.1, and 84.5 ppm.

In confirmation of this, three types of anomeric hydrogens are observed in the $^1H$ NMR spectrum (Figure 1): one at 5.11



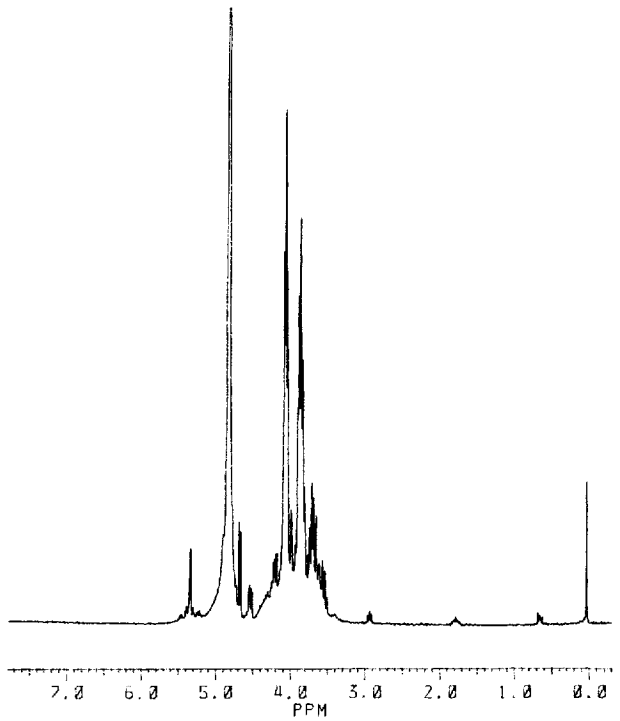



**Figure 1**—The $^1H$ NMR spectrum (300 MHz) of galactose-6-mustard-HCl.

ppm (alpha-$H_1$, $J_{1,2}$ = 3.6 Hz, 0.218 H), one at 4.464 ppm (beta-$H_1$, $J_{1,2}$ = 7.9 Hz, 0.224 H), and several small peaks over the 4.90 to 5.42 range for the various boat conformations or furanose forms (0.464 H). These have been cross-correlated with a HETCOSY ($^{13}C$-$^1H$) spectrum. No specific impurity peaks are observable in either the proton or $^{13}C$ NMR spectra. Water or HCl are the only possible impurities, as these would not be observed in the carbon-13 data and would appear in the HOD peak in the proton data.

**Biological Data**—*Murine Antitumor and Toxicity Studies—Acute Toxicity Studies*—The single ip dose of 6-bis-(2-chloroethyl)amino-6-deoxy-D-galactose hydrochloride (C6-galactose mustard) that produced deaths in 10% of normal $CD2F_1$ mice ($LD_{10}$ dose) is 15.0 mg/kg or 43 μmol/kg. The corresponding single $LD_{10}$ dose for nitrogen mustard is 2.9 mg/kg (15.1 μmol/kg) and that for L-PAM is 12 mg/kg (39 μmol/kg); these doses for nitrogen mustard and L-PAM are in agreement with previously published data from our laboratory.[6]

In a structure–activity analysis of chemical and biological parameters of chloroethylnitrosourea alkylating agents in mice,[9] our laboratory demonstrated a significant linear relationship between the molar $LD_{10}$ dose and chemical alkylating activity, as measured by reaction with NBP: the greater the alkylating activity, the lower the molar $LD_{10}$. For C6–galactose mustard, nitrogen mustard, and L-PAM, drug concentrations of 0.02–3.0 μmol were used to estimate NBP alkylating activity. The activity of C6–galactose mustard was 12% of nitrogen mustard, and that of L-PAM was 15% of nitrogen mustard. The molar $LD_{10}$ dose for nitrogen mustard is 15.1 μmol/kg compared with 43 μmol/kg for C6–galactose mustard and 39 μmol/kg for L-PAM.

