Biological Properties of $N^4$- and $N^1,N^6$-Spermidine Derivatives in Cultured L1210 Leukemia Cells

Carl W. Porter, Jr., Paul F. Cavanaugh, Jr., Neal Stolowich, Barry Ganis, E. Kelly, and Raymond J. Bergeron

ABSTRACT

Eleven novel spermidine (SPD) derivatives were synthesized as potential anticancer agents and evaluated for their ability to compete with $[^3H]$SPD for cellular uptake, to inhibit cell growth, to affect polyamine biosynthesis, to suppress enzyme activity, and to substitute for SPD in supporting growth of cultured L1210 leukemia cells. The compounds included a series of $N^4$-SPD derivatives ($N^4$-methyl-SPD, $N^4$-ethyl-SPD, $N^4$-acetyl-SPD, $N^4$-hexyl-SPD, $N^4$-hexanoyl-SPD, $N^4$-benzyl-SPD, and $N^4$-benzoyl-SPD) and a series of $N^1,N^6$-SPD derivatives ($N^1,N^6$-bis(ethyl)-SPD, $N^1,N^6$-bis(acetyl)-SPD, $N^1,N^6$-bis(propyl)-SPD, and $N^1,N^6$-bis(propionyl)-SPD). Uptake studies revealed $N^4$-alkyl derivatives to be the most effective competitive inhibitors of $[^3H]$SPD uptake ($K_i$, 26 to 43 $\mu M$) followed by $N^1,N^6$-alkyl derivatives ($K_i$, 71 to 115 $\mu M$), then $N^4$-acyl derivatives ($K_i$, 115 to $>500 \mu M$), and $N^1,N^6$-acyl derivatives ($K_i$, $>500 \mu M$). The data indicate the relative importance of the terminal amines and of charge as determinants of cellular uptake. Of the 11 derivatives, only $N^4$-hexyl-SPD, $N^1$, $N^1,N^6$-bis(ethyl)-SPD, and $N^1,N^6$-bis(propyl)-SPD demonstrated antiproliferative activity at 0.1 mM with 50% inhibitory concentration values at 48 h of 30, 40, and 50 $\mu M$, respectively. In the case of the $N^1,N^6$-SPD derivatives, recovery from growth inhibition was enhanced considerably by exogenous SPD, suggesting involvement of polyamine depletion. At 10 to 30 $\mu M$, both $N^1,N^6$-bis(ethyl)-SPD and $N^1,N^6$-bis(propyl)-SPD inhibited polyamine biosynthesis as indicated by significant reductions in polyamine pools and in biosynthetic enzyme activities. The more effective of the two, $N^1,N^6$-bis(ethyl)-SPD, depleted intracellular putrescine and spermidine and reduced spermine by ~50% at 96 h and decreased ornithine and S-adenosylmethionine decarboxylase activities by 98 and 62%, respectively. Since neither derivative (at 5 $\mu M$) directly inhibited these enzymes from untreated cell extracts by significantly more than SPD itself, it is suspected that they act by regulating enzyme levels. As a measure of regulatory potential of the derivatives, ornithine decarboxylase was assayed in cells treated for 24 h and compared to the effects of 10 $\mu M$ SPD which reduced the enzyme activity by 80%. None of the $N^4$-SPD derivatives affected ornithine decarboxylase activity, while $N^1,N^6$-bis(ethyl)- and (propyl)-SPD were nearly as effective as SPD. Apparently, the central amine of the molecule is critical for regulatory function. Neither of the $N^1,N^6$-SPD derivatives was capable of functionally substituting for the depleted SPD pools as determined by their inability to prevent $\alpha$-difluoromethylornithine-mediated cytostasis. By contrast, several of the $N^4$-SPD derivatives were effective in this activity, suggesting that the $N^1,N^6$-terminal amines are critical for SPD function. It is concluded that inhibition of cell growth via polyamine depletion occurs with those SPD derivatives (i.e., $N^1,N^6$-bis(ethyl)- or (propyl)-SPD) which regulate biosynthetic enzyme activities in a manner similar to polyamines but which, unlike the natural polyamines, are incapable of performing in functions essential for cell growth.

