Polyamine−Iron Chelator Conjugate

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The current study demonstrates unequivocally that polyamines can serve as vectors for the intracellular delivery of the bidentate chelator 1,2-dimethyl-3-hydroxypyridin-4-one (L1). The polyamine−hydroxypyridinone conjugate 1-(12-amino-4,9-diazadodecyl)-2-methyl-3-hydroxy-4(1*H*)-pyridinone is assembled from spermine and 3-*O*-benzylmaltol. The conjugate is shown to form a 3:1 complex with Fe(III) and to be taken up by the polyamine transporter 1900-fold against a concentration gradient. The K_i of the conjugate is 3.7 μM vs spermidine for the polyamine transporter. The conjugate is also at least 230 times more active in suppressing the growth of L1210 murine leukemia cells than is the parent ligand, decreases the activities of the polyamine biosynthetic enzymes ornithine decarboxylase and *S*-adenosylmethionine decarboxylase, and upregulates spermidine-spermine N^1-acetyltransferase. However, the effect on native polyamine pools is a moderate one. These findings are in keeping with the idea that polyamines can also serve as efficient vectors for the intracellular delivery of other iron chelators.

Introduction

The sustained increases in polyamine biosynthesis in pre-neoplastic and neoplastic tissues have led to a great deal of attention being focused on the polyamine biosynthetic network as a target in antineoplastic therapy.[1−3] In fact, the observation of Jänne et al.[4] that tumor cells grown in exogenous spermine shut down polyamine biosynthesis set the stage for further attempts at targeting the polyamine biosynthetic network as an antineoplastic design approach.

In the course of developing our strategy of using *N*-alkylated analogues of the natural polyamines as antineoplastic devices,[5−17] we had the occasion to map out the structural boundary conditions set by the polyamine transporter on these substrates. All of the findings from other laboratories,[18] as well as our own,[6,13,14] are consistent with the idea that charge is critical to transporter recognition of the polyamine analogues. For example, consider the pair N^1,N^{12}-diethylspermine (DESPM) and N^1,N^{12}-bis(2,2,2-trifluoroethyl)spermine (FDESPM). The first of these tetraamines forms a tetracation at physiological pH, competes well with spermidine for uptake, and is effective at controlling cell growth. The latter compound, FDESPM, is nearly identical to DESPM sterically, but is dicationic at pH 7.4, competes poorly with spermidine for uptake, and is not active. There are numerous other examples that illustrate this.[14,19]

Studies in these laboratories also indicated that the ability of these analogues to impact on cell growth was dependent on (1) the number of nitrogens in the molecule, (2) the distance between the nitrogens, and (3) the nature of the terminal alkyl substituents. Tetraamines, e.g., DESPM, were typically more effective than the corresponding triamine analogues, e.g., N^1,N^8-diethylspermidine.[6,15] In a family of tetraamines with the same terminal alkyl groups, the impact of altering either one or both terminal alkyl groups on the compound's activity is most obvious when looking at the K_i values, where the K_i is a measure of the ability of an analogue to compete with spermidine for the polyamine transport apparatus. On moving from smaller substituents, e.g., methyl to *tert*-butyl groups, the K_i rises sharply.[13] Two alternative explanations for the polyamine transport apparatus recognition of these analogues are possible: either the *tert*-butyl substituents were so large that the compound could not "fit" into the transport apparatus, or the *tert*-butyl group inhibited interaction of the protonated terminal nitrogen with a biological counteranion in the transport apparatus. A large group such as *tert*-butyl would certainly be expected to increase the distance between the two ions, thus weakening their interaction and diminishing "polyamine recognition". This idea is reinforced further by the fact that N^1,N^{14}-di-*tert*-butylhomospermine competitively inhibits radiolabeled spermidine uptake much less than N^1-ethyl-N^{14}-*tert*-butylhomospermine does.[13,20] Most important, when the terminal nitrogens of homospermine analogues are captured in aliphatic six-membered rings, these molecules also bind well to the polyamine transport apparatus.[14]

This work clearly established both the charge and structural boundary conditions for utilizing polyamines as vectors.[6,13−15,20] Other investigators have exploited these findings by attaching a variety of antineoplastics to the spermine backbone[18,21−23] or have affixed amino acids to the spermine backbone in an effort to successfully compete against extracellular polyamines for uptake by the polyamine transport apparatus, thereby augmenting the efficacy of DFMO.[24] The present report describes the further exploitation of these findings, utilizing polyamines to vector iron chelators into cells.

