mixture was evaporated to dryness, 40 mL of oxygen-free water and 2 g of Raney nickel catalyst was added to the residue, and the mixture was refluxed for 1.5 h. The catalyst was removed by filtration and evaporated in vacuo to a residue, which was chromatographed on a silica gel column. Elution with $CHCl_3$–MeOH–NH_3 (80:19:1) gave 110 mg (50%) of **10** as a pure solid: mp 188–190 °C; IR ν_{max} 1660 (C=O, amide); ^{1}H NMR (Me_2SO-d_6) δ 1.10 (t, 3 H, CH_2CH_3), 3.23 (m, 2 H, CH_2CH_3), 4.12 (d, 1 H, H-3′), 4.32 (s, 1 H, H-4′), 4.71 (m, 1 H, H-2′), 5.98 (d, J = 8.5 Hz, 1 H, H-1′), 6.48 (d, $J_{1,2}$ = 5.5 Hz, 1 H, H-1), 6.60 (s, 2 H, NH_2), 7.91 (d, $J_{2,1}$ = 5.5 Hz, 1 H, H-2), 8.30 (s, 1 H, H-8), 9.44 (m, 1 H, NH). Anal. ($C_{13}H_{17}N_5O_4$) C, H, N.

Biological Studies. Membrane Preparation. Membranes from rat brain, rat striatum, and bovine striatum were prepared as described.[13] Rat striatum was dissected according to Glowinski and Iversen.[16] Human platelet membranes were prepared by the method of Hoffman et al.[17] Rat fat cells were isolated as described by Honnor et al.,[18] and their membranes were prepared according to McKeel and Jarett.[19]

Adenylate Cyclase Assay. The activity of adenylate cyclase of the respective membranes was determined as previously described.[20]

Radioligand Binding Assays. The binding of [^{3}H]PIA to A_1 adenosine receptors of rat brain membranes was measured at 37 °C in the presence of adenosine deaminase as described.[13] The binding of [^{3}H]-5′-*N*-ethylamino)carbonyl]adenosine ([^{3}H]NECA) to the A_2 aenosine receptors of rat and bovine striatal membranes was measured at 37 °C according to Bruns et al.,[11] with the following modifications: the incubation volume was reduced to 250 μL containing 100 μg of protein; the A_1 receptor was saturated with 50 nM cyclopentyladenosine.

Data Analysis. Concentration–response curves containing at least eight different concentrations in duplicate were fitted by nonlinear regression to the Hill equation as described.[21] Binding data were analyzed by the curve-fitting program SCTFIT.[22] With this program, the simplest model (one site) was considered to be valid unless fitting with a more complex model resulted in a significantly better fit ($p < 0.01$, F test).

Platelet Aggregation Assay. Inhibition of platelet aggregation induced by ADP was determined as previously described.[15] IC_{50} values for each inhibitor were estimated from graphical plots of present inhibition vs log molar concentration (at least three to five different concentrations) and were defined as the concentrations that produce 50% inhibition of the aggregatory response to ADP. Data represent means and 95% confidence intervals of three independent determinations.

Acknowledgment. This work was supported by a grant (60%) from the Ministero Pubblica Istruzione. We thank M. Brandi and F. Lupidi for technical assistance and P. Boncagni for typing the manuscript.

Registry No. 1, 56707-85-8; **2a**, 113628-01-6; **26**, 113646-62-1; **2c**, 113628-11-8; **3a**, 113628-02-7; **36**, 113628-03-8; **3c**, 113628-04-9; **4**, 109151-84-0; **5**, 113628-05-0; **6**, 113646-63-2; **6** (acid chloride), 113628-06-1; **7**, 113628-07-2; **8**, 113628-08-3; **9**, 113628-09-4; **10**, 113628-10-7; (*R*)-$H_2NCH(CH_3)CH_2Ph$, 156-34-3; (c-C_5H_9)NH_2, 1003-03-8; (c-C_6H_{11})NH_2, 108-91-8; $EtNH_2$, 75-04-7; adenylate cyclase, 9012-42-4.

(16) Glowinski, J.; Iversen, L. L. *J. Neurochem.* **1966**, *13*, 655.
(17) Hoffman, B. B.; Michel, T.; Brenneman, T. B.; Lefkowitz, R. *J. Endocrinol.* **1982**, *110*, 926.
(18) Honnor, R. C.; Dhillon, G. S.; Londos, C. *J. Biol. Chem.* **1985**, *260*, 15122.
(19) McKeel, D. W.; Jarett, L. *J. Cell. Biol.* **1970**, *44*, 417.
(20) Klotz, K. N.; Cristalli, G.; Grifantini, M.; Vittori, S.; Lohse, M. J. *J. Biol. Chem.* **1985**, *260*, 14659.
(21) Lohse, M. J.; Klotz, K.-N.; Schwabe, U. *Mol. Pharmacol.* **1986**, *30*, 403.
(22) De Lean, A.; Hancock, A. A.; Lefkowitz, R. *J. Mol. Pharmacol.* **1982**, *21*, 5.

Synthetic Polyamine Analogues as Antineoplastics

Raymond J. Bergeron,*† Allen H. Neims,‡ James S. McManis,† Thomas R. Hawthorne,† John R. T. Vinson,† Rita Bortell,‡ and Michael J. Ingeno†

Department of Medicinal Chemistry, College of Pharmacy, and Deparment of Pharmacology, College of Medicine, University of Florida, Gainesville, Florida 32610. *Received September 2, 1987*

In this paper, we report on the synthesis and biological activity of a number of N-alkylated spermine compounds. The dialkylspermines N^1,N^{12}-dimethylspermine (DMSPM-2), N^1,N^{12}-diethylspermine (DESPM-3), and N^1,N^{12}-dipropylspermine (DPSPM-4) are all shown to inhibit the growth of L1210 cells in culture with IC_{50} values of less than 1 μM at 96 h. Furthermore, DESPM-3 is shown to be similarly active against Daudi and HL-60 cells in culture. A structure–activity relationship is shown to exist between the position at which spermine is alkylated and its antiproliferative properties. The activity of 10 μM DESPM-3 against L1210 cells was shown to be cytostatic, with greater than 90% cell viability by trypan blue exclusion, even after a 144-h exposure. When L1210 cells were treated with 10 μM DESPM-3 over a 144-h period, their size and mitochondrial DNA content were gradually but substantially diminished. However, flow cytometric measurements of the nuclear DNA content of these treated cells at 96 h indicated only slightly reduced S and G_2 populations and significant changes only after 144 h. A cloning assay performed on the cells after 96 h of exposure to this drug (10 μM) indicated that the cells were not growing. Finally, when male DBA/2 mice, inoculated with L1210 leukemia cells, were treated with DESPM-3, their life span was increased in excess of 200% relative to untreated controls. Moreover, many long-term survivors were apparently tumor free at the end of the experiment (60 days).

The role of polyamines in proliferative processes has received considerable attention in recent years.[1–5] In fact, interruption of the polyamine metabolic network has been at least partially successful in controlling the growth of cancer cells.[6,7] The antineoplastic drugs, α-(difluoromethyl)ornithine (DFMO) and methylglyoxal bis(guanylhydrazone) (MGBG), are both potent inhibitors of enzymes that are critical to polyamine biosynthesis. DFMO

†Department of Medicinal Chemistry.
‡Department of Pharmacology.

(1) Heby, O. *Differentation* **1981**, *19*, 1–20.
(2) Tabor, C. W.; Tabor, H. *Annu. Rev. Biochem.* **1984**, *53*, 749–790.
(3) Bachrach, U.; Kaye, A.; Chayen, R., Eds. *Advances in Polyamine Research*; Raven: New York, 1983; Vol. 4, p 808.
(4) Janne, J.; Poso, H.; Raine A. *Biochim, Biophys. Acta* **1978**, *473*, 241–293.

0022-2623/88/1831-1183$01.50/0 © 1988 American Chemical Society

is an ornithine decarboxylase inibitor[8] while MGBG inhibits spermine–spermidine acyl-transferase, the polyamine FAD-dependent oxidase system, and AdoMet decarboxylase among others.[9] Although it is not clear which of MGBG's roles is most important in inhibiting the growth of cancer cells, uptake of the drug by the polyamine transport apparatus seems critical. Unfortunately, the success of both DFMO and MGBG in a clinical setting has been somewhat marginal.[10] However, the fact that these agents do have anticancer activity, albeit limited, encouraged us and others to pursue the idea of impacting on polyamine metabolism as a rational approach to the control of proliferative processes.

