Comparison of Quantitation Methods for L-1210 Cell Populations and Evaluation of Selected Cytotoxic Agents in Leukemic Mice

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Abstract □ A more rapid cell-counting technique using an electronic cell counter was developed as an improvement over the slower hemocytometer method. The electronic counting method produced cell counts that had a smaller standard deviation but were not significantly different from the hemocytometer method. The acetate, hexanoate, and decanoate esters of *p*-*N*,*N*-bis(2-chloroethyl)aminophenol were investigated for acute toxicity in mice, effects on survival times, and effects on L-1210 cell populations in L-1210 leukemic mice. The decanoate ester was the least toxic compound and was most effective in lengthening the lifespan of the mice. The acetate and hexanoate esters were more effective in reducing L-1210 cell populations.

Keyphrases □ *p*-*N*,*N*-Bis(2-chloroethyl)aminophenol—ester derivatives synthesized and evaluated for antileukemic activity and toxicity, comparison of two cell-counting methods □ Antileukemic activity—*p*-*N*,*N*-bis(2-chloroethyl)aminophenol ester derivatives, synthesis and evaluation for antileukemic activity and toxicity, comparison of two cell-counting methods

Leukemia is a malignant tumor characterized by an overproduction of leukocytes and the presence of abnormal cells in hemopoietic organs (1). The proliferating leukemia cells fill the bone marrow spaces and prevent maturation of the red blood cells. These cells spill over into widely scattered areas of the body and accumulate even in organs with no inherent blood-forming capacity such as the spleen, liver, and lymph nodes (2). Lymphoid leukemia L-1210, a form of ascitic tumor, was induced in the spleen and lymph nodes of mice by painting the skin with 3-methylcholanthrene (3). The BDA/2 strain of mice is a reliable host in which to propagate this tumor system (4). An important characteristic of L-1210 leukemia is a widely disseminated proliferation resulting in death within 8–11 days (3).

A measure of the effectiveness of a cytotoxic agent against a neoplastic disease is its ability to kill or retard tumor cell growth at a dose that is not toxic to the host. A compound can be evaluated quantitatively by measuring its ability to increase the average survival time of L-1210 leukemic mice and to reduce the number of leukemic cells.

A model series of antineoplastic agents was synthesized in these laboratories for investigating the relationship of lipophilicity and antineoplastic activity. Three representative compounds, the acetate, hexanoate, and decanoate esters of *p*-*N*,*N*-bis(2-chloroethyl)aminophenol, were selected from the series for this investigation. Cyclophosphamide was used for comparison.

The specific objectives of this investigation were to: (*a*) determine the acute toxicity as measured by the LD_{50} for each compound studied, (*b*) determine the effects of each compound on L-1210 cell populations and the prolongation of life in leukemic mice, and (*c*) develop a rapid technique for the determination of tumor cell populations and compare this technique with the conventional hemocytometer method.

EXPERIMENTAL

Test Animals—DBA/2 mouse strain[1], BDF_1 mouse strain[1], HA/ICR mouse strain[2], and L-1210 leukemic mice (tumor source)[3] were used.

Instruments—The necessary equipment included an electronic cell counter[4], a channelizer[4], a dilutor[4], an *x*–*y* recorder[4], a hemocytometer[5], and a microscope[6].

Materials—Counting diluent[4], red blood cell-lysing reagent[4], crystal violet[7], Giemsa stain[8], isotonic diluting solution[9], trypan blue[10], Wright's stain[7], and cyclophosphamide[11] were obtained commercially. The fatty acid esters of *p*-*N*,*N*-bis(2-chloroethyl)aminophenol were prepared in these laboratories, and their physical and analytical data are reported in Table I[12]. These compounds will be referred to as the acetate, hexanoate, and decanoate esters.

Pretoxicity Testings—Groups of two healthy (HA/ICR) male mice were injected intraperitoneally with 500, 100, 10, 1, or 0.1 mg of test drug/kg in propylene glycol to evaluate the dose range for the pharmacological screen and LD_{50} determination.