INTRODUCTION

It is now apparent from a variety of experimental and clinical studies with the polyamine inhibitor, DFMO, that interference with polyamine biosynthesis and/or function represents a viable strategy in the treatment of proliferative disorders (30). In a previous paper (26), we described the biological properties of certain $N^4$-SPD derivatives and the potential of these compounds as anticancer agents. The derivatives used in that study represented available intermediates from the synthesis of bacterial siderophores (1, 2). While ineffective as antiproliferative agents, those particular derivatives served as model compounds to establish the following rationale for the design of additional SPD derivatives. Specifically, by utilizing the SPD carrier mechanism, the derivatives might (in similarity to the anticancer agent, MGBG) be concentrated in relatively high quantities (i.e., $\mu M$ range) in neoplastic cells and tissues (15, 29). There, they could evoke antiproliferative effects by (a) interfering with polyamine biosynthesis (i.e., direct enzyme inhibition, feedback regulation, or antizyme induction); (b) competing as analogues for polyamine binding sites involved in cell proliferation; (c) binding at polyamine sites and subsequently disrupting macromolecular structure and/or function; or (d) serving as vector molecules for delivering biologically active moieties (i.e., alkylating groups) or antineoplastics.

There have been previous attempts to identify potential anticancer agents among synthetic polyamine derivatives. Israel et al. (9) synthesized a series of linear aliphatic triamines and tetramines as homologues of SPD and SPM, respectively. These products retained the 3-aminopropyl-terminal function but showed variation in the PUT portion of the molecule, having from 2 to 12 methylene units. Four of the 32 compounds displayed antitumor activity against transplantable mouse tumors. The most active was the SPM homologue, $N,N^1$-bis(3-aminopropyl)nonane-1,9-diamine, which is believed to decompose into toxic metabolites via a plasma enzyme system. More recently, Weinstock et al. (31) synthesized and tested a number of homologues and acylated derivatives of SPD and SPM, some of which were able to inhibit cell growth.
which possessed in vitro activity against B16 melanoma and human epidermoid carcinoma of the nasopharynx. All of the above compounds are fundamentally different from the derivatives described here, and none of the active compounds was shown to inhibit polyamine biosynthesis or function.

In the present study, a series of N⁴-SPD derivatives and one of N¹,N⁸-SPD derivatives (see Table 1) were synthesized and evaluated for cell uptake and biological activity. Of the 11 SPD derivatives evaluated, 3 inhibited cell growth at concentrations of 50 μM or less, and 2 of these appear to do so by regulating polyamine biosynthesis in a manner similar to SPD itself. While they are primarily studied here as potential anticancer agents, they might also be useful as antiparasitic agents (30) or as experimental probes for examining polyamine metabolism and function in the context of cell proliferation.

MATERIALS AND METHODS

SPD Derivatives and DFMO. N⁴-Methyl-SPD, N⁴-ethyl-SPD, N⁴-acetyl-SPD, N⁴-hexyl-SPD, N⁴-hexanoyl-SPD, N⁴-benzyl-SPD, N⁴-benzyloxy-SPD, N¹,N⁸-bis(ethyl)-SPD, N¹,N⁸-bis(acetyl)-SPD, N¹,N⁸-bis(propyl)-SPD, and N¹,N⁸-bis(propionyl)-SPD were all synthesized specifically for this study following synthetic schemes and methodologies described elsewhere (1, 2). Their structures are presented in Table 1. DFMO was generously provided by the Merrell Dow Research Institute, Cincinnati, OH. MGBG was obtained from Aldrich Chemical Co., Milwaukee, WI.

Uptake Determinations. The SPD derivatives were studied for their ability to compete with [³H]SPD for uptake into ascites L1210 leukemia cells. The anticancer agent, MGBG, was included as a standard for comparison. L1210 leukemia cells were maintained as weekly i.p. passages in female DBA/2J mice. Cells were harvested 4 days after inoculation of 10⁶ cells by peritoneal lavage with RPMI-1640 cell culture medium.

The cells were washed twice with RPMI-1640, counted electronically, and adjusted to a density of 8 x 10⁸ cells/ml.