In the past we have considered two approaches to the design of polyamine antiproliferatives: (1) utilization of the polyamine uptake apparatus as a means of delivering currently available antineoplastics and (2) the design and synthesis of polyamine analogues that will not only accumulate in the cell utilizing the polyamine transport system but also interfere with polyamine metabolism and/or function.

N^1,N^8-Diethylspermidine and a series of analogues previously designed and synthesized in this laboratory were found to be active against cultured L1210 cells.[11-14] These spermidine analogues were shown to decrease the intracellular concentrations of ornithine decarboxylase and of the polyamines spermidine and putrescine.[13-15] These results encouraged us to synthesize additional N-alkylated polyamines in order to establish structure–activity relationships both in vitro and in vivo and to further evaluate the mechanism by which the analogues operate. In this study we focus largely on a group of dialkylated analogues of spermine.

Results and Discussion

Synthesis of Polyamine Analogues. The polyamine analogues synthesized in this study include (1) the N^1,N^{12} terminally dialkylated spermines, DMSPM-**2**, DESPM-**3**, DPSPM-**4**, (2) the N^4,N^9 internally alkylated spermine, IDESPM-**6**, (3) the N^1,N^1,N^{12},N^{12} terminally tetraalkylated spermine, TESPM-**5**, (4) the N^1,N^4,N^9,N^{12}-tetraalkylated spermine, NTESPM-**7**, (5) the piperazine spermine analogue, DEPIP-**8**, (6) the hexaamine, YANK-**9**, and (7) N,N'-diethyl-1,12-diaminododecane, DEDAD-**1**. The N^1,N^{12} terminally alkylated spermine compounds **2–4** were synthesized by first monosulfonating all of the spermine nitrogens with *p*-toluenesulfonyl chloride, leaving only the terminal nitrogens to be alkylated. The resulting sulfonamides were next treated with sodium hydride in dimethylformamide followed by reaction with excess alkylating agent, e.g., methyl iodide, ethyl iodide, or *n*-propyl iodide. This results in terminally dialkylated sulfonamides. The sulfonyl protecting groups were next removed by treatment of the sulfonamides with sodium in liquid ammonia for 12 h. The final resulting amines were purified as the hydrochloride salts. The synthesis of IDESPM-**6** also involved an alkylation. The commercially available diamine N,N'-diethyl-1,4-diaminobutane was bis-cyanoethylated with acrylonitrile and the resulting bisnitrile, **15**, hydrogenated over a Raney nickel catalyst to the corresponding tetraamine, **6**.

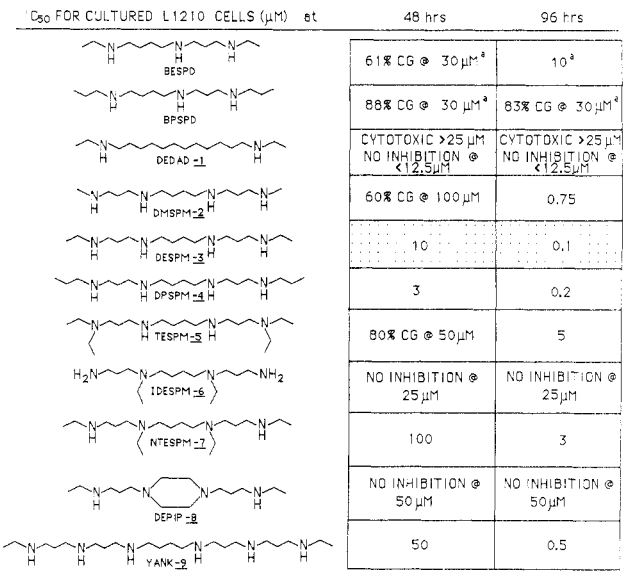


Figure 1. IC_{50} values (μM) of various polyamine analogues at 48 and 96 h for cultured L1210 cells. (a) Reference 12.

The preparation of the amines TESPM-**5** and DEPIP-**8** involved simple acylation reactions. For the synthesis of TESPM-**5**, 2 equiv of the amine N,N-diethyl-1,3-propanediamine was allowed to react with 1 equiv of succinyl chloride in methylene chloride in the presence of triethylamine. The resulting bisamide **10** was next reduced in tetrahydrofuran with lithium aluminum hydride to the corresponding terminally alkylated amine **5**. The last compound in the tetraamine series, DEPIP-**8**, also employed acylation as a key step in its synthesis. The diamine, 1,4-bis(3-aminopropyl)piperazine, was first acylated with acetyl chloride in methylene chloride in the presence of triethylamine followed by reduction of the resulting diamide **16**, with lithium aluminum hydride.

The synthesis of 1,20-bis(N-ethylamino)-4,8,13,17-tetraazaeicosane (YANK-**9**) began with the alkylation of N^1,N^4,N^9,N^{12}-tetrakis(*p*-tolylsulfonyl)spermine **11**. This sulfonamide was monoalkylated on each of the terminal nitrogens with N-ethyl-N-(3-chloropropyl)-*p*-toluenesulfonamide, **17**, in dimethylformamide in the presence of sodium hydride and potassium iodide. The resulting product, **18** was next exposed to sodium in liquid ammonia in order to remove the sulfonyl protecting groups. The product hexaamine, **9**, as with all of the other amines, was isolated as the hydrochloride salt. Finally, synthesis of the last of the amines, N,N'-diethyl-1,12-diaminododecane (DEDAD-1), involved a simple acetylation of 1,12-diaminododecane with acetic anhydride in pyridine, followed by reduction of the resulting diamide with diisobutylaluminum hydride to furnish the corresponding diamine 1.

In Vitro Structure–Activity Relationships. The compounds described in this study are all in some way related to the natural product spermine. They were designed to evaluate relationships that might exist between

(5) Luk, G. D.; Goodwin, G.; Marton, L. J.; Baylin, S. B. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 2355–2358.
(6) Seppanen, P.; Alhonen-Hongisto, L.; Janne, J. *Eur. J. Biochem.* **1980**, *110*, 7–12.
(7) Sjoerdsma, A.; Schechter, P. J. *Clin. Pharm. Ther.* **1984**, *35*, 287–300.
(8) Metcalf, B. W.; Bey, P.; Danzin, C.; Jung, M. J.; Casara, P.; Vevert J. P. *J. Am. Chem. Soc.* **1978**, *100*, 2551–2553.
(9) Pegg, A. E. *Biochem. J.* **1986**, *234*, 249–262.
(10) Janne, J.; Alhonen-Hongisto, L.; Seppanen, P.; Siimes, M. *Med. Biol.* **1981**, *59*. 448–457.
(11) Casero, R.; Bergeron, R. J.; Porter, C. W. *J. Cell. Physiol.* **1984**, *121*, 476–482.
(12) Porter, C. W.; Cavanaugh, P. F.; Stolowich, N.; Ganis, B., Kelly, E.; Bergeron, R. J. *Cancer Res.* **1985**, *45*, 2050–2057.
(13) Porter, C. W.; Ganis, B.; Vinson, T.; Marton, L. J.; Kramer, D.; Bergeron, R. J. *Cancer Res.* **1986**, *46*, 6279–6285.
(14) Porter, C. W.; McManis, J.; Casero, R.; Bergeron, R. J. *Cancer Res.* **1987**, *47*, 2821–2825.
(15) Porter, C. W.; Berger, F. G.; Pegg, A. E.; Ganis, B.; Bergeron, R. J. *Biochem. J.* **1987**, *242*, 433–440.

the structure of various alkylated spermines and their activity against tumor cells in culture. The results with L1210 cells clearly suggest that monoalkylation of both of the terminal primary nitrogens of spermine is important for antiproliferative activity (Figure 1). The polyamines N^1,N^{12}-dimethylspermine (DMSPM-2), N^1,N^{12}-diethylspermine (DESPM-3), and N^1,N^{12}-dipropylspermine (DPSPM-4) are all more active than either the N^1,N^1,N^{12},N^{12}-tetraalkylated spermine (TESPM-5) or the internally N^4,N^9-dialkylated spermine (IDESPM-6).