Pharmacological Screen and LD_{50} Evaluation—Five groups of six (HA/ICR) mice each were selected. A gross screen was conducted for acute toxicity, and results were recorded during the 3 hr following injection. The mice were observed and weighed every day for 21 days, and the mortalities were recorded daily. A linear regression and correlation coefficient program (5) and a graphic method (6) were applied to determine the LD_{50} for each drug test.

Transplantation Procedures—Sterile equipment was used under aseptic conditions. DBA/2 (6–9-week-old) host mice bearing L-1210 leukemia for 6–7 days were the tumor donors; they were sacrificed under ether vapor, immersed in 0.1% benzalkonium chloride, and swabbed with 70% ethanol. A 10-ml hypodermic syringe equipped with a 20-gauge needle (flushed with heparin at 1000 USP units/ml) was inserted into the abdominal cavity of the mouse and aspirated to obtain the lymphoid leukemia L-1210 cells (7).

The leukemia cells were placed in a container over ice. One drop of ascitic fluid was prepared for microscope examination. Cell morphology was determined by staining with Wright's or Giemsa stain to differentiate the leukocytes and lymphoblasts (8). Ascitic fluid containing at least 95% lymphoblasts or lymphocytes was used for transplantation in DBA/2 mice for tumor maintenance and in BDF_1 mice for antileukemic studies. All inoculations of L-1210 cells were made within 1 hr after cell removal from the host mouse to ensure a viable transplant.

Determination of Cell Counts—*With Electronic Cell Counter*—Ascitic fluid dilutions were made using a dilutor. A 40-μl sample of ascitic fluid was diluted with 20 ml of counting diluent to make a 1:500 dilution for the white blood cell counts. A 200-μl sample of the 1:500 dilution was used to make a 1:50,000 dilution in counting diluent for the red blood cell counts. Six drops of the red blood cell-lysing reagent were added to the

[1] Jackson Laboratories.
[2] ARS/Sprague–Dawley.
[3] National Cancer Institute, National Institutes of Health, Besthesda, Md.
[4] Model ZB counter and accessories, Coulter Electronics.
[5] American Optical Co.
[6] Model RA, Carl Zeis, West Germany.
[7] Matheson, Coleman & Bell.
[8] Fisher Scientific Co.
[9] Microbiological Associates.
[10] Allied Chemical Co.
[11] Cytoxan, Mead Johnson Co.
[12] Taken in part from M. B. Caldwell, M.S. thesis, College of Pharmacy, University of South Carolina, Columbia, S.C., 1974.

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Table I—Physical Data and Elemental Analysis for the Esters of *p*-*N*,*N*-Bis(2-chloroethyl)aminophenol

Derivative	Mol. Wt.	Observed Melting Point	Analysis, % Calc.	Found
Acetate[a,b]	276	Oil	C 50.53	50.62
			H 5.61	5.28
			N 4.93	4.83
Hexanoate	332	40.5–41°	C 57.83	58.02
			H 6.93	6.85
			N 4.22	4.12
Decanoate	388	Semisolid	C 61.86	61.81
			H 7.99	8.01
			N 3.61	3.47

[a] The calculated carbon, hydrogen, and nitrogen percentages include an average of 0.5 mole of water/mole of the derivative. [b] W. C. J. Ross, G. P. Warwick, and J. J. Roberts, *J. Chem. Soc.*, **1955**, 3110.

remaining 1:500 dilution of ascitic fluid, and white blood cell counts were performed using the electronic cell counter.

After completion of the white blood cell counts, the red blood cell counts of the 1:50,000 dilution were determined. Six readings for each dilution were recorded and corrected, and the mean cell values were calculated. Peripheral blood samples, obtained by puncture of the ophthalmic venous plexus of the mouse eye using a heparinized capillary tube, were treated in a similar manner.