Prewarmed L1210 cell suspensions (5 x 10⁶/ml) were incubated in 1 ml of RPMI-1640 medium containing 2% 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid-3-(N-morpholino)propanesulfonic acid and 0.2, 0.5, 1.0, 2.0, 5.0, or 10 μM [³H]SPD (New England Nuclear Corp., Boston, MA) alone or in the presence of 10 or 100 μM polyamine or SPD derivative. The cells were incubated for 20 min at 37°C except for one tube containing 10 μM [³H]SPD which was not prewarmed and was incubated at 4°C to measure nonspecific binding. At the end of the incubation, the tubes were centrifuged at 900 x g for 5 min at 4°C. A 200-μl aliquot of supernatant was removed for scintillation counting, and the remainder of the supernatant was discarded. The pellet was washed twice with 5 to 7 ml of cold RPMI-1640 containing 1 mM SPD to displace nonspecifically bound [³H]SPD. The pellet was then dried with a cotton swab and dissolved in 200 μl of n NaOH at 60°C for 20 to 60 min. The material was neutralized with n HCl, diluted to 1 ml with distilled water, and transferred to a vial for scintillation counting. Results were expressed as pmol [³H]SPD per min per mg protein. Protein concentrations were determined by the method of Lowry et al. (13). Uptake data were analyzed for kinetic characteristics by using a Hewlett Packard HP-85 microcomputer programmed for nonlinear regression curve fitting (6).

Cell Growth. Murine L1210 leukemia cells were maintained in logarithmic growth as a suspension culture in RPMI-1640 medium containing 2% 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid-3-(N-morpholino)propanesulfonic acid, 1 mwa amino guanidine, and 10% NuSerum (Collaborative Research, Inc., Lexington, MA). Cells were grown in either glass culture tubes in a total volume of 2 ml or 25- or 75-sq cm tissue culture flasks in a total volume of 15 and 50 ml, respectively, under a humidified 5% CO₂ atmosphere at 37°C. Cultures were treated while in logarithmic growth as a suspension culture in RPMI-1640 medium containing 2% 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid-3-(N-morpholino)propanesulfonic acid, 1 mwa amino guanidine, and 10% NuSerum (Collaborative Research, Inc., Lexington, MA). Cells were grown in either glass culture tubes in a total volume of 2 ml or 25- or 75-sq cm tissue culture flasks in a total volume of 15 and 50 ml, respectively, under a humidified 5% CO₂ atmosphere at 37°C. Cultures were treated while in logarithmic growth (0.5 to 1 x 10⁶ cells/ml) with the SPD derivatives, or SPD at 0.1 mM. After 24 or 48 h, cells were removed from tubes for counting and viability determinations. Cell number was determined by electronic particle counting (Model ZF Coulter Counter; Coulter Electron...
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Competition with ^[3H]SPD for uptake into ascites L1210 cells was used as an indication of the ability of the SPD derivatives to gain entry into cells. We recognize that this measurement is not a certain indication of cell permeation by the drug and may represent competition at the membrane carrier. However, we find a general correlation has been observed between K_<i> values and IC_<50> values with paired compounds (Chart 2; also see Refs. 25 to 27) and have also noted a correlation with HPLC data when comparing relative peak heights. These were not presented here because of problems in completely separating the derivatives from existing polyamines.

RESULTS

Competition with ^[3H]SPD for uptake into ascites L1210 cells was used as an indication of the ability of the SPD derivatives to gain entry into cells. We recognize that this measurement is not a certain indication of cell permeation by the drug and may represent competition at the membrane carrier. However, we find a general correlation has been observed between K_<i> values and IC_<50> values with paired compounds (Chart 2; also see Refs. 25 to 27) and have also noted a correlation with HPLC data when comparing relative peak heights. These were not presented here because of problems in completely separating the derivatives from existing polyamines.

Competition with ^[3H]SPD for 20 min at 37°C, take up an average of 85 pmol SPD per 10^7 cells per min. Under these conditions, the K_<m> for ^[3H]SPD uptake was ~2 4 M, and the V_<max> was 117 pmol per mg protein per min. When cells were incubated at 4°C, cell-associated radioactivity was less than 10% of the value seen at 37°C.