Although several of the analogues had 48 h 50% inhibitory concentrations (IC_{50} values) of less than 50 μM, the polyamine analogues generally took a significant period of time to exhibit their maximal effects on cell proliferation. We therefore selected the 96-h IC_{50} values for comparison and observed the activity order: spermine analogues > yank > spermidine analogues. The effect of the *N*-alkyl chain length on the activity of SPM analogues showed ethyl > propyl > methyl. Furthermore, when the two central nitrogens of spermine are replaced by methylenes, while each of the terminal nitrogens maintains its alkyl group (DEDAD-1), there is a substantial loss in activity against L1210 cells even though the distance between the terminal nitrogens is approximately maintained. When the distance between the two central nitrogens of DESPM-3 is shortened by incorporating the nitrogens in a 1,4 piperazine system, the compound's antiproliferative activity essentially disappears despite the fact that an analogue with internal tertiary nitrogen atoms, NTESPM-7, does retain discernible activity. Finally, and most interesting, is the observation related to the extension of the DESPM-3 backbone and the activity of the resulting hexaamine, YANK-9. The ability of this hexaamine to inhibit the growth of L1210 cells is very similar to that of DMSPM-2, DESPM-3, and DPSPM-4.

In comparing the spermine compounds with their spermidine counterparts (Figure 1), it is clear that the spermine compounds are substantially more active against L1210 cells. This is in keeping with the findings of Porter et al.[14] that DESPM-3 has a far greater impact on polyamine biosynthesis and the polyamine biosynthetic enzymes than the spermidine analogues. The spermine compounds reduce the level of intracellular polyamine pools as well as decreasing both ornithine decarboxylase and adenosylmethionine decarboxylase levels.

Biological Effects of DESPM-3 on Cultured Tumor Cells. Of the spermine analogues tested, DESPM-3 was the most effective inhibitor of growth of L1210 cells, having 48-h and 96-h IC_{50} values of 10 and 0.1 μM, respectively. Because of the this, it was decided to evaluate the in vitro activity of this amine against both Daudi cells and HL-60 cells and to measure its effect on cell size, nuclear DNA (nDNA) distribution and mitochondrial DNA (mtDNA) accumulation.

The activity of DESPM-3 against Daudi cells and HL-60 cells is comparable to that against L1210 cells. Because of the differences in doubling times between L1210 cells (ca. 10–12 h) and Daudi or HL-60 (ca. 25 h), the IC_{50} of DESPM-3 at 48 and 96 h against L1210 cells is best compared with the IC_{50} values at 72 and 144 h against HL-60 (10 and 0.3 μM) and Daudi cells (>40 and 0.5 μM).

L1210 cells grown in the presence of 10 or 1 μM DESPM-3 showed similar growth patterns (Figure 2). The inhibitory effects of DESPM-3 at these concentrations did not become apparent for approximately 48 h, and after 96 h there was little cell growth. Though cell division had nearly ceased by 144 h, more than 90% of the cells remain viable by trypan blue exclusion. However, the fraction of

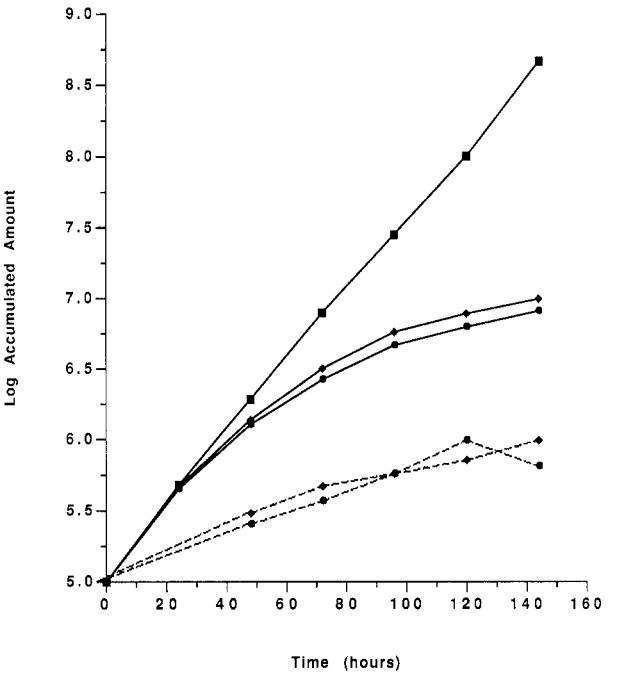


Figure 2. Kinetic study of accumulated L1210 cell growth (based on number of cell doublings) of untreated L1210 cells (■–■), cells treated with 10 μM DESPM-3 (●–●), and 1 μM DESPM-3 (♦–♦). The time course of mtDNA accumulation (accumulated cell counts × percent control mtDNA/cell) for untreated cells follows the untreated cell growth curve, while mtDNA accumulation for cells treated with 10 μM DESPM-3 (●--●) and 1 μM DESPM-3 (♦--♦) was consistent with lack of mtDNA synthesis coupled with cell division.

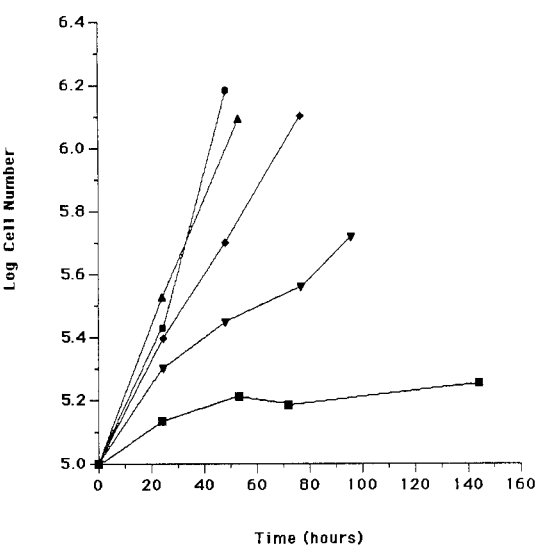


Figure 3. Growth curve of untreated L1210 cells (●) and cells treated with 10 μM DESPM-3 for 24 h (▲), 48 h (♦), 72 h (▼), and 96 h (■). Cells were washed and reseeded in fresh complete media at 0 h.

Table I. Percent of Control Clonagenic L1210 Cells after Treatment with 10 μM DESPM-3 for Up to 96 h

DESPM-3 10 μM treatment	% of clones relative to controls
24 h	100
48 h	75
72 h	21
96 h	2

clonogenic cells decreased significantly during the treatment (Table I).

The extent of treatment reversibility on cell growth upon removal of the drug depends on the duration of the treatment (Figure 3). The longer the cells are exposed

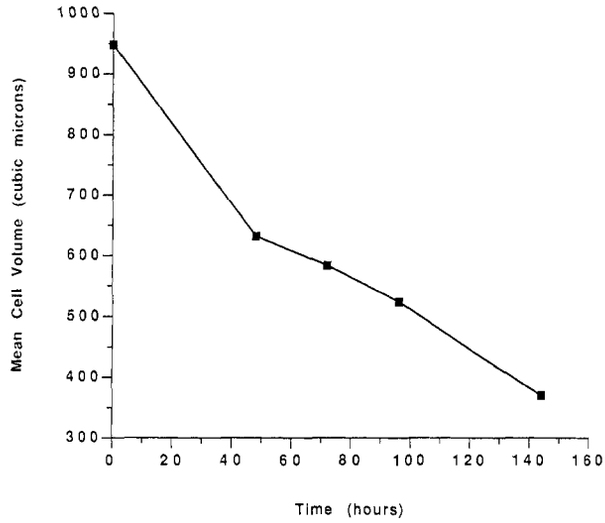


Figure 4. Time course for the effect of 10 μM DESPM-3 on the size (mean cell volume) of L1210 cells.

to DESPM-3, the slower they grew after transferring them into fresh media without drug. We also observed, during these studies, that cells substantially decreased in size while exposed to the drug. Utilizing electronic particle sizing, we determined that treatment of L1210 cells with 10 μM DESPM-3 resulted in a progressive decrease in cell diameter from 12.2 μm, for control L1210 cells, to 8.9 μm after exposure to the drug for 144 h. This corresponds to a decrease in cell volume of approximately 60% (948 to 369 μm^3). The time course of this phenomenon is presented in Figure 4.