*P388 Antitumor Studies*—The antitumor activity of C6-galactose mustard was evaluated for murine P388 leukemia in comparative studies with nitrogen mustard and L-PAM. Mice ($CD2F_1$ male) were implanted intraperitoneally with $1 \times 10^6$ P388 cells, and the test drug was administered as a single ip dose on the following day. For each of the three mustard compounds, the $LD_{10}$ proved to be the maximally effective single dose. C6-Galactose mustard increased the mean survival time to 19.5 d, compared with 9.5 d for tumor-bearing mice that received no treatment or drug vehicle only; this is a 106% increase in life span (ILS). Nitrogen mustard produced a 15.2-d mean survival time, or a

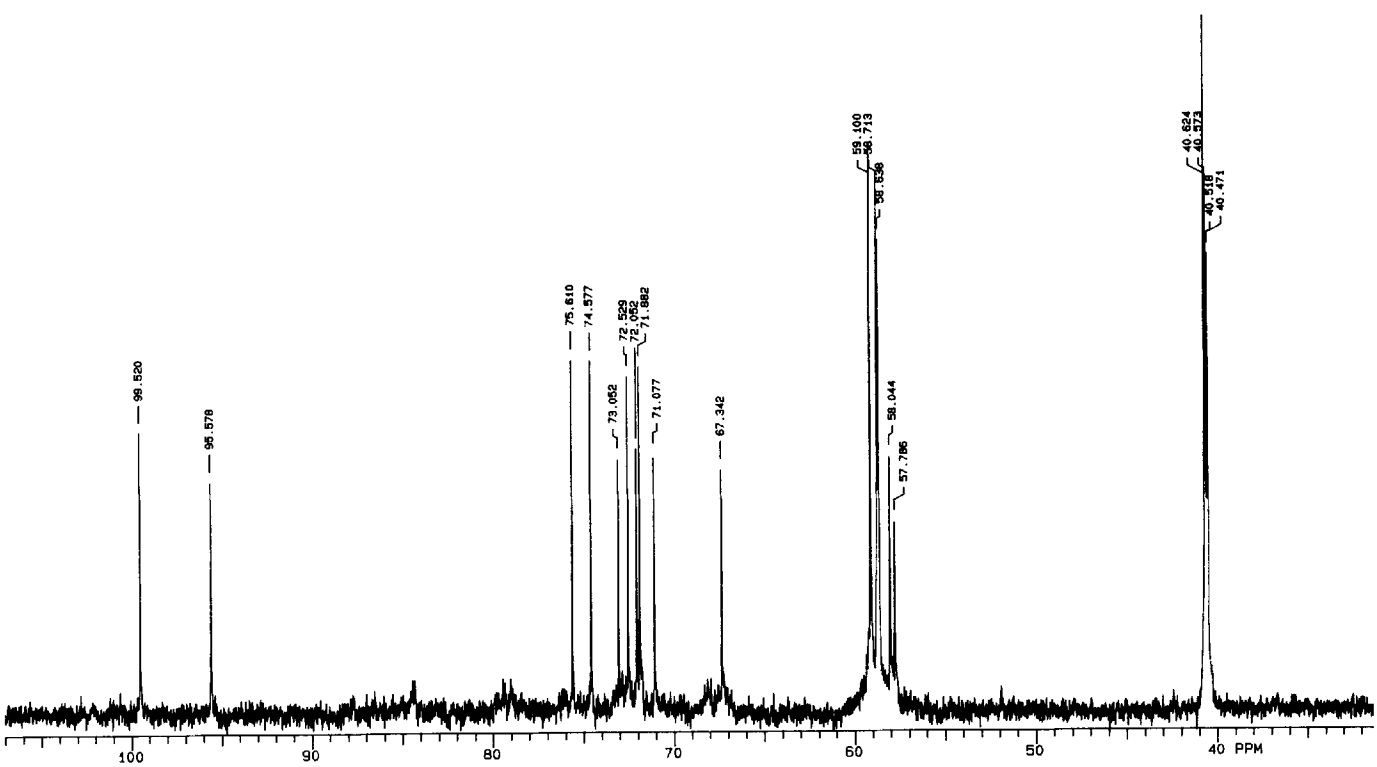



**Figure 2**—The $^{13}C$ NMR spectrum (75 MHz) of galactose-6-mustard-HCl.

60% ILS. The survival achieved with C6-galactose mustard was significantly longer than that obtained with nitrogen mustard ($p \leq 0.01$), and L-PAM produced a mean survival time in excess of 21.5 d.

