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Hexahydropyrimidines as Masked Spermidine Vectors in Drug Delivery

RAYMOND J. BERGERON AND HOWARD W. SELIGSOHN

Department of Medical Chemistry, University of Florida, Florida 32610

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N-(4-Aminobutyl)hexahydropyrimidine and *N*-(3-aminopropyl)hexahydropyrimidine are shown to compete with spermidine for uptake by L1210 cells. This observation is in keeping with the idea that spermidine may adopt a hydrogen-bonded cyclic structure in the course of transport. Furthermore, the differences in the ability of spermidine, homospermidine, and norspermidine to utilize the spermidine uptake apparatus is analyzed in terms of the charge on the triamines, as calculated from pK_a data.

INTRODUCTION

Spermidine and its analogs have received considerable attention in recent years as potential vectors for antineoplastics (*1, 2*). The investigations to date have been largely concerned with ascertaining the kinds of structural modifications which can be made on the spermidine molecule without diminishing the molecule's ability to act as a substrate for the spermidine uptake apparatus of neoplastic cells (*1–6*).

The concept is predicated on the observations that (1) proliferating cells produce substantially more polyamines than differentiated resting cells (*7–11*) and (2) when polyamine biosynthesis is shut off with difluoromethyl ornithine (DFMO), proliferating cells will take up exogenous polyamines at a substantially increased rate (*1*).

Initial studies suggested that the formal charge on the polyamines norspermidine, spermidine and homospermidine at physiological pH is of considerable importance in uptake by L1210 cells (*1–6*). For example when both terminal nitrogens of spermidine are acetylated the resulting derivative can no longer effectively compete with spermidine for the L1210 cell spermidine uptake apparatus. However, when this diamide is reduced, the corresponding N^1,N^8-diethylamino compound competes favorably with spermidine for the polyamine uptake apparatus. Clearly, at physiological pH the amines are protonated and the amides are not. What is most interesting is the fact that 1,7-diaminooctane (K_i = 21.1 μM) and 1,6-diaminoheptane (K_i = 18.2 μM) compete very well with spermidine for uptake. This raises the question as to the role of spermidine's central nitrogen in uptake.

If spermidine's central nitrogen is acetylated, competition for uptake decreases

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(K_i = 115 μM) while again reduction to the corresponding N^4-ethylspermidine improves on competition (K_i = 3.1 μM). The differences in competition for the spermidine uptake apparatus between an acyl and alkyl N^4-substituted spermidine is even more apparent in the case of N^4-benzoylspermidine (K_i > 500 μM) vs N^4-benzylspermidine (K_i = 36 μM). The differences between the benzyl and benzoyl vs ethyl and acetyl suggest that both charge and steric factors play a role in substrate recognition. The remarkable differences in uptake between acetyl/ethyl, benzoyl/benzyl spermidines encouraged us to look more closely at the roles of both charge and steric factors in polyamine uptake.

RESULTS AND DISCUSSION

In an attempt to more accurately define the role of charge in polyamine uptake, the protonation state of N^4-benzylspermidine, as well as the homo- and norspermidine analogs, was studied. To achieve this, the pK_a's of each nitrogen of the N^4-benzyl analogs of the triamines norspermidine (BNSPD), spermidine (BSPD), and homospermidine (BHSPD) and homospermidine (HSPD) itself were determined potentiometrically. The benzyl analogs were chosen because considerable uptake data has been accumulated on these compounds. The results of the pK_a studies are presented in Table 1 along with other selected polyamine pK_a's. Knowing the pK_a's, it is possible to calculate the concentration of the mono-, di-,

TABLE 1

pK_a's FOR THE TRIAMINES R_1—NH—$(CH_2)_a$—$\underset{\displaystyle R_2}{\overset{|}{N}}$—$(CH_2)_b$—NH—$R_3$ AS DETERMINED BY POTENTIOMETRIC TITRATION