With Hemocytometer—Ascitic fluid was diluted 20-fold with a white cell-diluting fluid using a white blood cell pipet. A 1% acetic acid solution containing 0.001% trypan blue was used to establish cell viability. A hemocytometer was used to determine the cell counts (2, 9). White blood cell and red blood cell counts were recorded as the mean of the five observations in each test from ascitic or blood samples. After determining cell viability and cell counts, the ascitic fluid was diluted with isotonic diluting solution. The inoculum contained 1×10^5 tumor cells in 0.1 ml. A 1-ml tuberculin syringe equipped with a 25-gauge needle was used to inject 0.1 ml of diluted ascitic L-1210 cells into the DBA/2 or BDF_1 mice.

***In Vivo* Determination of Antileukemic Activities by Survival Times *versus* Cell Differentiation**—On Day 0, the tumor was implanted into BDF_1 male mice; the number of survivors was recorded daily during the test period. The doses used were below the LD_{10} value of each test drug to minimize drug toxicity (4). The test solutions were injected within 15 min after preparation at a dose volume not to exceed 0.01 ml/g of body weight. The test drug solutions were administered intraperitoneally to each test group on Days 2 and 5. The experiment was evaluated on the day of death for the last animal in a test group or after 30 days (4).

For leukemia cell differentiation, the drugs were administered in the same manner as in the survival study; these experiments were evaluated on Day 7. The test animals were sacrificed, the ascitic fluid was collected, and the leukemia cells were counted.

RESULTS AND DISCUSSION

The gross appearance of leukemic mice was observed after inoculation of 1×10^5 leukemia cells in six BDF_1 mice. The body weight, hair appearance, motor activity, food intake, and size of the abdominal cavity remained normal until around the 4th or 5th day after inoculation. After 5–6 days, the abdominal cavity appeared enlarged and distended from an accumulation of ascitic fluid. Body weight generally increased 5–10% after 7 days or by the time of death. Piloerection and alopecia were observed as the disease approached the lethal stage. Beginning on the 5th or 6th day, the leukemic mice were rejected by their healthier cage mates.

Table II—Summary of Acute Toxicity of Cyclophosphamide and the Fatty Acid Ester Derivatives Administered Intraperitoneally to Male HA/ICR Mice

Drug[a]	LD_{50}, mg/kg	LD_{50}, μmoles/kg	95% Confidence Limits, μmoles/kg	Slope, probit log dose
Decanoate ester	19.7	55.4	32.31–78.4	2.5045
Hexanoate ester	17.7	53.3	38.19–68.43	3.2822
Acetate ester	14.0	50.8	30.5–70.8	2.4724
Cylcophosphamide	281.4	1078	880.5–1272.0	4.6279

[a] Administered in propylene glycol.

Table III—Comparison of Doses Producing Optimal Survival Times (% T/C) of Treated L-1210 Leukemic BDF_1 Mice

Drug[a]	Dose, μmoles/kg	Mean Survival Time, days (±*SE*) Test Group	Control Group	Survival, % T/C[b]
Cyclophosphamide	499.00	23.0 (2.5)[c]	8.83 (0.31)	255.6
Acetate	5.44	9.66 (2.74)	8.33 (0.21)	116.0
Hexanoate	3.01	10.16 (2.03)	8.67 (0.42)	117.3
Decanoate	4.7	12.0 (1.57)[d]	8.00 (0.26)	150.0

[a] Administered in propylene glycol to six mice/group. [b] The % T/C represents the ratio of the sum of number of days (T) each treated animal survives to the sum of the number of days each control animal (C) survives multiplied by 100. [c] $p < 0.01$. [d] $p < 0.05$.

The LD_{50} values for the drugs were obtained by probit analysis and linear regression (Table II) (5, 6). In the HA/ICR mice, the LD_{50} for the decanoate ester was 55.4 μmoles/kg. Compared to cyclophosphamide, the decanoate ester was more toxic, but it was the least toxic of the fatty acid ester derivatives. The LD_{50} values for the hexanoate and acetate esters were 53.3 and 50.8 μmoles/kg, respectively. Cyclophosphamide was the least toxic compound, having an LD_{50} value of 1078 μmoles/kg. This value is less than literature values (1152 and 1425 μmoles/kg), which were determined in different strains (10, 11).