Kinetic plots (Chart 1) and nonlinear regression analysis of uptake data (6) indicated uptake inhibition of the competitive type for most of the derivatives. From K_<i> values in Chart 2, it is apparent that the N^4-SPD derivatives were far better inhibitors of ^[3H]SPD uptake than the N^3,N^4-SPD derivatives. The larger the substituent at the N^3 position, the less effective the derivative was found to be in competing for uptake. Thus, N^4-methyl-SPD and N^4-ethyl-SPD were the most effective of the 7 N^4-SPD derivatives with K_<i> values of ~3.1 4M. When this central nitrogen
was made uncharged by acyl linkages, such as in the case of N4-acetyl-SPD, N4-hexanoyl-SPD, or N4-benzoyl-SPD, the derivatives competed much less effectively than their charged (at physiological pH) counterparts. This is clearly illustrated in the kinetic plot for N4-ethyl-SPD and N4-acetyl-SPD (Chart 1). The inhibitory characteristics of MGBG are included for comparison purposes. All of the N4-SPD derivatives containing a charged central nitrogen, including N4-benzyl-SPD, compete more effectively for uptake than the anticancer agent, MGBG (Kc, 51 μM).

As noted above, the N1,N8-SPD derivatives inhibited [3H]SPD uptake much less effectively than the N4-SPD derivatives (Chart 2). The Kc values for alkyl derivatives of the N4-SPD series ranged from 2.6 to 43.4 μM whereas those for the N1,N8-SPD series were 71 to 115 μM. As with the N4-SPD series, the acyl derivatives of the N1,N8-SPD series competed very poorly for uptake. The effects of the 11 SPD derivatives on the growth of cultured L1210 cells were initially screened at 0.1 mM (Chart 3). IC50 determinations were performed on compounds showing significant growth-inhibitory activity at 0.1 mM. Of the N4-SPD derivatives, only N4-hexyl-SPD significantly inhibited growth at 0.1 mM. It was determined that the IC50 for this derivative was 30 μM at 48 h. Of the 4 compounds belonging to the N1,N8-SPD series, the 2 alkyl derivatives, N1,N8-bis(ethyl)-SPD and N1,N8-bis(hexyl)-SPD, demonstrated significant antiproliferative activity at <0.1 mM with IC50 values at 48 h of 40 and 50 μM, respectively (Chart 3). N4-Hexyl-SPD, N1,N8-bis(ethyl)-SPD, and N1,N8-bis(hexyl)-SPD were studied in greater detail to determine whether growth inhibition was accompanied by perturbations of polyamine metabolism. At 30 μM, N4-hexyl-SPD inhibited cell growth by 43% at 48 h but did not seem to significantly alter intracellular polyamine pools (Table 2). By contrast, the derivatives N1,N8-bis(ethyl)-SPD and N1,N8-bis(hexyl)-SPD seemed to have a profound effect on polyamine biosynthesis as indicated by reductions in polyamine pools and decreases in cellular ornithine and AdoMet decarboxylase activities. Of the 2 derivatives, the N1,N8-bis(ethyl)-SPD was more active when compared on an equimolar basis. At the most effective concentration and time tested (30 μM, 96 h), this derivative depleted nearly all PUT and SPD, decreased SPM by 50%, and reduced ornithine and AdoMet decarboxylase activities by 98 and 62%, respectively, while inhibiting cell growth by 74%. Despite extensive growth inhibition, cell viability remained >95% as determined by trypan blue dye exclusion. At 10 or 30 μM, N1,N8-bis(propyl)-SPD also altered polyamine metabolism but was considerably less effective than the ethyl derivative.

Whether the reduction in polyamine pools by these 2 derivatives is directly related to growth inhibition could not be studied with prevention experiments using exogenous PUT or SPD, since either polyamine would compete with the derivative for cellular uptake (27). The problem could be approached, however, with recovery studies in which cells treated for 48 h were placed in drug-free medium or in medium containing 10 μM SPD (Chart 4). Under these conditions, recovery was slow to occur in drug-free medium with cells treated with N1,N8-bis(ethyl)-SPD. In the presence of SPD, however, the cells rapidly recovered at a rate similar than that of control cells. Identical findings were obtained with N1,N8-bis(hexyl)-SPD.