R_1	R_2	R_3	a	b	pK_1	pK_2	pK_3
H	H	H	2	2	9.79	8.98	4.25[a]
			2	3	10.44	9.36	6.37[b]
			2	4	10.67	9.59	6.98[c]
			3	3	10.65	9.57	7.72[d]
			3	4	10.83	9.83	8.35[e]
			4	4	10.77(2)	10.01(3)	8.92(1)[f]
H	—$CH_2C_5H_6$	H	3	3	10.58(6)	9.79(9)	6.51(7)
			3	4	10.64(3)	9.84(7)	7.12(11)
			4	4	10.84(4)	10.11(7)	7.75(15)
H	—(C=O)C_5H_6	H	3	3	9.98(19)	7.76(35)	
			3	4	10.51(6)	8.97(8)	

[a] Ref. (*18*).
[b] Ref. (*19*).
[c] Ref. (*20*).
[d] Ref. (*21*).
[e] Ref. (*22*).
[f] The number in parentheses is the standard deviation associated with the last significant figure.

TABLE 2

CORRELATION BETWEEN THE POLYAMINE INHIBITION CONSTANT FOR SPD UPTAKE AND THE FRACTION OF POLYAMINE EXISTING AS THE TRICATION AT PHYSIOLOGICAL pH

Compound	K_i[a] (μM)	α (3)	$K_i \times \alpha$ (3) (μM)
BNSPD	135	0.114	15.4
BSPD	36	0.343	12.3
BHSPD	14	0.691	9.7
NSPD	8.4	0.674	5.7
HSPD	3.5	0.971	3.4

[a] Ref. (*1*).

and trication of a polyamine in solution at any given pH, using the equations:

$$\alpha\,(3) = \frac{[H^+]^3}{[H^+]^3 + K_1[H^+]^2 + K_1K_2[H^+] + K_1K_2K_3}$$

$$\alpha\,(2) = \frac{K_1[H^+]^2}{[H^+]^3 + K_1[H^+]^2 + K_1K_2[H^+] + K_1K_2K_3}$$

$$\alpha\,(1) = \frac{K_1K_2[H^+]}{[H^+]^3 + K_1[H^+]^2 + K_1K_2[H^+] + K_1K_2K_3}$$

$$\alpha(0) = \frac{K_1K_2K_3}{[H^+]^3 + K_1[H^+]^2 + K_1K_2[H^+] + K_1K_2K_3},$$

where α (i) is the fraction of polyamine existing as the $+i$ cation. Recall that the order of uptake inhibition is homospermidine > spermidine > norspermidine. It is clear that there are substantial differences in the relative concentrations of polycations at a particular pH. For example, at pH 7.4 only 67.4% of norspermidine is in the form of the trication while 89.9% of spermidine is in this form and 97.1% of homospermidine is tripronated (Fig. 1). Similar trends are seen with the benzyl compounds, where 11.4, 34.3, and 69.1%, respectively, exist as the trication (Fig. 2).

The values for α (3) seem to correlate well with previous *in vitro* measurements of inhibition constants (*1, 3*). By multiplying the actual concentration of polyamine by the fraction of the polyamine which exists as the +3 cation at pH 7.4, one can determine the "effective" concentration of polyamine. When one recalculates the K_i's of various polyamines based on these corrected concentrations, the large differences in affinity for the transport apparatus are greatly diminished (Table 2).