Results and Discussion

Design Concept. Previous studies directed at the use of polyamines as vectors to deliver antineoplastics

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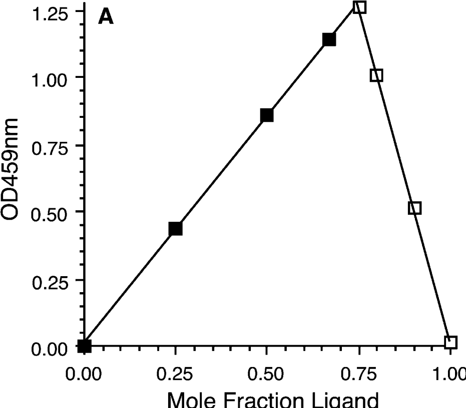
focused on improving the incorporation of intercalating agents, such as acridine,[18,23] or of alkylating agents, such as chlorambucil,[21,22] into tumor cells. Alternatively, polyamines have been used as agents to vector amino acids, such as lysine, into the polyamine uptake apparatus, where the amino acid presumably stymies the uptake of extracellular polyamines.[24] The current study explores the use of polyamines as drug delivery systems for the transport of iron chelators, in particular, a bidentate hydroxypyridinone–polyamine conjugate [1-(12-amino-4,9-diazadodecyl)-2-methyl-3-hydroxy-4(1*H*)-pyridinone (**3**)], into L1210 murine leukemia cells.

It is well-established by this laboratory and others that iron chelators can significantly reduce the growth of tumor cells in vitro.[25–28] We further demonstrated that this reduction could be accounted for by the inhibition of ribonucleotide reductase, as evidenced by thymidine uptake studies and arrest of cell division at the G1-S border.[29–31] One major shortcoming with iron chelators as antineoplastics is that most of these ligands [e.g., desferrioxamine (DFO)] do not cross the cell membrane particularly well.[32]

In addition, although chelators are effective as growth inhibitors of tumor cells in culture, where the iron sources are limited, this has not been the case in tumor xenografts in whole animals.[33] In the latter instance, there is a continual and ample supply of protein-bound iron, e.g., as transferrin. In contrast, two scenarios are unfolding when iron chelators are introduced to cells in a culture flask: (1) extracellular iron in the culture medium is ligated, preventing uptake of the metal, and (2) mobile intracellular iron is bound by any intracellular chelator, preventing transformation of apo-ribonucleotide reductase to its active form. For the latter process to be therapeutically significant, higher levels of chelator must be transported into the cell. To assess whether this goal is achievable, we elected to first visit with the delivery of a simple bidentate ligand, 1,2-dimethyl-3-hydroxypyridin-4-one (L1). This chelator does not achieve high concentrations (~1 μM) in cultured cells and thus is a good test of the concept. Further, L1 promotes the iron-mediated oxidation of ascorbate (Fenton chemistry),[34–36] providing another potential means of cytotoxicity. After assembly of the conjugate, the stoichiometry of its ferric complex was established using a Job's plot. The conjugate and comparitor polyamine fragments were then evaluated in L1210 cells for (1) their effect on cell proliferation (IC_{50} values), (2) their ability to compete with radiolabeled spermidine for the polyamine transport apparatus (K_i), (3) their uptake into cells, (4) their impact on polyamine pools, and (5) their effects on the polyamine enzymes L-ornithine decarboxylase (ODC), *S*-adenosyl-L-methionine decarboxylase (AdoMetDC), and spermidine/spermine N^1-acetyltransferase (SSAT).