Because of the unusual growth properties induced in the L1210 cells by DESPM-3, the nDNA histograms of the treated cells were evaluated. Flow cytometric analysis of nDNA content of L1210 cells after treatment with 10 μM DESPM-3 for 48 and 96 h showed only a slightly decreased S phase and G_2 phase cell populations compared with controls (Figure 5). This was not consistent with a decrease in cell growth caused by blocking progression of cells through any particular phase of the cell cycle. A significant number of cells accumulating at the G_1/S border was seen only after 144 h of treatment.

There was a striking decrease in mtDNA content per L1210 cell after treatment with DESPM-3 (Figure 6). This decrease in mtDNA content is compatible with an almost immediate cessation of mtDNA synthesis coupled with dilution due to cell division for three or four doublings. Control L1210 cells grew in log-linear fashion with a 10–12-h doubling time. Cells treated with DESPM-3 showed no significant growth inhibition at 24 h. By 48 h, L1210 cells treated with DESPM-3 contained about 20% of the normal amount of mtDNA and the rate of cell doublings had just begun to decrease. The mtDNA content of treated cells had decreased to about 10% of control by 96 h at which point cell doubling time nearly tripled (30–35 h). Cells treated with DESPM-3 remained constant at 10% of control mtDNA content up to 144 h at which time cell division had essentially ceased. Exposure of L1210 cells to 10 μM DESPM-3 rather than 1 μM DESPM-3 had little, if any, significant difference in effects on cell growth or mtDNA content, a finding consistent with the almost complete inhibition of mtDNA accumulation caused by the lower concentration of drug. The relationship between cell doubling and mtDNA content, seen in Figure 2, is consistent with a mechanism in which DESPM-3 inhibition of mtDNA replication causes a depletion in mtDNA content with cell division until a

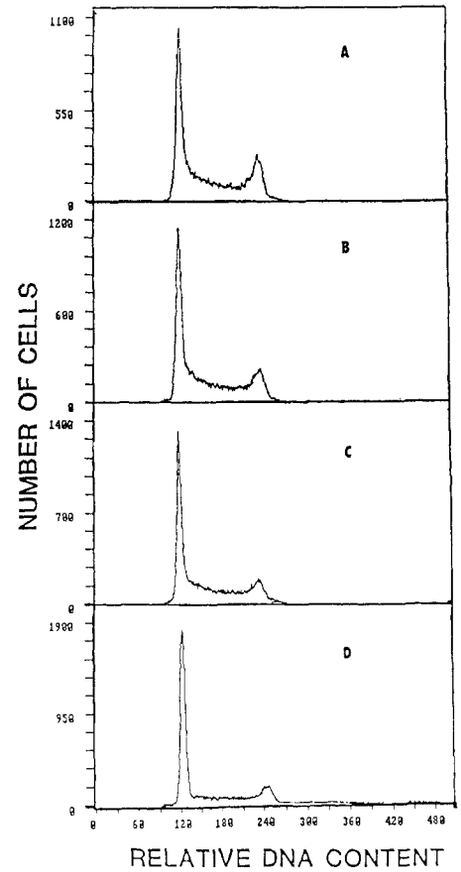


Figure 5. Flow cytometric analysis of nDNA content for control L1210 cells (A) and cells treated with 10 μM DESPM-3 for 48 h (B), 96 h (C), and 144 h (D).

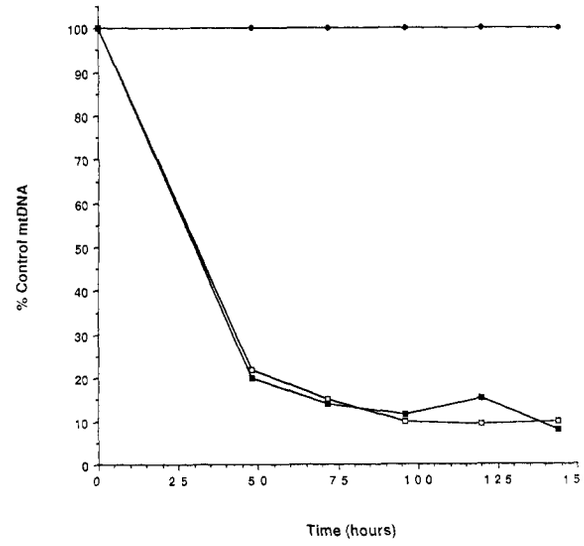


Figure 6. Comparison of the time course for the effect of 1 μM (□) and 10 μM (■) DESPM-3 on percent of control mtDNA in L1210 cells. The amount of mtDNA in untreated cells (♦) was assumed 100% at each time point.

quantity insufficient to support continued cell growth remains. It is possible that secondary effects on energy production contribute to the decrease in cell size. However, it is clear that the impact of these drugs on mitochondria demands a study of their effects on the growth of multidrug-resistant cells, which often employ highly energy dependent efflux mechanisms, e.g., the P170 glycoprotein, as well as their effects in combination with other drugs that act on the mitochrondria. We are currently investigating both of these areas.

Table II. Activity of DESPM-3 against Intraperitoneally Implanted L1210 Leukemia in DBA/2 Mice

test no.	dose[a] mg/kg per injection	schedule		mean survival[b] ± SD, days	%ILS	S/T[c]
1[d]	control			9.7 ± 0.7		0/47
2[d]	5	Q8H	days 1–6	12.3 ± 2.0	25	0/8
3[d]	15	Q12H	days 1–6	14.9 ± 1.2	55	0/6
4[d]	10	Q8H	days 1–6	35.3 ± 12.0	264	0/8
5[d]	15	Q8H	days 1–6	46.6 ± 17.6	376	9/16
6[d]	17.5	Q8H	days 1–6	52.3 ± 11.0	439	4/8
7[e]	control			8.9 ± 0.6		0/13
8[e]	20	Q8H	days 1–3	14.0 ± 0.4	57	0/4
9[e]	20	Q8H	days 1–4	18.1 ± 4.9	103	0/10

[a] Drug administered by ip injection. Control mice given no treatment. [b] Experiment ended and animal survival evaluated on day 60. Mice alive at this time had no sign of tumor. [c] The number of mice that survived until day 60 was recorded and expressed as a ratio of survivors to the total in the test group (S/T). [d] Mice injected with 10^5 L1210 cells i.p. on day 0. [e] Mice injected with 10^6 L1210 cells ip on day 0.

Similar results have been obtained in studies of the action of MGBG on L1210 cells in vitro.[16] The important difference between DESPM-3 and MGBG in this regard was that L1210 cells treated with MGBG began to reaccumulate mtDNA after the growth rate decreased. Indeed, when the mtDNA had reaccumulated to nearly the control value, cell growth also recovered. This resistance to MGBG was not inherited, but reflected a marked decrease in the intracellular content of MGBG (for given concentration of drug in the incubation medium). We have observed no such phenomenon with DESPM-3.

The addition of 100 μM aminoguanidine, an amine oxidase inhibitor, to DESPM-3-treated cells had no significant effects on cell growth or mtDNA content.

In Vivo Activity of DESPM-3 against L1210 Cells in DBA/2 Mice. The spermine analogue DESPM-3 was screened for its ability to inhibit the growth of L1210 ascites tumor growth in DBA/2 mice and thus to increase their life span. DESPM-3 is a highly charged, very hydrophilic compound. Since it is expected to be excreted quickly and efficiently, we decided to give a split dosing schedule over several days. Test animals were injected with either 10^6 or 10^5 L1210 cells on day 0 and treatment was initiated 24 h later. The results (Table II) clearly demonstrated a dose–response relationship, with ip administration of 15 mg/kg every 8 h for 6 days having the optimum effect of the schedules tested. At this dose animals had an average increase in life span (ILS) of greater than 376%. At the writing of this text, 60% of the test animals were still alive at 60 days, with no sign of tumor. Although the 17.5 mg/kg dosing schedule gave a greater percentage of long-term survivors, there was some drug toxicity as indicated by a number of early deaths. Also, after the sixth day of dosing, the 17.5 mg/kg animals appeared to be rather gaunt with an average weight loss of greater than 10%. The animals that recovered, however, appeared normal 4–5 days after the drug treatment was completed. In going from a dose of 5 mg/kg every 8 h for 6 days (a total dose of 90 mg/kg) to 15 mg/kg every 8 h for 6 days (a total dose of 270 mg/kg), we observed an increase from 25% to 376% in life span. Recall that the drug dose was only increased three times while the life span increased at least 15 times.