However, the role of a +3 cation is unclear in view of the fact that long chain diamines, which would be +2 cations, compete so effectively for uptake. Furthermore, in looking at the concentration of the +2 cation of triamines and competition with spermidine for uptake an inverse relationship seems to exist. Finally, there is so little +1 cation and free base that their roles would seem insignificant. It may be that the role of the trication is to bring the polyamine to the surface of

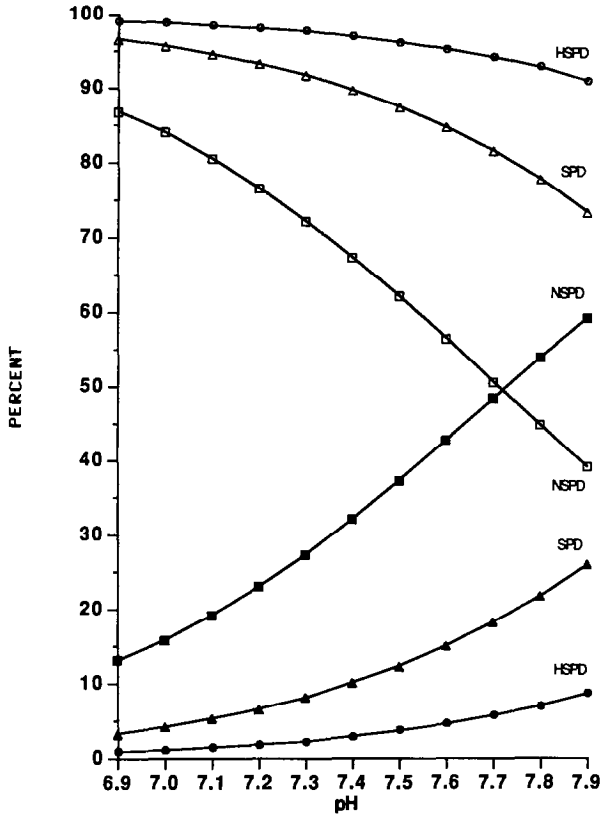


FIG. 1. The percentage of the polyamines NSPD, SPD, and HSPD which exists as the dication (closed symbols) or trication (open symbols) as a function of pH.

the negatively charged cell membrane. Once associated with the cell membrane other factors may come into play. Clearly, charge is not the sole explanation for the selectivity in polyamine uptake.

Both the N^4-acetyl and N^4-benzoyl compound have restricted rotation about the amide C—N bond while the corresponding reduced compounds have more freedom of rotation. This implies that although the position in which the acetyl group is held does not substantially hinder uptake, the benzoyl group does. A consideration of how charges on the terminal nitrogens of spermidine might be utilized to hold the polyamine in a particular conformation and the possible conformations which might allow a benzoyl vs a benzyl functionality—or, to a lesser extent, an acetyl vs ethyl functionality—to interfere with this binding led us to postulate the importance of a hydrogen-bonded cyclic conformation for polyamine transport. It is clear that a trication cannot form intramolecular hydrogen bonds and that spermidine exists mainly as a trication in an aqueous environment at pH 7.4. It may be that the trication is energetically unfavorable at the cell membrane and less charged species exist.

It has been shown that the strength of a hydrogen bond is maximal when the

bond is colinear; i.e., when the X--H--X angle formed by the nuclei is 180 degrees. In the case of intramolecular hydrogen bonds the optimal angle cannot always be achieved, resulting in a weaker bond. For example, hydrogen bonding of the terminal hydroxyls of compounds of the general structure HO—$(CH_2)_n$—OH have been measured by infrared spectroscopic analysis (*12*). The data indicates that 1,4-butanediol (n = 4) forms a stronger intramolecular hydrogen bond than does 1,3-propanediol (n = 3). For terminal diamines H_2N—$(CH_2)_n$—NH_2 acid-base titrimetry has provided similar data; i.e., 1,4-diaminobutane is more basic than 1,3-diaminopropane (*13*). This may be due to the increased electrostatic repulsion in the case of 1,3-diaminopropane and/or due to the presence of a stronger intramolecular hydrogen bond in the case of 1.4-diaminobutane; a seven-membered intramolecularly hydrogen-bonded ring can more closely achieve a conformation which optimizes the N--H--N bond angle.