Synthesis. Spermine-L1 conjugate **3** was accessed by the method of Scheme 1. 3-Benzyloxy-2-methyl-4-pyrone (**1**)[37] was stirred with spermine·4HCl (1.1 equiv) and NaOH (7 equiv) in aqueous ethanol at room temperature, giving monoadduct **2** in 12% yield after chromatography and treatment with HCl. To direct ring formation to a single primary amine, N^1,N^4,N^9-tris(*tert*-butoxycarbonyl)spermine[38] was heated with pyridinone synthon **1** and NaOH in aqueous ethanol, generating



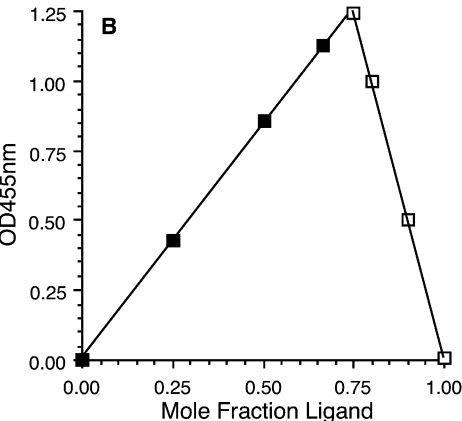


Figure 1. Job's plots of **3** (panel A) and L1 (panel B). Solutions containing different ligand/Fe(III) ratios were prepared so that [ligand] + [Fe(III)] = 1.0 mM. The data points were fitted to the mole fractions (1) from 0 to 0.75 and (2) from 0.75 to 1.00; r^2 = 1.000 and 1.000, respectively, for **3** and r^2 = 0.999 and 1.000, respectively, for L1. The theoretical mole fraction maximum for a 3:1 ligand/Fe complex of 0.75 was observed in both instances.

Scheme 1. Synthesis of the L1-spermine Conjugate (**3**)[a]

a,b

· 4HCl

1   **2** R = Bn   **3** R = H

[a] Reagents: (a) spermine·4 HCl (1.1 equiv), 2 N NaOH (aq) (7 equiv)/EtOH, then HCl, 12%; (b) H_2/Pd–C/EtOH, 76%.

the tris(BOC) derivative of **2** in only 9% yield. An explanation for these low yields is not apparent, since 3-*O*-benzylmaltol (**1**) condensed efficiently with methylamine and 1,3-diaminopropane, providing precursors to L1 (82%)[39] and 1-(3-aminopropyl)-2-methyl-3-hydroxy-4(1*H*)-pyridinone (71%),[40] respectively. Hydrogenolysis of the benzyl protecting group of **2** (10% Pd–C, aqueous CH_3CH_2OH, 1 atm) furnished L1 homologue **3** in 76% chromatographed yield.

Stoichiometry of the Adduct–Iron(III) Complex. The stoichiometry of the ferric complex of **3** was determined spectrophotometrically at its λ_{max} and compared with the stoichiometry of the parent drug at its λ_{max} (459 nm, Figure 1A, and 455 nm, Figure 1B, respectively). The two plots are essentially identical,

Table 1. L1210 Cell Growth Inhibition and Transport for Selected Monosubstituted Polyamines, L1, and the SPM-L1 Conjugate

compound structure/abbreviation	IC_{50} (μM)[a]		
	48 h	96 h	K_i (μM)[b]
AcSPM	> 100	> 100	10
MESPM[c]	99	0.33	1.7
MPSPD(N^8)[d]	> 100	55	8.5
L1	46	55	>500
3	0.2	0.2	3.7

[a] The IC_{50} was estimated from growth curves for L1210 cells grown in the presence of nine different concentrations of drug spanning four logarithmic units: 0, 0.03, 0.1, 0.3, 1.0, 3, 10, 30, and 100 μM. IC_{50} data are presented as the mean of at least two experiments with variation from the mean typically 10−25% for the 96-h IC_{50} values. [b] K_i determinations were made by following analogue inhibition of spermidine transport. All polyamine analogues exhibited simple substrate-competitive inhibition of [^{3}H]SPD transport by L1210 cells. Values reported in the table represent the mean of at least two or three experiments with a variation typically less than 10%. [c] Reproduced from ref 13. [d] Reproduced from ref 15.

demonstrating that both compounds form a 3:1 ligand/metal complex. This clearly indicates that the spermine fragment of the L1-polyamine conjugate does not alter the stoichiometry of the ferric complex.