To determine whether the effects on polyamine biosynthesis involved direct inhibition of ornithine and/or AdoMet decarboxylases, the N1,N8-SPD derivatives were incubated for 90 min with an enzyme-containing cell extract from untreated L1210 cells. The enzyme reaction was found to proceed in a linear fashion for >2 h. At a 5 mM concentration, ornithine decarboxylase was inhibited by 22% with SPD, 25% with N1,N8-bis(ethyl)-SPD, and 28% with N1,N8-bis(propyl)-SPD. AdoMet decarboxylase was inhibited 18% with SPD, 10% with N1,N8-bis(ethyl)-SPD, and 16% with N1,N8-bis(hexyl)-SPD.

It was of interest to determine why the N4-SPD derivatives failed to inhibit cell growth, especially those which entered cells very effectively. All derivatives were screened at 10 μM (24 h) for their ability to suppress ornithine decarboxylase activity and

**Chart 2. Comparison of growth inhibition of [3H]SPD uptake by various SPD derivatives and MGBG based on Kc values.**

**Chart 3. Comparison of growth inhibition by the various N4- and N1,N8-SPD derivatives at 0.1 mM in cultured L1210 cells treated for 48 h.**

**Chart 4. Comparison of growth inhibition by the various N4- and N1,N8-SPD derivatives at 10 μM in cultured L1210 cells treated for 48 h.**

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Table 2

Effects of selected SPD derivatives and DFMO on growth, polyamine pools, and polyamine biosynthetic enzyme activities in cultured L1210 cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time (h)</th>
<th>% of control growth</th>
<th>% viable (trypan blue)</th>
<th>PUT (pmol/10^6 cells)</th>
<th>SPD (pmol/10^6 cells)</th>
<th>SPM (pmol/10^6 cells)</th>
<th>Ornithine (% of control)</th>
<th>AdoMet (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>48</td>
<td>100</td>
<td>100</td>
<td>420</td>
<td>3280</td>
<td>920</td>
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<td>48</td>
<td>100</td>
<td>100</td>
<td>400</td>
<td>3410</td>
<td>880</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>30 µM N^4-hexyl-SPD</td>
<td>48</td>
<td>57</td>
<td>87</td>
<td>&lt;50</td>
<td>&lt;300</td>
<td>820</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>10 µM N^4,N^4-bis(ethyl)-SPD</td>
<td>48</td>
<td>86</td>
<td>94</td>
<td>&lt;50</td>
<td>&lt;180</td>
<td>600</td>
<td>ND</td>
<td>ND</td>
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<td>96</td>
<td>&lt;50</td>
<td>&lt;230</td>
<td>640</td>
<td>8</td>
<td>66</td>
</tr>
<tr>
<td>30 µM N^4,N^4-bis(ethyl)-SPD</td>
<td>48</td>
<td>61</td>
<td>96</td>
<td>&lt;50</td>
<td>&lt;90</td>
<td>440</td>
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<td>38</td>
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<tr>
<td>10 µM N^4,N^4-bis(propyl)-SPD</td>
<td>48</td>
<td>97</td>
<td>98</td>
<td>300</td>
<td>2580</td>
<td>800^c</td>
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<td>91</td>
<td>1220</td>
<td>1200</td>
<td>750^c</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>30 µM N^4,N^4-bis(propyl)-SPD</td>
<td>48</td>
<td>88</td>
<td>94</td>
<td>130</td>
<td>1790</td>
<td>760^c</td>
<td>56</td>
<td>79</td>
</tr>
<tr>
<td>5 mM DFMO (for comparison)</td>
<td>48</td>
<td>55</td>
<td>96</td>
<td>&lt;50</td>
<td>&lt;50</td>
<td>1130</td>
<td>6</td>
<td>272</td>
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<tr>
<td></td>
<td>48</td>
<td>11</td>
<td>91</td>
<td>&lt;50</td>
<td>&lt;50</td>
<td>990</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

^a Control levels were 7.85 nmol CO₂ per 10^7 cells per h for ornithine decarboxylase and 2.27 nmol CO₂ per 10^7 cells per h for AdoMet decarboxylase.

^b ND, not determined.

^c Derivative eluted near SPD or SPM. The value represents an estimate.

^d An unknown compound coeluted with the PUT peak. The value represents a combination of both.