In previous uptake experiments, HSPD inhibited [^{3}H]SPD uptake better than NSPD, implying that the aminobutyl sidechain is more easily recognized by the polyamine uptake apparatus than the aminopropyl sidechain. Since the aminobutyl sidechain can form a more stable intramolecular hydrogen bond, a greater percentage of the dication of HSPD may exist as the cyclic hydrogen-bonded

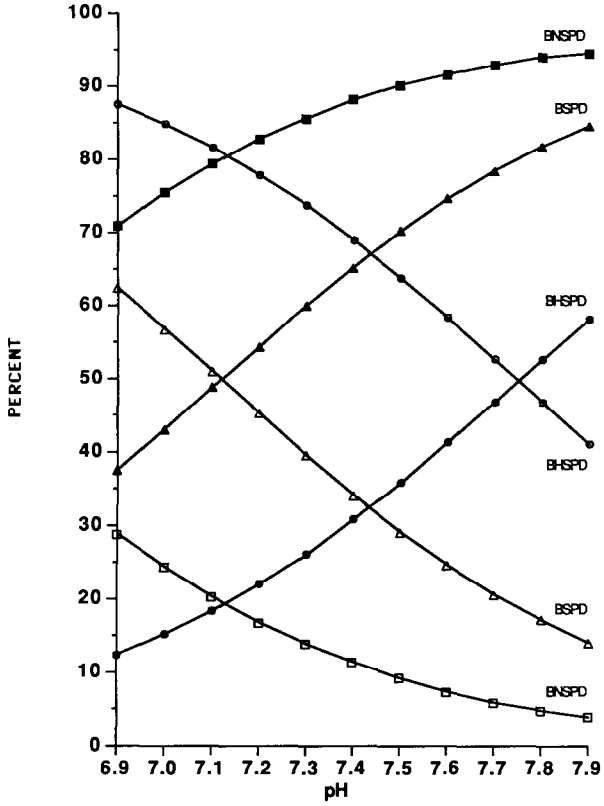
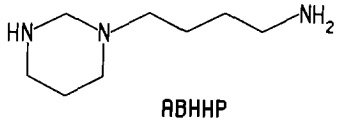


FIG. 2. The percentage of the polyamines BNSPD, BSPD, and BHSPD which exists as the dication (closed symbols) or trication (open symbols) as a function of pH.

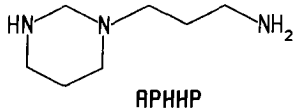
conformer on the cell surface, leading to better recognition and inhibition. Although in the case of N^1,N^7-diaminoheptane and N^1,N^8-diaminooctane no central nitrogen with which to form an intramolecular hydrogen bond exists, a pseudocyclic conformation may still be achieved by folding of these molecules. In addition, N^4-benzoyl SPD cannot compete effectively for uptake. This may reflect the inability of this molecule to achieve a cyclic conformation for steric reasons. However, the corresponding benzyl compound can compete very well. Although this analog will exert steric restrictions similar to the benzoyl analog, the lone electron pair associated with the central amine may assist formation of a cyclic conformer by forming an intramolecular hydrogen bond. Also, because of increased freedom of rotation which exists about the bonds unlike that for the corresponding amide, the amine may be able to more easily adopt the required conformation.

^{1}H-NMR measurements were considered in order to show that polyamines do indeed form cyclic conformers; however, it was clear this would not definitively indicate whether or not the L1210 cell polyamine uptake apparatus transports six- and/or seven-membered hydrogen-bonded cyclic structures; thus, the importance of cyclic conformers in uptake had to be evaluated by another method. It was decided to prepare cyclic methylene-bridged analogs of intramolecularly hydrogen-bonded norspermidine and spermidine—hexahydropyrimidine analogs, Fig. 3—and determine if these analogs would be transported by L1210 cells. A hexahydropyrimidine analog is easily synthesized from the appropriate amine and formaldehyde (*14*). The product still possesses the polyamine backbone intact, but this backbone is "locked in" to a cyclic conformation by the methylene bridge. Since all three nitrogens remain amines, they can still bear positive charge. If cyclic conformers are involved in the uptake mechanism, these analogs should be readily recognized and transported by the polyamine carrier.