The dosing schedule is critical. When the animals received 20 mg/kg every 8 h for 4 days (a total dose of 240 mg/kg) there was a 90% ILS, while those treated with 10 mg/kg every 8 h for 6 days (a total dose of 180 mg/kg) exhibited a 262% ILS. This is in keeping with our observations in cell culture. Irreversibility of cell-growth inhibition in culture required that the cells be exposed to the drug for a minimum of 96 h, suggesting that in our short-term dosing animal experiments the cells were not exposed to the drug long enough to irreversibly inhibit their proliferation.

(16) Bortell, R.; Raynor, L. O.; Neims, A. H. *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **1987**, *46*, 999 (detailed manuscript in preparation).

Conclusion

The terminally bisalkylated spermines evaluated in this study were highly effective in controlling the growth of rapidly proliferating cells. DESPM-3 was the most potent compound with regard to inhibition of L1210 cells; it was also active against the human tumor lines, Daudi and HL-60 cells, in vitro with 96-h or 144-h IC_{50} values of less than 1 μM in all cases. Furthermore, this compound was highly effective in extending the life span of DBA/2 mice previously inoculated with L1210 cells; 60% of the treated animals were long-term survivors. The precise mechanism by which these compounds operate still remains somewhat obscure. Although the cells are viable after a 96-h exposure to 10 μM DESPM-3, as determined by trypan blue exclusion, and their nDNA histograms were essentially the same as control, a cloning assay indicated that the cells would not grow. The cells decreased in size substantially, a decrease that continued with exposure time. On exposure to DESPM-3, mtDNA was depleted. The decrease was consistent with a lack of synthesis coupled with continued cell division in the first few days after treatment. These results suggest that although the cells are alive, they simply do not have sufficient energy to support normal division, although other explanations are certainly feasible. The striking effects of the polyamine analogues on the mitochrondia of the L1210 cells may be independent of depletion of intracellular polyamine pools and polyamine biosynthetic enzymes, and other mechanisms are currently under investigation.

Experimental Section

Cell culture materials and proteinase K were obtained from Sigma Chemical Co. *N,N'*-Diethyl-1,4-butanediamine was purchased from Alfa Products Division, Morton, Thiokol, Inc. RNase T1 was obtained from BRL Scientific, Gaithersburg, MD. All other organic reagents and solvents were purchased from Aldrich Chemical Co. Sodium sulfate was employed as a drying agent, and solvents were routinely distilled. The stationary phase used for column chromatography was silica gel 60 (70–230 mesh) obtained from EM Science (Darmstadt, West Germany). Proton NMR spectra were recorded on a Varian EM-390 or a Nicolet NT-300 instrument. Unless otherwise noted, the samples were run in $CDCl_3$ with the chemical shifts given in parts per million downfield from a tetramethylsilane internal standard. The chemical shifts for samples run in D_2O are reported with either HOD (δ 4.80) or 3-(trimethylsilyl)propanesulfonic acid, sodium salt (δ 0.00) as standards. Mass spectra were carried out on a Kratos MS 80 instrument. Elemental analyses were performed by Atlantic Microlabs, Inc., Atlanta, GA.

***N,N'*-Bis[3-(diethylamino)propyl]succinamide (10).** Succinyl chloride (2.0 g, 12.9 mmol) in 50 mL of CH_2Cl_2 was added

to a stirred solution of *N,N'*-diethyl-1,3-propanediamine (5.0 g, 38.7 mmol) and triethylamine (2.9 g, 28.4 mmol) in 100 mL of anhydrous CH_2Cl_2 at 4 °C under N_2. The solution was allowed to warm to room temperature and stirred for 12 h. The reaction solution was washed with 5% NaOH (3 × 100 mL), and the washes were back-extracted with $CHCl_3$ (2 × 100 mL). The organic extracts were dried and concentrated to a brown oil, which was purified by silica gel (500 g, 5% concentrated NH_4OH/MeOH) to yield **10** as a yellow solid (1.5 g, 34% yield): NMR ($CDCl_3$) δ 1.1–1.3 (t, 12 H), 1.6–2.0 (m, 4 H), 2.5 (s, 4 H), 2.6–3.0 (m, 12 H), 3.2–3.5 (q, 4 H). Anal. ($C_{18}H_{38}N_4O_2$) C, H, N.

N^1,N^1,N^{12},N^{12}-Tetraethylspermine (TESPM-5). Compound **10** (1.5 g, 4.4 mmol) was dissolved in anhydrous, freshly distilled THF (100 mL) and the solution cooled to 4 °C. Lithium aluminum hydride (LAH; 1.0 g, 26.4 mmol) was added very slowly under N_2. The reaction mixture was allowed to warm to room temperature and then refluxed for 36 h. The reaction mixture was cooled to 4 °C followed by the cautious addition of water (1.0 mL), 15% NaOH (1.0 mL), and water (3.0 mL). The mixture was filtered, concentrated, and partitioned between $CHCl_3$ (150 mL) and 15% NaOH (40 mL). The layers were separated, and the aqueous layer was further extracted with $CHCl_3$ (3 × 100 mL). The organic extracts were evaporated to give a brown oil (1.2 g), which was purified on silica gel (100 g, 40% concentrated NH_4OH/MeOH), to yield 0.64 g of pure amine. The crystalline tetrahydrochloride salt was formed by the addition of concentrated hydrochloric acid (2 mL) to a solution (EtOH, 10 mL) of the above product and recrystallized from aqueous ethanol to afford 0.84 g of **5** (42% yield): NMR (D_2O) δ 1.2–1.4 (t, 12 H), 1.8–2.4 (m, 8 H), 3.0–3.4 (m, 20 H). Anal. ($C_{18}H_{46}Cl_4N_4 \cdot 2H_2O$) C, H, N.

N^1,N^4,N^9,N^{12}-Tetratosylspermine (11). *p*-Toluenesulfonyl chloride (9.98 g, 52.3 mmol) in dry CH_2Cl_2 (200 mL) was added dropwise with rapid stirring to spermine tetrahydrochloride (4.53 g, 13.0 mmol) and 10% aqueous NaOH (200 mL, 0.132 mol) at 0 °C. After 1 h the mixture was allowed to warm to room temperature and stirred 2 days. The organic phase was separated and washed with 0.5 N HCl (100 mL), H_2O (100 mL), and brine (50 mL), dried, and concentrated to afford 10.48 g of a glass, which was purified on silica gel (450 g, 3% MeOH/$CHCl_3$) to give 9.69 g (91% yield) of **11**: NMR δ 1.4–1.9 (m, 8 H), 2.4 (s, 12 H), 2.8–3.3 (m, 12 H), 5.4 (t, 2 H), 7.2–7.4 (m, 8 H), 7.6–7.8 (m, 8 H). Anal. ($C_{38}H_{50}N_4O_8S_4$) C, H, N.

N^1,N^{12}-Diethyl-N^1,N^4,N^9,N^{12}-tetratosylspermine (12). Sodium hydride (0.86 g of an 80% mineral oil suspension, 0.028 mol) was added to a solution of **11** (5.84 g, 7.13 mmol) in dry DMF (100 mL) at 0 °C. After the evolution of gas subsided, ethyl iodide (3.4 mL, 42.8 mmol) was added and the solution stirred for 1 h. The reaction apparatus was next fitted with a reflux condenser and heated (70–80 °C) for 12 h. Water (100 mL) and $CHCl_3$ (150 mL) were added, and the mixture was stirred. The layers were separated, the aqueous phase was further extracted with $CHCl_3$ (3 × 75 mL), and the combined organic extracts were washed with 2% aqueous Na_2SO_3 (3 × 75 mL), water (50 mL), and brine (50 mL). The extracts were concentrated in vacuo to give the crude product, which was purified by silica gel chromatography (350 g, 2% EtOH/$CHCl_3$) to yield 4.68 g (75% yield) of **12**: NMR δ 1.1 (t, 6 H), 1.5–2.1 (m, 8 H), 2.4 (s, 12 H), 3.0–3.3 (m, 16 H), 7.2–7.5 (m, 8 H), 7.6–7.8 (d, 8 H). Anal. ($C_{42}H_{58}N_4O_8S_4$) C, H, N.