*Toxicity to Bone Marrow*—The bone marrow toxic potential of $LD_{10}$ doses of C6-galactose mustard, nitrogen mustard, and L-PAM was evaluated in normal male $CD2F_1$ mice, and the data are summarized in Figure 3. The nadir white blood cell (WBC) count with C6–galactose mustard occurred on day 3, with a reduction to 74% of control values. This effect was concentrated on circulating lymphocytes, since granulocytes remained at 91% of control. With nitrogen mustard, the nadir white count was reduced to 57% of control, with an absolute neutrophil nadir of 70% of control. However, L-PAM proved significantly more myelotoxic, with a protracted WBC nadir of 40–45% of control on days 3–5, and a nadir absolute neutrophil count of 49% of control.

*Human Bone Marrow CFU-GM Studies*—The toxicity of the three mustards for human bone marrow CFU-GM, the stem cell committed to granulocytic and monocytic differentiation, was also assessed over a range of drug concentrations. At the highest concentration tested (0.01 mM), C6-galactose mustard reduced stem cells to 67% of control (44 ± 5.9 colonies and clusters versus 66 ± 6.4 for cells treated with drug vehicle, mean ± SD). In contrast, nitrogen mustard at the same millimolar concentration reduced colonies to 25% of control (16 ± 2.1, $p < 0.01$), and L-PAM produced a virtual elimination of colony formation.

In summary, a single $LD_{10}$ dose of 6-bis-(2-chloroethyl)-amino-6–deoxy-D-galactopyranose hydrochloride produced minimal WBC depression and no significant decrease in absolute neutrophil counts. This dose was significantly less myelosuppressive than an equitoxic dose of nitrogen mustard ($p < 0.05$) or L-PAM ($p < 0.01$). Further, at the $LD_{10}$ dose, the antitumor activity of C6-galactose mustard was superior to that achieved with an equitoxic dose of nitrogen mustard. These data strongly suggest that this and other sugar-containing analogues may represent a class of effective nitrogen mustard alkylating agents with reduced acute and chronic bone marrow toxicity, and a possible reduced long-term risk for malignant transformation of bone marrow.

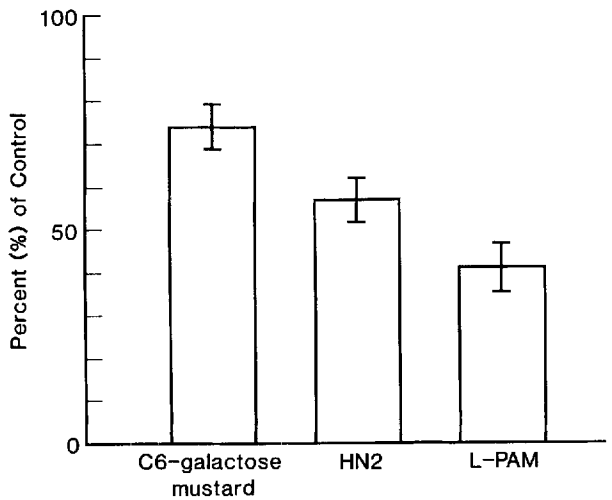



**Figure 3**—Peripheral leukocyte (WBC) count on day 3 following ip administration of an $LD_{10}$ dose of C6-galactose mustard (15 mg/kg), nitrogen mustard (HN2, 2.9 mg/kg), or L-PAM (12 mg/kg). The bars represent SD.

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