Effect of the Conjugate on Cell Proliferation (IC_{50}). Certainly, one of the major issues in assessing any advantages a polyamine−iron chelator conjugate may have over its constituent fragments is in choosing the correct comparitors. At present, it is unclear how the cell might see this conjugate in terms of charge, i.e., either as a kind of trication or as a genuine tetracation, and it remains to be determined what the pK_a value of each of the nitrogens of the conjugate **3** is. Accordingly, several different polyamines were selected as models (Table 1): N^1-acetylspermine (AcSPM), a trication; N^1-ethylspermine (MESPM), a tetracation; and N^8-propylspermidine [MPSPD(N^8)], another trication. In each of these models, one end remains a primary amine affixed to an aminopropyl group, as in the conjugate. In each case, the 48- and 96-h IC_{50} and K_i values are presented. The numbers for MESPM[13] and MPSPD(N^8)[15] are historical; AcSPM, L1, and **3** were tested in the current series of experiments. After 48 h of treatment, none of the three model polyamine analogues have impressive IC_{50} values, 99 μM for MESPM and >100 μM for MPSPD(N^8) and AcSPM. The situation is remarkably different at 96 h for MESPM (0.33 μM); neither MPSPD(N^8) nor AcSPM is particularly active (55 and >100 μM, respectively). The parent iron chelator, L1, is not especially efficacious at either 48 (46 μM) or 96 (55 μM) h. However, adduct **3** is quite effective. The IC_{50} value is 0.2 μM at both time points, at least 230 times more active than the parent ligand. At 48 h, **3** is nearly 500 times more active than any of the parent polyamine analogues, although by 96 h its IC_{50} value is comparable to that of MESPM. The most profound difference between the conjugate and the polyamine analogues is that whereas the IC_{50} values of the alkylated polyamines diminish as the length of treatment increases, the IC_{50} value for the conjugate is low at 48 h and remains the same at 96 h. This behavior is similar to that of the chelator moiety: L1 remains static in its performance, even though the corresponding IC_{50} values are much higher than those for **3**.

Competition for the Polyamine Transporter (K_i Values). Again, consideration of the appropriate comparitors is an important issue; the arguments are the same as those presented above. Not surprisingly, each of the polyamine analogues competed effectively for the polyamine transport apparatus; the K_i values were 10, 1.7, and 8.5 μM for AcSPM, MESPM, and MPSPD(N^8), respectively (Table 1). Although the parent ligand L1 was not a transport competitor, its spermine conjugate **3** was a very effective transport competitor (K_i, 3.7 μM, Table 1), suggesting that the polyamine does vector the ligand.

Impact on Polyamine Pools and Accumulation of Analogues. In each case, cells were treated with the compound of interest at the 48-h IC_{50} concentration. The effects on the native polyamines are reported as the percent of the polyamine found in untreated control cells, and the analogue accumulation is reported as millimolar (Table 2). Of all four polyamine analogues, including the conjugate **3**, MESPM was the most effective at reducing polyamine pools. At a treatment concentration of 100 μM, putrescine, spermidine, and spermine were diminished to 0%, 1%, and 21% of control, respectively. At the same treatment concentration, MPSPD(N^8) lowered putrescine and spermidine, although spermine was unaffected (103%). However, at the treatment concentration of 100 μM, AcSPM had a substantial effect on putrescine, generated a slight decrease in spermidine, and increased spermine levels. Of the three model polyamines, MPSPD(N^8) achieved

Table 2. Effect of Selected Monosubstituted Polyamines, L1, and the SPM-L1 Conjugate on Polyamine Pools in L1210 Cells

compd	treatment concn (μM)	PUT[a]	SPD[a]	SPM[a]	analogue[b]
AcSPM	100	24	71	111	1.62
MESPM[c]	100	0	1	21	1.24
MPSPD(N^8)[d]	100	0	33	103	3.52
L1	50	104	117	127	~0.001[e]
3	0.2	33	64	139	0.39

[a] Putrescine (PUT), spermidine (SPD), and spermine (SPM) levels after 48 h of treatment are given as percent polyamine found in untreated controls. The control values in pmol/10^6 L1210 cells are PUT = 183 ± 15, SPD = 2694 ± 232, SPM = 774 ± 81. [b] Analogue amount is expressed as nmol/10^6 cells. Untreated L1210 cells (10^6) correspond to about 1 μL volume; therefore, the concentration can be estimated as millimolar. [c] Reproduced from ref 13. [d] Reproduced from ref 15. [e] A detectable peak coelutes with a spike of authentic L1.