N^1,N^{12}-Diethylspermine (DESPM-3). A solution of **12** (2.78 g, 3.18 mmol) in dry, distilled THF (200 mL) was cooled to −78 °C and liquid NH_3 (300 mL) was condensed in the flask with a dry ice condenser. Sodium spheres (3.0 g, 0.13 mol) were then added in portions, and the reaction was stirred at −78 °C for 4 h. Next the cold bath was taken away from the reaction flask while the dry ice condenser was kept cold for ca. 3 h. Then the NH_3 was allowed to boil off under a stream of N_2. Ethanol (40 mL) was then cautiously added dropwise under N_2 and then H_2O (20 mL) was added to fully quench the reaction. After most of the organic solvent was removed, the aqueous layer was extracted with Et_2O (4 × 50 mL) and then $CHCl_3$ (4 × 50 mL). The extracts were combined and the solvent was removed. Kugelrohr distillation (up to 230 °C, high vacuum) of the residue gave the free amine, which was dissolved in 1:1 EtOH/Et_2O and treated with 10% excess concentrated HCl. After solvent evaporation, the tetrahydrochloride salt was purified by recrystallization (aqueous EtOH) to give 0.79 g (61% yield) of **3**: NMR (D_2O) δ 1.4 (t, 6 H), 1.9 (m, 4 H), 2.25 (m, 4 H), 3.25 (m, 16 H). Anal. ($C_{14}H_{38}Cl_4N_4$) C, H, N.

N^1,N^{12}-Dimethyl-N^1,N^4,N^9,N^{12}-Tetratosylspermine (13). Compound **11** (1.5 g, 1.8 mmol) was alkylated with methyl iodide by using the general procedure for the preparation and purification of **12** to give 1.23 g of **13** (79% yield): NMR δ 1.4–2.0 (m, 8 H), 2.4 (s, 12 H), 2.7 (s, 6 H), 2.9–3.3 (m, 12 H), 7.3 (d, 8 H), 7.7 (dd, 8 H). Anal. ($C_{40}H_{54}N_4O_8S_4$) C, H, N.

N^1,N^{12}-Dimethylspermine (DMSPM-2). Compound **13** (1.23 g, 1.45 mmol) was detosylated by the general procedure for the synthesis and purification of **3**, to give 151.2 mg of **2** as the tetrahydrochloride salt (28% yield): NMR (300 MHz, D_2O) δ 1.8 (br s, 4 H), 2.1 (m, 4 H), 2.75 (s, 6 H), 3.1 (m, 12 H). Anal. ($C_{12}H_{34}Cl_4N_4$) C, H, N.

N^1,N^{12}-Dipropyl-N^1,N^4,N^9,N^{12}-Tetratosylspermine (14). Compound **11** (1.5 g, 1.8 mmol) was alkylated with *n*-propyl iodide by using the general procedure for the preparation and purification of **12** to give 1.53 g of **14** (93% yield): NMR (300 MHz) δ 0.85 (t, 6 H), 1.4–1.6 (m, 8 H), 1.8–1.9 (m, 4 H), 2.4 (s, 12 H), 3.0–3.2 (m, 16 H). Anal. ($C_{44}H_{62}N_4O_8S_4$) C, H, N.

N^1,N^{12}-Dipropylspermine (DPSPM-4). Compound **14** (1.5 g, 1.7 mmol) was detosylated by using the general procedure for the synthesis and purification of **3** to give 0.36 g of **4** (49% yield) as the tetrahydrochloride salt: NMR (300 MHz, D_2O) δ 1.0 (t, 6 H), 1.7 (dd, 4 H), 1.8 (br s, 6 H), 3.1 (m, 16 H). Anal. ($C_{16}H_{42}Cl_4N_4$), C, H, N.

***N,N'*-Diethyl-*N,N'*-bis(β-cyanoethyl)-1,4-butanediamine (15).** Acrylonitrile (8 g, 0.15 mol) was added dropwise to *N,N'*-diethyl-1,4-butanediamine (9.4 g, 70 mmol) with stirring at 0 °C. The solution was allowed to warm to room temperature and to stir for 48 h. The product was purified by column chromatography (450 g of silica gel, 5% $CHCl_3$/MeOH) to give 16.63 g of **15** (95% yield): NMR δ 1.0 (t, 6 H), 1.5 (m, 4 H), 2.4–2.9 (m, 16 H). Anal. ($C_{14}H_{26}N_4$) C, H, N.

N^4,N^9-Diethylspermine (IDESPM-6). Compound **15** (16.28 g, 65.0 mmol) was hydrogenated with Raney nickel (4 g) in a solution of ethanol (250 mL), NaOH (10 g), and H_2O (13 mL). After the catalyst was filtered and washed with EtOH, the bulk of the organic solvent was removed. Next 10% NaOH (100 mL) was added to the concentrate, which was extracted with $CHCl_3$ (5 × 50 mL). The organic extracts were concentrated to give a crude product, which was purified by column chromatography (500 g of silica gel, 30% concentrated NH_4OH/MeOH) to give **6** as the free amine (4.8 g, 29% yield). The tetrahydrochloride salt was formed by adding a 20% excess of concentrated HCl to the free amine in EtOH/Et_2O (1:1). After solvent removal, the salt was recrystallized from aqueous ethanol to give 1.36 g of **6** (5% yield) as a hygroscopic product: NMR (D_2O δ 1.3 (t, 6 H), 1.7–2.0 (m, 4 H), 2.0–2.4 (m, 4 H), 3.1–3.5 (m, 16 H). Anal. ($C_{14}H_{38}Cl_4N_4$) C, H, N.

1,4-Bis(3-acetamidopropyl)piperazine (16). A solution of acetyl chloride (19.5 mL, 0.274 mol) in dry CH_2Cl_2 (50 mL) was added dropwise to a solution of 1,4-bis(3-aminopropyl)piperazine (25.0 g, 0.125 mol) and triethylamine (27.8 g, 0.274 mol) in CH_2Cl_2 (100 mL) at 0 °C and the solution allowed to slowly warm to room temperature and to stir for 24 h. Water (50 mL) was added and saturated NaOH added until the pH was >12. The layers were separated, and the aqueous phase was extracted with $CHCl_3$ (4 × 50 mL). The combined $CHCl_3$ extracts were evaporated to give an oil, which was purified by column chromatography (230 g of silica gel, 1% concentrated NH_4OH/MeOH) to give 7.48 g (22% yield) of crystalline product, **16**: mp 136–137 °C; NMR δ 1.6–1.8 (m, 4 H), 1.95 (s, 6 H), 2.4–2.6 (m, 12 H), 3.3–3.4 (m, 4 H), 7.0 (br s, 2 H); CIMS calcd for $C_{14}H_{28}N_4O_2$ 284.221, found 284.218 (M^+).

1,4-Bis[3-(ethylamino)propyl]piperazine (DEPIP-8). Intermediate **16** (6.1 g, 21 mmol) was dissolved in dry THF (50 mL) and LAH (9.7 g, 0.24 mol) was added in portions to the solution cooled to 0 °C. After removal of the ice bath, the mixture was stirred for 4 h and then refluxed for 6 h. The mixture was cooled to 0 °C and quenched by careful addition of water (10 mL), 15% NaOH (10 mL), and water (30 mL). After being stirred for 1 h, the mixture was concentrated on the rotovap and then Et_2O was added. The inorganic salts were filtered and exhaustively washed with Et_2O, and the filtrate was evaporated to yield a yellow oil. Kugelrohr distillation (up to 230 °C, high vacuum) of the crude

oil gave **8** as the free tetraamine: NMR δ 1.1 (t, 6 H), 1.5–2.0 (m, 6 H), 2.3–2.8 (m, 20 H). Amine **8** was dissolved in a 1:1 solution of EtOH/Et_2O and a 20% excess of concentrated HCl added. After solvent evaporation, the tetrahydrochloride salt was recrystallized from aqueous ethanol to give 0.74 g of pure **8**. Anal. ($C_{14}H_{36}Cl_4N_4$) C, H, N.