Two questions were addressed relative to these compounds: (1) Are they stable in solution? (2) Do they compete for the polyamine uptake apparatus? The stability question was addressed simply because of the potentially reversible nature of hexahydropyrimidine formation under the conditions of uptake. The question was answered utilizing ^{1}H-NMR. A deuterated version of the culture medium employed in the polyamine uptake studies (RPMI 1640) was prepared. *N*(4-Aminobutyl)hexahydropyrimidine (ABHHP) was added to the medium and a 300-MHz ^{1}H-NMR spectrum recorded immediately and after 4 h. A singlet characteristic of the



ABHHP



APHHP

FIG. 3. Cyclic spermidine analogs.

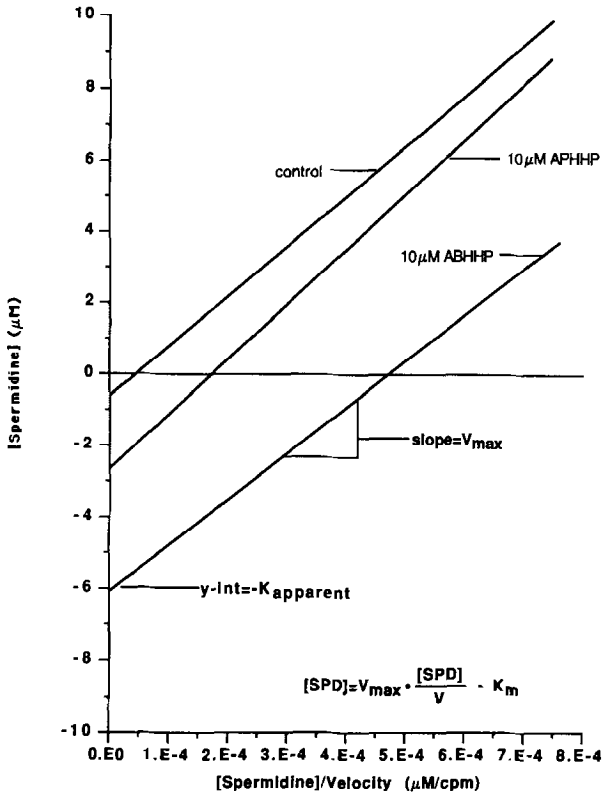


FIG. 4. Hofstee plot for APHHP and ABHHP. 5×10^6 cells/ml of cultured L1210 cells were incubated for 20 min at 37°C with ^{14}C-labeled SPD and 10 μM SPD analog.

hexahydropyrimidine methylene bridge exhibits a chemical shift of 3.4 ppm downfield from TMS. The two spectra indicated that the methylene bridge of ABHHP was stable during the course of the biological studies. Since the mechanism of hydrolysis is the same for ABHHP and *N*-(4-aminopropyl)hexahydroypyrimidine (APHHP), it was deemed unnecessary to repeat the experiment for the latter.

Once having established the stability of the cyclic compounds under the experimental conditions, spermidine competition studies were initiated, following established procedures (*3*). Briefly, L1210 cells in log growth phase were exposed to ^{14}C-labeled spermidine at concentrations varying from 0.2 to 10 μM in the absence or presence of 10 μM hexahydropyrimidine. ^{14}C-Spermidine cellular uptake was measured utilizing scintillation procedures. The data were plotted according to the Hofstee method (Fig. 4), and the K_i's calculated from

$$K_{\text{apparent}} = K_m(1 + [i]/K_i),$$

utilizing a K_m of 0.77 μM for spermidine (Table 3). Although the absolute magnitude of K_m for SPD and K_i for NSPD are somewhat different than those reported previously, the relative magnitudes are similar. This difference can be attributed to the fact that our experiments utilized L1210 cells from tissue culture, whereas