The survival of treated groups (T) to untreated control groups (C) was compared. A calculated % T/C value exceeding 100% implied that the drug treatment increased the lifespan. The doses producing optimal survival in leukemic mice for cyclophosphamide and the fatty ester derivatives are summarized in Table III.

The decanoate ester produced a maximum mean survival time (% T/C) of 150% at 4.7 μmoles/kg (Table III). A small increase in survival time was observed for the hexanoate ester (Table III); the % T/C value at the optimal survival dose level of 3.01 μmoles/kg was 117%. The optimal dose for the acetate ester was 5.44 μmoles/kg, which increased the % T/C value to 116%. Significant increases in survival times (255.5%) were observed for cyclophosphamide (Tables III and IV). Two mice lived beyond 30 days, and 30% of the animals showed a complete regression of the tumor at a dose of 400 μmoles/kg. After treatment with cyclophosphamide, leukemic mice showed little evidence of ascitic tumor cells and weight loss.

Table IV—Summary of Survival Times and Leukemia Cell Counts in Treated L-1210 BDF_1 Leukemic Mice

Drug, μmoles/kg	Survival Time, % T/C	Cells/mm³ (±*SE*)[a] Test	Control	Cell Counts, % T/C[b]
Cyclophosphamide				
685	152	2,750 (135)	137,150 (6837)	2.0
616	166	2,810 (722)	107,300 (3640)	2.6
552	170	2,700 (111)	107,300 (3650)	2.5
499	256[c]	4,010 (44)	137,150 (4792)	2.9
448	231	6,800 (179)	137,150 (4792)	4.9
Acetate ester				
8.15	95	46,300 (1193)	135,750 (4784)	34.1
7.34	106	47,000 (5718)	135,750 (4784)	34.6
5.44	115	66,100 (2914)	114,175 (4573)	57.9
3.62	108	83,600 (2475)	114,175 (4573)	73.2
1.81	102	88,800 (3411)	114,175 (4573)	77.8
Hexanoate ester				
7.53	98	50,625 (3787)	150,900 (7296)	33.5
6.09	112	81,700 (2643)	150,900 (7296)	54.1
3.01	117	71,500 (2394)	150,900 (7296)	47.4
1.51	113	81,700 (2568)	150,900 (7296)	54.1
Decanoate ester				
5.28	112	60,300 (2776)	135,750 (4784)	44.5
4.70	150	56,600 (3352)	135,750 (4784)	41.7
3.86	131	76,600 (2427)	148,100 (6815)	48.5
2.57	123	79,600 (1292)	158,100 (7354)	50.3
1.27	110	90,200 (2323)	158,100 (7354)	57.1

[a] Electronic cell-counting procedure. [b] The % T/C represents the ratio of cell counts test (T) to control (C) multiplied by 100. Samples taken on Day 7. [c] $p < 0.01$.

Table V—Comparison of L-1210 Cell Counts of Ascitic Fluid from Untreated Leukemic BDF_1 Mice Determined on the Electronic Cell Counter or Hemocytometer on Day 7 after Intraperitoneal Inoculation of 1×10^5 Cells [a]

Mouse	Cell Count, cells/mm³ ± *SD*	
	Electronic Cell Counter	Hemocytometer
1	130,160 ± 384.7	125,740 ± 1573
2	108,520 ± 645.8	113,520 ± 2570
3	118,880 ± 178.9	122,500 ± 1584
4	158,000 ± 1593.7	151,972 ± 972
5	108,920 ± 657.3	127,540 ± 1181
6	137,880 ± 1507.3	125,312 ± 2917

[a] Correlation coefficient = 0.943 based on the average of five individual determinations for each method ($n_1 = n_2 = 5$) and $p < 0.05$.

A difference in drug toxicity between tumor- and non-tumor-bearing animals is common (4, 12), although quantitating this difference often is difficult because the tumor may induce early death. In the higher dosage ranges, the mice died early from drug toxicity; at the lower dosages, the animals died from the tumor.