N^1,N^4,N^9,N^{12}-Tetraethylspermine (NTESPM-7). Compound **7** was synthesized from spermine according to the literature procedure.[17]

1,12-Bis(*N*-ethylamino)dodecane (DEDAD-1). Compound **1** was synthesized by modification of a known procedure.[18] 1,12-Diacetamidododecane (1.29 g, 4.54 mmol) was placed in a dry flask equipped with a condenser (N_2 inlet), and the flask and contents were cooled to 0 °C. Diisobutylaluminum hydride (1.0 M in toluene, 30 mL, 30 mmol) was added cautiously by syringe. The solution was heated at 60 °C for <9 h, then at 85 °C for 1 day. Brine (1.0 mL) was added to the reaction at 0 °C, and then the mixture was added carefully with stirring to 6 N HCl (20 mL) and ice, resulting in vigorous gas evolution. The suspension was basified with 10% NaOH (60 mL) and then extracted with ether (3 × 100 mL). After a brine wash (50 mL), the organic layer was concentrated to give 1.18 g of oil, which was chromatographed on silica gel (70 g, 10% concentrated NH_4OH/CH_3OH) to give 0.76 g of **1** as the free diamine. The product was dissolved in ethanol (100 mL) and acidified with concentrated HCl (1 mL) and the solvent stripped off. Recrystallization from absolute ethanol afforded 0.873 g of **1** (58% yield): NMR (D_2O) δ 1.29–1.97 (m, 26 H), 3.03–3.30 (m, 8 H). Anal. ($C_{16}H_{38}Cl_2N_2$) C, H, N.

***N*-Ethyl-*N*-(3-chloropropyl)-*p*-toluenesulfonamide (17).** Sodium hydride (80% in oil, 0.51 g, 17 mmol) was added to *N*-ethyl-*p*-toluenesulfonamide (2.50 g, 12.6 mmol) in dry DMF (36 mL). After gas evolution subsided (20 min), 1,3-dichloropropane (7.0 mL, 73.7 mmol) was added by syringe. The mixture was heated at 57 °C for 16 h and then cooled and poured into ice water (200 mL), which was extracted with ether (2 × 200 mL). The combined extracts were washed with water (4 × 100 mL) and brine (100 mL). Removal of solvent by rotary evaporation and then under high vacuum gave 3.49 g crude product, which was chromatographed on silica gel (225 g, 30% hexane/$CHCl_3$) to furnish 2.78 g of **17** as an oil (80% yield): NMR δ 1.15 (t, 3 H), 1.9–2.2 (m, 2 H), 2.42 (s, 3 H), 3.12–3.35 (m, 4 H), 3.6 (t, 2 H), 7.3–7.8 (dd, 4 H); FABMS calcd for $C_{12}H_{18}ClNO_2S$ 275, found 276 (M + 1, base).

3,7,11,16,20,24-Hexakis(*p*-tolylsulfonyl)-3,7,11,16,20,24-hexaazahexacosane (18). Sodium hydride (80% in oil, 0.21 g, 7.0 mmol) and potassium iodide (53 mg, 0.32 mmol) were added to **11** (1.82 g, 2.22 mmol) in dry DMF (10 mL). After 30 min, **17** (2.9 g, 10.5 mmol) in DMF (10 mL) was introduced and the mixture stirred for 20 h at room temperature and then heated at 40–50 °C for 2 h. The cooled mixture was poured into ice-cold 5% NaOH (100 mL), which was extracted with $CHCl_3$ (3 × 50 mL). A water wash (100 mL) and then solvent removal gave 4.69 g of crude product. Silica gel chromatography (400 g, 1% EtOH/$CHCl_3$) yielded 1.73 g of **18** (60% yield): NMR δ 1.08 (t, 6 H), 1.45–2.10 (m, 12 H), 2.34 (s, 18 H), 2.96–3.37 (m, 24 H), 7.2–7.8 (m, 24 H); FABMS calcd for $C_{62}H_{84}N_6O_{12}S_6$ 1296, found 1297 (M + 1).

1,20-Bis(*N*-ethylamino)-4,8,13,17-tetraazaeicosane (YANK-9). A solution of **18** (0.79 g, 0.61 mmol) in distilled THF (45 mL) was added to a dry 500-mL three-necked flask, equipped with a dry ice condenser. The solution was cooled to about −40 °C under N_2, and ammonia gas (200 mL), after passage through NaOH, was condensed into the reaction flask. Sodium spheres (0.99 g, 43 mmol), which had been rinsed twice in hexane and cut in half, were added cautiously. After the cold temperature was maintained for 4–5 h, ammonia was allowed to evaporate under a stream of N_2. Excess absolute ethanol (50 mL) was carefully added to the residue at 0 °C, and the mixture was concentrated. Next 10% NaOH (15 mL) was added, and extraction with $CHCl_3$ (10 × 20 mL), while the aqueous layer was salted out, drying, and evaporation of the organic phase gave crude free amine (0.31 g). Kugelrohr distillation (up to 169 °C/0.005 mm) provided 0.216 g of **9** as the free hexaamine, which was dissolved in ethanol (100 mL) and treated with 0.5 mL of concentrated HCl. After solvent removal, the solid was recrystallized from 17% aqueous ethanol (120 mL) and washed with cold, absolute EtOH (2 × 3 mL) to afford 0.131 g of **9** for a 36% yield: NMR (300 MHz, D_2O) δ 1.31 (t, 6 H), 1.74–1.84 (m, 4 H), 2.05–2.19 (m, 8 H), 3.07–3.25 (m, 24 H). Anal. ($C_{20}H_{54}Cl_6N_6$) C, H, N.

Cell Culture. Murine L1210 leukemia cells, human Burkitt lymphoma cells (Daudi), and promyelocytic leukemia cells (HL-60) were maintained in logarithmic growth as suspension cultures in RPMI-1640 medium containing 2% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid–3-morpholinopropanesulfonic acid and 10% fetal bovine serum. Cells were grown in 25 cm^2 tissue culture flasks (Corning) in a total volume of 10 mL under a humidified 5% CO_2 atmosphere at 37 °C.

IC_{50} Determinations. The cells were treated while in logarithmic growth (L1210 cells, 3×10^4 cells/mL; Daudi and HL-60, 10^5 cells/mL) with the polyamine derivatives diluted in sterile water and filtered through a 0.2-μm filter immediately prior to use. Following a 48-h incubation with L1210 cells and a 72-h incubation with Daudi or HL-60 cells, cells were reseeded (L1210 cells, 3×10^4 cells/mL; Daudi and HL-60 cells, 1×10^5 cells/mL) and incubated in the presence of the polyamine derivative for an additional 48 or 72 h, respectively.

Cell samples were removed at the indicated time periods for counting. Cell number was determined by electronic particle analysis (Coulter Counter, Model ZB1, Coulter Electronics, Hialeah, FL) and confirmed periodically with hemocytometer measurements. Cell viability was assessed by trypan blue dye exclusion.

The percentage of control growth was determined as follows:

$$\% \text{ of control growth} = \left(\frac{\text{final treated cell no.} - \text{initial inoculum}}{\text{final untreated cell no.} - \text{initial inoculum}}\right) \times 100$$

The IC_{50} is defined as the concentration of compound necessary to reduce cell growth to 50% of control growth after defined intervals of exposure. The IC_{50} values against L1210 cells for the most active analogues ($IC_{50} < 1$ μM at 96 h) were also measured in the presence of 100 μM aminoguanidine, a polyamine oxidase inhibitor.

Animal Studies. The murine L1210 leukemia cells were maintained in male DBA/2J mice. Cells, from a single mouse which was injected intraperitoneally (ip) with 2.5×10^4 cells 7 days earlier, were harvested and diluted with cold saline so that an inoculum of 10^5 or 10^6 cells could be administered by a 0.25 mL ip injection. In each study, mice were injected ip with the appropriate number of L1210 cells on day 0. The polyamine analogue was diluted in sterile saline within 24 h of use and the unused portion stored at 5 °C.

DESPM-**3** was administered by ip injection according to the appropriate dosing schedule. Concentrations of DESPM-**3** at each dose were adjusted so that the mice were injected with a volume of 1 mL/100 g (i.e., a 25-g mouse was injected with 0.25 mL of drug solution). Untreated mice served as controls.

The parameter used for treatment evaluation was mean survival time (percent increased life span, %ILS):

$$\%\text{ILS} = [(\text{mean survival time treated animals} - \text{mean survival time controls})/\text{mean survival time controls}] \times 100$$

Analysis of Drug Effects on mtDNA. L1210 cells (in complete media at 10^5 cells/mL) were incubated at 37 °C in the presence of 1 or 10 μM DESPM-**3**. Every 24 h, for 144 h, cell samples were removed for counting, and the cells were reseeded in fresh medium and drug at 1×10^5 cells/mL. Cells were assayed daily for mtDNA content.