Chart 4. Recovery studies with untreated cells (CON) and cells treated with 30 µM N^4,N^4-bis(ethyl)-SPD (BES). Following a 48-h incubation, control and treated cells were reseeded in the presence or absence of 10 µM SPD. Note that those reseeded in SPD were able to recover from drug treatment.

Chart 5. Comparison of suppression of ornithine decarboxylase (ODC) activity by the various SPD derivatives and the polyamines, PUT, SPD, and SPM. Cells were treated for 24 h with 10 µM SPD derivative or polyamine. Column height correlates positively with enzyme suppression. The dotted line indicates enzyme activity in cells not treated with derivatives or polyamines.

compared in this activity with the natural polyamines (Chart 5). None of the N^4-SPD derivatives lowered enzyme activity by more than 25%. Apparently, the N^4 locus of SPD is critical for this cellular effect. By contrast, the 2 N^4,N^4-alkyl derivatives, N^1,N^4-bis(ethyl)- and (propyl)-SPD, decreased cellular ornithine decarboxylase substantially under identical conditions. In fact, their ability to do so was comparable to that of the natural polyamines, PUT and SPD, and only slightly less than SPM. Under these treatment conditions, AdoMet decarboxylase was not significantly (<10%) decreased by any of the analogues (data not shown).

All SPD derivatives were then tested for their ability to prevent DFMO-induced cytostasis and, hence, to replace SPD in supporting cell growth (Chart 6). Cells were incubated for 48 h with DFMO alone (50% growth inhibition, 0% prevention), DFMO plus SPD (2% growth inhibition, 100% prevention), or DFMO plus a derivative. Prevention of cytostasis at greater than 50% of that accomplished by SPD was taken to indicate that the derivative was an effective functional substitute for SPD in supporting cell growth. Of the N^4 derivatives, methyl-, ethyl-, and acetyl-SPD, all prevented cytostasis at greater than 50% of that accomplished by SPD itself. By contrast, none of the N^4,N^4 derivatives was capable of preventing DFMO cytostasis or of enhancing it.
DISCUSSION

As the initial step in the biological evaluation of the SPD derivatives, uptake competition studies with [3H]SPD were performed to determine the ability of the derivatives to utilize the SPD carrier mechanism, a property which might relate to their entry into cells and potentially to their selectivity for tumor tissue as described previously (26). Basically, the findings indicate that N1'-Spd derivatives compete better for [3H]SPD uptake than the N1,N8-SPD compounds and that the alkyl derivatives of either the N1'- or N1,N8-SPD series are much more effective competitors than the acyl derivatives (Chart 2). Whether certain of the 5 acyl derivatives enter cells at all is doubtful.

Clearly, the primary amines (N1' and N8) of SPD are more critical than the secondary amine (N4') as determinants of uptake, since the N1',N8 derivatives compete less effectively than N4'-derivatives. The presence of a positive charge at any of the amines also plays an important role in uptake specificity as evidenced by the relative inability of acyl derivatives to compete effectively with [3H]SPD (Charts 1 and 2).

Of the 7 N1'-Spd derivatives tested for antiproliferative effects (Chart 3), only N1'-hexyl-SPD displayed significant activity with an IC50 value of 30 μM at 48 h. Both polyamine pool size analysis and enzyme data suggest that it does not act by perturbing polyamine metabolism. Two of the 4 N1',N8-SPD derivatives, N1', N1,N8-bis(ethyl)-SPD and N1',N8-bis(propyl)-SPD, displayed meaningful growth-inhibitory properties with IC50 values of 40 and 50 μM, respectively. The greater activity of the ethyl derivative may relate to its apparent ability to enter cells more effectively as evidenced by the relatively inability of acyl derivatives to compete effectively with [3H]SPD (Charts 1 and 2).

The reductions in polyamine pools were apparently due to a lowering of the activities of the biosynthetic enzymes, ornithine and AdoMet decarboxylase. At 30 μM and after 96 h, N1',N8-bis(ethyl)-SPD eliminated intracellular PUT and SPD, reduced SPM by 50%, and decreased ornithine and AdoMet decarboxylase activities by 98 and 62%, respectively. It is likely that these enzyme effects are brought about by regulatory mechanisms (16) since, at concentrations approximating those achieved in the cell (5 mM), the derivatives failed to directly inhibit the decarboxylases from a cell extract of untreated cells by significantly more than SPD itself.