Histological determinations were conducted on leukemic mice (BDF_1) to note changes in the peripheral blood and ascitic fluid. The red blood cell and white blood cell counts from peripheral blood were comparable to those of the normal controls (average count of 5×10^6 and 1×10^4 cells/mm³, respectively) until about Day 5. After this time, the red blood cell counts decreased to an average of 3.9×10^6 cells/mm³. The volume of ascitic fluid in the abdominal cavity increased to ~2 ml by Day 7 or by the time of death. By Day 7, the ascitic fluid usually contained in excess of 1×10^5 leukemia cells (white blood cell)/mm³. In the normal mice, only a fraction of 1 ml of ascitic fluid having few red blood cells or white blood cells could be recovered. Autopsies were performed on normal and leukemic mice to compare the involved organs (liver, spleen, and lymph nodes). The livers became enlarged and lost the color associated with healthy organs. The lymph nodes and spleens also were enlarged. In advanced stages of leukemia, the spleens increased greatly in weight, thickness, and length.

The white blood cell counts in the antileukemic tests were obtained using both the hemocytometer and the electronic cell counter. A significant correlation between the cell counts obtained from the two methods is illustrated in Table V. The electronic cell-counting method proved to be more statistically precise, *i.e.*, smaller standard deviations. The increased speed of this method is important in that it reduces the time interval that the leukemia cells must be outside of an animal host during the transplant procedure. This maximizes the number of viable cells in the inoculum. Even though using the electronic cell counter proved to be reliable for the determination of white blood cell counts, it still was necessary to determine morphological structures and viability of the leukemia cells by microscopic examination.

The inhibitory effects on the growth rate of L-1210 leukemia cells (% T/C cell count) and the effects on survival (% T/C survival) following administration of cyclophosphamide and the fatty acid ester derivatives are summarized for the various doses in Table IV. The white blood cell counts of the ascitic fluid samples taken from the leukemic mice on Day 7 after inoculation gradually decreased when the dose was increased. A % T/C cell count ratio of <100% implies that the drug reduced the growth rate of leukemia cells.

The reduction of cell growth rates in leukemic mice receiving the fatty acid ester derivatives varied with the dose. All doses of the decanoate ester reduced the growth rate of leukemia cells. With the decanoate ester, the growth rate of leukemia cells when determined on the electronic cell counter was reduced from 57.1 (% T/C cell count) to 41.7% (Table IV) over a dosage range of 1.27–5.28 μmoles/kg, with the optimum reduction and survival occurring at 4.7 μmoles/kg. When the dose was decreased, the growth rate of leukemia cells increased (Table IV).

All doses of the hexanoate ester inhibited the growth rate of leukemia cells. The growth rate was reduced to 33.5% at 7.53 μmoles of the hexanoate ester/kg. For the hexanoate ester, the optimum cell growth inhibition was 47.4% and occurred at the optimal dose (117% T/C survival) of 3.01 μmoles/kg.

Decreased cell counts also were observed in the leukemic mice treated with the acetate ester. A 34.1% growth rate was obtained at 8.15 μmoles/kg. At the optimal level (% T/C survival) for the acetate ester of 5.44 μmoles/kg, the leukemia cell counts were depressed to 57.9%. All doses of the acetate ester inhibited the leukemic cell growth rate; however, this compound exhibited greater host toxicity.

Significant reductions in the leukemia cell populations were observed in the cyclophosphamide studies. The % T/C cell count value decreased to 2.0% (Table IV) at a dose of 685 μmoles/kg. Cyclophosphamide was included for comparison and for establishing further the reliability of the animal model in determining antileukemic activity of new agents.

Comparison of the maximum reductions in leukemia cell counts produced with cyclophosphamide and the fatty acid derivatives revealed that the hexanoate and acetate esters were effective at higher doses. At an optimal survival dose of the fatty acid esters, the decanoate ester showed a greater reduction of the leukemia cell growth rate than the hexanoate or acetate esters. The results of this study indicate that additional compounds in this series may possess antileukemic activity, and the trend suggests that a further increase in the fatty acid side chain could lead to greater reduction in toxicity and an increase in survival time.

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