Table 3. Impact of Selected Monosubstituted Polyamines, L1, and the SPM-L1 Conjugate on Ornithine Decarboxylase (ODC), *S*-Adenosylmethionine Decarboxylase (AdoMetDC), and Spermidine/Spermine N^1-Acetyltransferase (SSAT) in L1210 Cells[a]

compound	ODC	AdoMetDC	SSAT
AcSPM	25 ± 11	86 ± 11	180 ± 11
MESPM[b]	10	27	150
MPSPD(N^8)[c]	14	64	500
L1	114 ± 5	120 ± 4	108 ± 58
3	20 ± 4	77 ± 11	275 ± 6

[a] Enzyme activity is expressed as percent of untreated control for ODC (1 μM at 4 h), AdoMetDC (1 μM at 6 h), and SSAT (10 μM at 48 h for all of the above analogues except **3**, which is 0.2 μM). Each experiment included a positive control, which had a known, reproducible impact on enzyme activities (mean ± SD): 1 μM DEHSPM lowered ODC to 6.7 ± 2.6% of the untreated control; 1 μM DEHSPM decreased AdoMetDC to 40.7 ± 6.2% of the untreated control; and 2 μM DENSPM increased SSAT to 3877 ± 76% of the untreated control. Data shown in the table represent the mean of at least three experiments and have variances consistent with those suggested by the positive control data presented above. [b] Reproduced from ref 13. [c] Reproduced from ref 15.

the highest intracellular concentration, 3.52 mM; AcSPM and MESPM reached concentrations less than half of this, 1.62 and 1.24 mM, respectively. Perhaps not surprisingly, treatment of cells with L1 (50 μM) had little impact on native polyamines (Table 2); putrescine and spermidine remained close to control levels, and spermine rose to 127% of control values. The behavior of conjugate **3** when given at its IC_{50} concentration (0.2 μM) was vastly different from that of L1 and very similar to that of AcSPM. Putrescine was diminished to 33% of the control, spermidine was reduced to 64% of the control, and spermine increased to 139% of control values. Interestingly, the adduct **3** was transported quite efficiently into the cells; the intracellular concentration of 0.39 mM is more than 1900-fold higher than the extracellular treatment concentration and is also several hundred times higher than the intracellular concentration attained by the parent ligand L1.

Effect on Polyamine Metabolic Enzymes. Both MESPM and MPSPD(N^8) exerted rather significant effects on all three polyamine enzymes (Table 3). MESPM diminished ODC and AdoMetDC to 10% and 27% of the control, respectively, and slightly increased SSAT activity to 150% of the control.[13] Although its impact on ODC was similar to that of MESPM, MPSPD(N^8) decreased AdoMetDC to a lesser extent (64% of control) and substantially augmented SSAT activity, to 500% of the control.[15] In the case of AcSPM, its impact on ODC was comparable to that of the other two polyamines, 25 ± 11% of the control, but its effect on AdoMetDC was not as great (86 ± 11%); this polyamine boosted SSAT activity to 180 ± 11% of the control. It was not unexpected that the parent chelator L1 did not materially influence the activity of any of the polyamine enzymes (ODC, 114 ± 5%; AdoMetDC, 120 ± 4%; SSAT, 108 ± 58%). The conjugate **3** behaved much like MPSPD(N^8), reducing ODC and AdoMetDC to 20 ± 4% and 77 ± 11% of the control, respectively, and increasing SSAT activity to 275 ± 6% of the control. These results further support the idea of incorporation of the conjugate.

Conclusion

The data presented are consistent with earlier results from our laboratory[14,20] and the more recent work by other investigators.[18,23] The polyamine transporter will recognize and process polyamine derivatives, even if the terminal substituent is quite large, for example, *tert*-butyl[20] or an integrated L1 molecule in the present work, as long as the charge distribution is appropriately in place. The polyamine–L1 conjugate is far (at least 230 times) more active at controlling cell growth than is the parent L1 chelator. This conjugate is concentrated in the cell by more than 1900-fold against a gradient, i.e., nearly 400 times more effectively than L1. As expected, it suppresses ODC and AdoMetDC and upregulates SSAT. The conjugate suppresses putrescine substantially and spermidine only moderately. In short, this chelator conjugate offers an attractive system for further study of the impact of iron chelators, particularly those that promote Fenton chemistry, on tumor cell growth both in vitro and in vivo.