TABLE 3

SUMMARY OF *IN VITRO* EXPERIMENTAL RESULTS FOR CYCLIC SPERMIDINE ANALOGS

Compound	SPD uptake inhibition		IC_{50} (μM)
	% of control[a]	K_I (μM)	
None	100		
NSPD	29	4.67 ± 0.33	0.7
APHHP	58.9	3.91 ± 2.57	0.5
SPD	9.5	0.77 ± 0.31(K_m)	1000.0[b]
ABHHP	11.4	1.41 ± 1.15	40.0

[a] 10 μM SPD, 100 μM SPD analog.
[b] Ref. *1*.

previous work employed Ascites cells. Consequently, one would anticipate that the Ascites cells would take up the hexahydropyrimidines somewhat differently than cultured L1210 cells. However, the relative uptake should be the same.

In order to verify that the variance could not be assigned to the fact that we employed different concentrations of polyamines in our studies, a Dixon plot was generated, using NSPD as a model. The data was plotted using a rearrangement of the Michealis–Menton equation for competitive inhibition;

$$\frac{1}{V} = \left[\frac{K_m}{S \cdot V_m \cdot K_i}\right] \cdot [i] + \left[\frac{S + K_m}{S \cdot V_m}\right].$$

The linearity of this plot (Fig. 5) suggests that the mode of inhibition is the same over the concentration range examined; i.e., K_i is constant.

The ability of the hexahydropyrimidines to prevent the growth of L1210 cells was also measured. These analogs were found to be very effective at preventing cell division, as evidenced by their IC_{50} values (Table 3). This may be due to interference with or disruption of polyamine metabolism or function. Whatever the mode of action, it is clear from these data that the possibility of the polyamines adopting cyclic conformations during transport is quite reasonable.

EXPERIMENTAL

APHHP and ABHHP were synthesized as described below. Other polyamines were synthesized by methods developed in this laboratory (*15, 16*). ^{14}C-Labeled SPD was obtained from Amersham. Solvents were obtained from Fisher Scientific Company. All other reagents were supplied by Aldrich Chemical Company.

1. Potentiometric Measurements

A 20.00-ml sample which contained 0.1 M KCl and approximately 0.001 M polyamine was placed in a water-jacketed cell and titrated with 1.06 M HCl at 25°C

using a Radiometer-Copenhagen DTS833 Digital Titration System in conjunction with a Lauda K-2/R circulating constant-temperature bath. The data were analyzed by the computer program PHFIT (*17*). Each polyamine was titrated at least five times.

2. *Synthesis of Cyclic Analogs*

The method of McManis was used (*14*). Briefly, one equivalent of formaldehyde was added to 1 eq of SPD or NSPD in aqueous solution and stirred 24 h. The aqueous layer was then made basic and extracted into $CHCl_3$. The crude is distilled (80°C, 0.02 Torr) to yield the pure product.

3. *Cyclic Analog Stability*

The culture medium (RPMI-1640 containing 2% Hepes–Mops buffer and 1.0 mM aminoguanidine) was lyophylized and reconstituted with D_2O several times. The pD of the resultant solution was adjusted to 7.4 with D_2SO_4. ABHHP was added to the deuterated medium which was prewarmed to 37°C, at a concentration