Because recovery of the organelles might vary with drug treatment, we have recently developed a dot-blot procedure for assay of mtDNA that involves analysis of cell lysates rather than preparations of mitochondria.[19] Cells [(5×10^4 to 2×10^5)/mL] were lysed in 2% SDS in the presence of proteinase K (5 μg) and

(17) Schopp, E.; Hesse, M. *Helv. Chim. Acta* **1973**, *56*, 124–139.
(18) Kuehne, H.; Hesse, M. *Helv. Chim. Acta* **1982**, *65*, 1470–1481.
(19) Raynor, L. O.; Bortell, R.; Neims, A. H. *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **1987**, *46*, 1001 (detailed manuscript in preparation).

RNase T1 (100 units). The total cell lysate was applied to nitrocellulose paper with use of a 96-well, vacuum operated dot-blot apparatus. The blots were hybridized to a ^{35}S-labeled d-ATP nick translated probe made by inserting full-length mouse mtDNA into pSP64 vector at the SacI site. The vector itself was used to generate a standard curve for each experiment. Dot blots were visualized by autoradiography and cut out, and radioactivity was determined by scintillation counting.

Flow Cytometric Analysis. Flow cytometric analysis of nuclear DNA content was performed with the RATCOM flow cytometer (RATCOM Inc., Miami, FL) interfaced with a microcomputer (IBM-XT). Cultured L1210 cells in log growth were incubated at 37 °C with 10 μM DESPM-3 and samples removed at 0, 48, 96, and 144 h were analyzed for nuclear DNA content distributions after being stained with diamidinophenylindole in a nuclear isolation media (NIM-DAPI).[20]

Cloning Assay. L1210 cells maintained between 10^5 and 10^6 cells/mL were incubated with 10 μM DESPM-3 for 96 h. At 24-h intervals, treated cells were washed (2 × 10 mL) and diluted in fresh complete media and plated in triplicate 96-well microtiter plates at 0.4 cell/well with each well containing 100 μL of sample. The plates were incubated at 37 °C in a humidified incubator in an atmosphere of 5% CO_2 and 95% air. The plates were examined with an inverse-phase microscope at 100× magnification. The final number of colonies per plate was quantitated 7 days after plating. Groups of 50 or more cells/well were identified as having been cloned from a single viable cell. Also, at each 24-h interval, treated cell samples were washed and the cells resuspended in fresh media at 10^5 cells/mL in duplicate 10-mL flasks. The regrowth of the treated cells was followed for up to 144 h.

Cell Size. Cell size was determined directly by the method of Schwartz et al.[21] In brief, uniform polymeric microspheres ranging from 4.72 to 10.2 μm in diameter (Polysciences, Warrington, PA) were diluted in Hematall (Fisher Scientific Co.). Electronic size was measured on the FACS Analyzer (Becton Dickinson, Sunnyvale, CA) with the amplifier in the log mode. The peak channel number for each size microbead was plotted against the corresponding calibrated diameter and calculated volume to obtain a calibration curve. L1210 cells were treated with 10 μM DESPM-3 for 0–144 h and samples of 10^6 cells were removed at 24-h intervals and pelleted. The cells were resuspended in 0.5 mL of Hematall and analyzed. The peak channel number of the treated cells was plotted on the calibration curve to obtain the approximate cell size directly.

Registry No. 1, 70655-37-7; 1·2HCl, 113812-27-4; 2, 113812-18-3; 2·4HCl, 113812-17-2; 3, 61345-84-4; 3·4HCl, 113812-15-0; 4, 113812-20-7; 4·4HCl, 113812-21-8; 5, 113812-11-6; 5·HCl, 113812-12-7; 6, 113812-23-0; 6·4HCl, 113812-24-1; 7, 40563-84-6; 8, 113830-94-7; 8·4HCl, 113812-26-3; 9, 113812-30-9; 10, 63958-61-2; 11, 113812-13-8; 12, 113812-14-9; 13, 113812-16-1; 14, 113812-19-4; 15, 113812-22-9; 16, 113812-25-2; 17, 113812-28-5; 18, 113812-29-6; SPM·4HCl, 306-67-2; $C_2H_5NHCCH_2)_3NHC_2H_5$, 10061-68-4; $ClCO(CH_2)_2COCl$, 543-20-4; $CH_2{=}CHCN$, 107-13-1; $C_2H_5NH(CH_2)_4NHC_2H_5$, 19435-68-8; $CH_3CONH(CH_2)_{12}NHCOCH_3$, 31991-77-2; $CH_3C_6H_4$-p-$SO_2NHC_2H_5$, 80-39-7; $Cl(CH_2)_3Cl$, 142-28-9; 1,4-bis(3-aminopropyl)piperazine, 7209-38-3.

(20) Thornwaite, J. T.; Sugerbaker, E. V.; Temple, W. J. *Cytometry* **1980**, *1*, 229–237.

(21) Schwartz, A.; Sugg, H.; Ritter, T. W.; Fernandez-Repollet, E. *Cytometry* **1983**, *3*, 456–458.

An Examination of *O*-2-Isocephems as Orally Absorbable Antibiotics

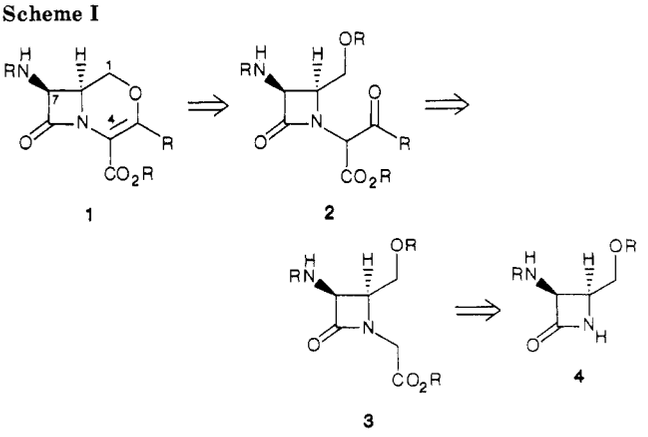
Harold Mastalerz,*† Marcel Menard,† Vivianne Vinet,† James Desiderio,‡ Joan Fung-Tomc,‡ Robert Kessler,*‡ and Yuan Tsai‡

Antiinfective Research, Bristol-Myers Pharmaceutical Group, Candiac, Quebec, Canada, J5R 1J1, and the Department of Microbiology, Bristol-Myers Pharmaceutical Group, Wallingford, Connecticut 06492-7660. *Received August 26, 1987*

The synthesis and structure–activity relationships of a series of orally absorbed *O*-2-isocephems are described. These compounds possessed a D-[(*p*-hydroxyphenyl)glycyl]amino substituent at the 7-position while the substituent at the 3-position was varied. Relative to the analogous cephems, the *O*-2-isocephems exhibited comparable to better activity against Gram-positive organisms. Against Gram-negative organisms, their activity was variable but did indicate a lower β-lactamase stability. Following oral administration, the *O*-2-isocephems generally exhibited longer half-lives but lower C_{max}'s and urinary recoveries.

The perception[1] that the use of orally administered antibiotics can be more cost-effective has spurred the search for new, long-acting, orally active β-lactam antibiotics. *O*-2-Isocephems, e.g. 1, are nuclear analogues of the cephalosporins and have been extensively examined in these laboratories[2a] as parenterally administered antibacterials. We have conducted a reexamination of this class of compounds to see if they would show promise as orally administered antiinfectives. This consisted of examining the activity of a group of *O*-2-isocephems bearing a D-[(*p*-hydroxyphenyl)glycyl]amino substituent at the 7-position as a function of the nature of the substituent at the 3-position. The results of this effort together with the details of a new, enantioselective *O*-2-isocephem synthesis are described in this paper.

Scheme I



Chemistry

Since existing syntheses[2] were too long and did not provide much room to vary the 3-substituent, we devised a new synthesis (Scheme I), which would be more suited to our needs. We believed that the *O*-2-isocephem 1 could

† Antiinfective Research.
‡ Department of Microbiology.

(1) Scrip, 1985 Chemotherapy Report; PJB Publications: Surrey, U. K., 1985; p 22.

0022-2623/88/1831-1190$01.50/0 © 1988 American Chemical Society