Experimental Section

Reagents were purchased from Aldrich Chemical Co. (Milwaukee, WI), and Fisher Optima-grade solvents were routinely used. Silica gel 32–63 from Selecto Scientific, Inc. (Suwanee, GA) was used for flash column chromatography, and Sephadex LH-20 was obtained from Amersham Biosciences (Piscataway, NJ). Melting points are uncorrected. NMR spectra were obtained at 300 MHz (^1H) or 75 MHz (^{13}C) on a Varian Unity 300 in D_2O, with chemical shifts (δ) given in parts per million referenced to sodium 3-(trimethylsilyl)propionate-2,2,3,3-d_4 (0.0) or 1,4-dioxane (67.19), respectively. Coupling constants (*J*) are in hertz. Elemental analyses were performed by Atlantic Microlabs (Norcross, GA).

1,2-Dimethyl-3-hydroxypyridin-4-one (L1) was a generous gift from Dr. H. H. Peter (Ciba-Geigy, Basel, Switzerland). N^1-Acetylspermine (AcSPM) as its trihydrochloride salt (A-2679) was purchased from Sigma (St. Louis, MO).

1-(12-Amino-4,9-diazadodecyl)-2-methyl-3-(phenylmethoxy)-4(1*H*)-pyridinone Tetrahydrochloride (2). Sodium hydroxide (2 N, 190 mL, 0.38 mol) was added in portions to a solution of **1** (11.49 g, 53.15 mmol) and spermine·4HCl (20.20 g, 58.01 mmol) in 39% aqueous CH_3CH_2OH (570 mL) with ice bath cooling. The reaction mixture was stirred at room temperature for 1 day, and its volume was reduced by rotary evaporation. Water (200 mL) was added, followed by several $CHCl_3$ extractions. The organic portion was washed with saturated NaCl, dried with sodium sulfate, and concentrated under reduced pressure. Flash chromatography (30% concentrated NH_4OH/CH_3OH) and acidification with concentrated HCl in CH_3CH_2OH gave **2** (3.35 g, 12%) as a white solid: mp

188−190 °C; ^{1}H NMR δ 1.7−1.8 (m, 4 H), 2.0−2.2 (m, 4 H), 2.28 (s, 3 H), 3.0−3.2 (m, 10 H), 4.23 (t, 2 H, J = 7.7), 5.12 (s, 2 H), 6.91 (d, 1 H, J = 7.2), 7.4−7.5 (m, 5 H), 7.98 (d, 1 H, J = 7.5); ^{13}C NMR δ 13.5, 23.4, 24.4, 26.8, 37.2, 44.8, 45.2, 47.6, 47.7, 53.7, 76.0, 114.3, 129.5, 129.9, 130.3, 135.8, 142.5, 143.7, 150.6, 165.9. Anal. ($C_{23}H_{40}Cl_4N_4O_2 \cdot 2H_2O$) C, H, N.

1-(12-Amino-4,9-diazadodecyl)-2-methyl-3-hydroxy-4(1*H*)-pyridinone Tetrahydrochloride (3). Distilled solvents and glassware that had been presoaked in 3 N HCl for 15 min were employed. Pd−C (10%, 400 mg) was added to **2** (1.43 g, 2.62 mmol) in 38% aqueous CH_3OH (130 mL). The reaction mixture was stirred under H_2 at 1 atm for 5 h and was filtered through Celite, which was washed with water (10 mL) and CH_3CH_2OH (30 mL). After removal of the solvents in vacuo, chromatography on Sephadex LH-20 (CH_3CH_2OH) furnished **3** (0.911 g, 76%) as a white solid: mp 216−218 °C; ^{1}H NMR δ 1.7−1.8 (m, 4 H), 2.02−2.15 (m, 2 H), 2.18−2.31 (m, 2 H), 2.57 (s, 3 H), 3.07−3.18 (m, 10 H), 4.35 (t, 2 H, J = 7.8), 6.89 (d, 1 H, J = 7.2), 7.91 (d, 1 H, J = 7.2); ^{13}C NMR δ 12.8, 23.4, 24.4, 26.9, 37.2, 44.9, 45.2, 47.6, 53.8, 112.0, 139.0, 142.5, 143.6, 160.1. Anal. ($C_{16}H_{34}Cl_4N_4O_2 \cdot H_2O$) C, H, N.