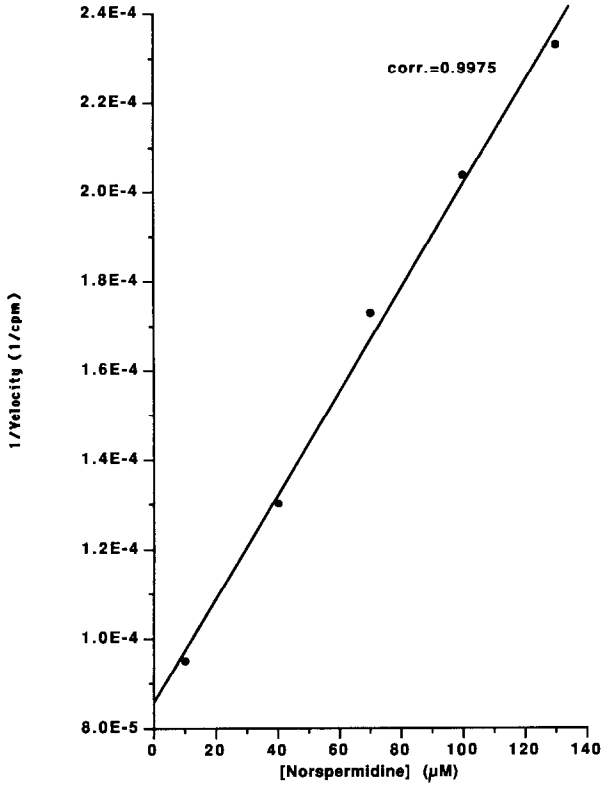


FIG. 5. Dixon plot for NSPD with cultured L1210 cells. Cells were incubated at 37°C with 10 μM ^{14}C-labeled SPD and 10 to 130 μM NSPD for 20 min.

of 0.3 M and a 300-MHz ^{1}H-NMR spectrum was recorded immediately. A characteristic singlet appears at 3.4 ppm downfield from TMS, and was monitored throughout the experiment. A second spectrum was recorded after 5 h and electronically subtracted from the initial spectrum. This difference spectrum indicated that the hexahydropyrimidine moiety remained intact during this time.

4. Inhibition of Spermidine Uptake

L1210 cells were maintained in log growth phase to a density of approximately 8 $\times$ 10^5 cells/ml in RPMI-1640 tissue culture medium containing 2% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid–4-morpholinepropanesulfonic acid, 1.0 mM aminoguanidine, and 10% fetal calf serum before centrifugation at 500*g* at 4°C for 7 min. The pellet was resuspended with cold culture medium to a density of 10^7 cells/ml. To each of our vessels containing [^{14}C]SPD and SPD analog in 0.5 ml culture medium was added 0.5 ml of 10^7 cells/ml. Three of the samples were prewarmed to 37°C and shaken at 37°C for 20 min, while the other was not prewarmed and was incubated at 0°C to measure nonspecific binding. All samples were then centrifuged at 500*g* at 0°C for 5 min and the supernatant discarded. The pellet was washed twice with 5 ml of cold culture medium containing 1.0 mM SPD to displace nonspecifically bound [^{14}C]SPD. The pellet was dissolved in 300 μl of 1.0 N NaOH at 60°C for 60 min and acidified with 700 μl 1.0 N HCl. Eight hundred microliters was transferred to a scintillation vial for counting.

a. L1210 cells were exposed to 10 μM [^{14}C]SPD and 100 μM SPD analog, and the inhibited uptake is expressed as percentage of uninhibited uptake. Each experiment was performed at least four times.

b. Cells were exposed to 10 μM SPD analog and [^{14}C]SPD at concentrations varying from 0.2 to 10 μM. The results are expressed as an inhibition constant (K_i) for the polyamine uptake apparatus. Each experiment was performed at least four times.

c. Cells were exposed to 10 μM [^{14}C]SPD and SPD analog at 10, 40, 70, 100, and 130 μM. The result is expressed as the linear correlation coefficient. The experiment was performed twice.

5. IC_{50} Measurements

L1210 cells were maintained in logarithmic growth in RPMI-1640 culture medium containing 2% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid–4-morpholinepropanesulfonic acid, 1.0 mM aminoguanidine, and 10% fetal calf serum. Cells (50 ml) were grown in 75-cm^2 tissue culture flasks at 37°C. Cells were treated while in logarithmic growth (3×10^4 cells/ml) with SPD analogs. After 48 h, cells were counted and the IC_{50} measured. Each experiment was performed at least four times.

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