A number of mechanisms have been implicated in the regulation of polyamine enzymes in eukaryotic cells (16). Those limiting enzyme activity include: (a) inhibition of enzyme synthesis at the level of transcription and/or translation (5, 10, 16); (b) changes in the rate of enzyme turnover (18); (c) posttranslational modification of the enzyme by reversible protein phosphorylation (11); (d) interconversion from an active to an inactive form (18); and (e) induction of the specific enzyme inhibitor, ornithine decarboxylase antizyme, which complexes with and inactivates the enzyme (3, 8). At present, we are uncertain which of these mechanisms might be activated by the N1',N8-SPD derivatives, but similarities in responses suggest it is the same as that elicited by the natural polyamines.

The lack of antiproliferative potential among the N4'-SPD derivatives (excepting N4'-hexyl-SPD) is probably attributable to their general inability to suppress ornithine decarboxylase activity (Chart 5). This is somewhat surprising, since certain of the N4'-SPD derivatives (i.e., N4'-methyl-SPD) are less chemically modified than either N1',N8-SPD derivative and should be more likely to behave as SPD in performing this function. Paradoxically, their close resemblance to SPD probably accounts for their superior uptake properties relative to the N1',N8-SPD derivatives, yet they are less effective in suppressing enzyme activities.

That exogenous SPD regulation of ornithine decarboxylase does not result in growth inhibition is due to replenishment of depleted endogenous SPD pools, so that cellular functions required for proliferation can proceed. By comparing the ability of the various SPD derivatives to prevent DFMO cytostasis during a limited (48-h) incubation, we have attempted to determine which of them can substitute for SPD in these same functions. While several of the N4'-SPD derivatives were very effective in this regard (Chart 6), none of the N4',N8'-SPD derivatives was capable of this activity. This distinguishes them from SPD and may account for their growth-inhibitory properties.

Recently (4), we reported that extended incubations (96 h) of cells in the presence of DFMO and one of the functional substitutes for SPD, such as N4'-methyl-SPD or N4'-ethyl-SPD, will eventually result in growth inhibition. Detailed experiments (4) indicate that growth inhibition under these circumstances is not due to the inability of the derivatives to substitute for SPD but rather to intracellular depletion of SPM and/or to the inability of the derivatives to be converted in significant quantities to a functional SPM analogue (4).

The biological responses of the various SPD derivatives have been summarized in Table 3. It seems plausible that those SPD derivatives which enter cells effectively might negatively regulate biosynthesis, DFMO (Ref. 13; Table 2) and S-adenosyl-1,8-diamino-3-thiooctane (24), which characteristically deplete PUT and/or SPD without lowering SPM.

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A number of mechanisms have been implicated in the regulation of polyamine enzymes in eukaryotic cells (16). Those limiting enzyme activity include: (a) inhibition of enzyme synthesis at the level of transcription and/or translation (5, 10, 16); (b) changes in the rate of enzyme turnover (18); (c) posttranslational modification of the enzyme by reversible protein phosphorylation (11); (d) interconversion from an active to an inactive form (18); and (e) induction of the specific enzyme inhibitor, ornithine decarboxylase antizyme, which complexes with and inactivates the enzyme (3, 8). At present, we are uncertain which of these mechanisms might be activated by the N1',N8-SPD derivatives, but similarities in responses suggest it is the same as that elicited by the natural polyamines.

The lack of antiproliferative potential among the N4'-SPD derivatives (excepting N4'-hexyl-SPD) is probably attributable to their general inability to suppress ornithine decarboxylase activity (Chart 5). This is somewhat surprising, since certain of the N4'-SPD derivatives (i.e., N4'-methyl-SPD) are less chemically modified than either N1',N8-SPD derivative and should be more likely to behave as SPD in performing this function. Paradoxically, their close resemblance to SPD probably accounts for their superior uptake properties relative to the N1',N8-SPD derivatives, yet they are less effective in suppressing enzyme activities.