Stoichiometry of the Ligand−Fe(III) Complex. The stoichiometry of each complex was determined spectrophotometrically for **3** and L1 at the λ_{max} (459 and 455 nm, respectively) of the visible absorption band of the ferric complexes by the method given in detail in an earlier publication.[41] Briefly, a 0.5 mM iron(III) nitrilotriacetate (NTA) solution was made immediately before use by dilution of a 50 mM Fe(III)−NTA stock solution with TRIS buffer. Solutions of the ferric complex containing different ligand/Fe(III) ratios were then prepared by mixing appropriate volumes of 0.5 mM ligand in 100 mM TRIS Cl, pH 7.4, and 0.5 mM Fe(III)−NTA such that [ligand] + [Fe] = 1.00 mM constant. The Job's plot for each set of mixtures was then derived.

Cell Culture. Murine L1210 leukemia cells were maintained in logarithmic growth as a suspension culture in RPMI-1640 medium (Gibco, Grand Island, NY) containing 10% fetal bovine serum (Gibco), 2% HEPES-MOPS buffer, 1 mM L-glutamine (Gibco), and 1 mM aminoguanidine at 37 °C in a water-jacketed 5% CO_2 incubator.

IC_{50} Determinations. Cells were grown in 25-cm^2 tissue culture flasks in a total volume of 10 mL. Cultures were treated during logarithmic growth ($0.5−1.0 \times 10^5$ cells/mL) with the compounds of interest, reseeded, and incubated as described previously.[16] Cell counting and calculation of percent of control growth were also carried out as given in an earlier publication.[16] 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.

Uptake Determinations. The molecules of interest were studied for their ability to compete with [^{3}H]SPD for uptake into L1210 leukemia cell suspensions in vitro as given in detail in previous publications.[9,13,16] Briefly, cell suspensions were incubated in 1 mL of culture medium containing radiolabeled SPD alone or radiolabeled SPD in the presence of graduated concentrations of chelator or derivative. At the end of the incubation period, the tubes were centrifuged; the pellet was washed, digested, and neutralized prior to scintillation counting. Lineweaver−Burk plots indicated simple competitive inhibition with respect to SPD.

Polyamine Pool and Compound Analysis. During logarithmic growth, cells were treated with the compounds. At the end of the treatment period, cell suspensions were sampled, washed three times in ice-cold, incomplete medium, and pelleted for extraction using 0.6 N perchloric acid,[13] then freeze-fractured in liquid nitrogen/hot water three times. Each supernatant was frozen at −20 °C until analysis of polyamine content by HPLC.[42] L1 was measured by an HPLC method with UV detection described in the literature[43] with the following modifications: mobile phase A, 20% buffer/80% CH_3OH; mobile phase B, 98% buffer/2% CH_3CN. The buffer consisted of potassium phosphate (10 mM) and EDTA (2 mM), pH 2.9. Under these conditions, the polyamines eluted on the chromatogram as follows (min): putrescine, 10.8; AcSPM, 27.3; spermidine, 33.1; compound **3**, 47.1; spermine, 48.0.

Enzyme Assays. Ornithine decarboxylase and AdoMetDC activities were determined according to the procedures of Seely and Pegg[44] and Pegg and Pösö,[45] respectively, on the basis of quantitation of $^{14}CO_2$ released from [^{14}C]carboxyl-labeled L-ornithine or *S*-adenosyl-L-methionine. Included in each assay were untreated L1210 cells as negative controls as well as cells treated with DEHSPM, a drug having a known reproducible effect on each enzyme, as positive controls.

Spermidine/spermine N^1-acetyl transferase activity was based on quantitation of [^{14}C]-N^1-acetylspermidine formed by acetylation of SPD with [^{14}C]acetyl coenzyme A according to the method of Libby et al.[10] Cells treated with DENSPM were positive controls.

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Supporting Information Available: Scheme of cyclocondensation of N^1,N^4,N^9-tris(*tert*-butoxycarbonyl)spermine with 3-benzyloxy-2-methyl-4-pyrone (**1**) to the tris(BOC) derivative of **2** (9%). This material is available free of charge via the Internet at http://pubs.acs.org.

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