That exogenous SPD regulation of ornithine decarboxylase does not result in growth inhibition is due to replenishment of depleted endogenous SPD pools, so that cellular functions required for proliferation can proceed. By comparing the ability of the various SPD derivatives to prevent DFMO cytostasis during a limited (48-h) incubation, we have attempted to determine which of them can substitute for SPD in these same functions. While several of the N4'-SPD derivatives were very effective in this regard (Chart 6), none of the N1',N8-SPD derivatives was capable of this activity. This distinguishes them from SPD and may account for their growth-inhibitory properties.

Recently (4), we reported that extended incubations (96 h) of cells in the presence of DFMO and one of the functional substitutes for SPD, such as N4'-methyl-SPD or N4'-ethyl-SPD, will eventually result in growth inhibition. Detailed experiments (4) indicate that growth inhibition under these circumstances is not due to the inability of the derivatives to substitute for SPD but rather to intracellular depletion of SPM and/or to the inability of the derivatives to be converted in significant quantities to a functional SPM analogue (4).

The biological responses of the various SPD derivatives have been summarized in Table 3. It seems plausible that those SPD derivatives which enter cells effectively might negatively regulate
polyamine biosynthesis by reducing the activities of the biosynthetic enzymes via one or more of the mechanisms described above. Under such conditions, growth inhibition would be expected to occur with derivatives that are incapable of substituting for SPD in functions required for cell proliferation. In Table 3, only N1,N8-bis(ethyl)-SPD and N1,N8-bis(propyl)-SPD fit this profile of biological activities. Although additional studies are clearly necessary to confirm this sequence of events, the possibility suggests an intriguing strategy for achieving growth inhibition by polyamine depletion. Such a regulatory approach might offer several advantages over one relying on specific enzyme inhibitors: (a) the derivatives should be active at relatively low concentrations as indicated by the ability of μM concentrations of SPD and SPM to effectively suppress enzyme activity (5, 17); (b) the levels of more than one biosynthetic enzyme may be affected at the same time; (c) compensatory increases in related enzymes may not occur as they do with enzyme inhibitors (22); and (d) depletion of all polyamines including SPM might be possible. That such a regulatory approach has not emerged as an antiproliferative strategy in other biosynthetic systems may be attributable to the unique properties of the key polyamine enzymes, ornithine and AdoMet decarboxylase. Both are highly inducible, extremely short lived, and sensitive to product regulation (20). Presently, we are attempting to substantiate this approach by further evaluating the N1,N8-alkyl derivatives and by designing and synthesizing new polyamine derivatives having greater binding affinities.

Certain diamine analogues of PUT, particularly 1,3-diaminopropane (28), have also been shown to produce a rapid decrease in ornithine decarboxylase activity. While 1,3-diaminopropane and the N1,N8-SPD derivatives seem to behave similarly in this respect, they differ distinctly from one another in other ways (8). Unlike 1,3-diaminopropane, N1,N8-bis(ethyl)- or (propyl)-SPD (a) acts at μM (as opposed to mM) concentrations, (b) does not assume the function(s) of natural polyamines in supporting cell growth, (c) is not converted to a higher polyamine, (d) decreases (as opposed to increases) AdoMet decarboxylase activity, (e) seems more effective in decreasing SPM pools, and (f) may not be readily metabolized in vivo, since primary amines are not available. The proven effectiveness of 1,3-diaminopropane in decreasing polyamines in in vivo systems (28) provides indication that the more potent N1,N8-bis(ethyl)-SPD may also be active in vivo.

Finally, distinct structure-activity relationships relevant to the SPD molecule have become apparent during this study (Table 3). Terminal amines of SPD are more critical in cellular uptake and functions required for cell proliferation than is the central amine. By contrast, the central amine seems to be more critically involved in the regulatory activities of the molecule than the terminal amines. This information will be particularly useful in designing new derivatives to affect specific aspects of polyamine metabolism and/or function.

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BIOLOGICAL PROPERTIES OF SPD DERIVATIVES


Biological Properties of $N^4$- and $N^1,N^8$-Spermidine Derivatives in Cultured L1210 Leukemia Cells

Carl W. Porter, Paul F. Cavanaugh, Jr., Neal Stolowich, et al.


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