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Synthesis of Bis-Spermine Dimers that are Potent Polyamine Transport Inhibitors

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Abstract—A series of novel spermine dimer analogues was synthesized and assessed for their ability to inhibit spermidine transport into MDA-MB-231 breast carcinoma cells. Two spermine molecules were tethered via their N^1 primary amines with naphthalenedisulfonic acid, adamantanedicarboxylic acid and a series of aliphatic dicarboxylic acids. The linked spermine analogues were potent polyamine transport inhibitors and inhibited cell growth cytostatically in combination with a polyamine synthesis inhibitor. Variation in the linker length did not alter polyamine transport inhibition. The amount of charge on the molecule may influence the molecular interaction with the transporter since the most potent spermidine transport inhibitors contained 5–6 positive charges.

The polyamines (Fig. 1), putrescine (PUT), spermidine (SPD), and spermine (SPM) contribute to many essential cellular functions through their interactions with DNA, RNA, proteins, and lipids.[1–6] Polyamines are essential for cell proliferation through their involvement in DNA replication, cell cycle regulation, and protein synthesis. The importance of their function is highlighted by the fact that specific biosynthesis, degradation, uptake and excretion pathways tightly control cellular polyamine levels.[7–9] Excessive cell growth has been correlated with high levels of intracellular polyamines.[1] Numerous tumor cell types have been analyzed and shown to have higher polyamine levels than normal, non-tumorigenic cells. Within a single tumor type, the more highly malignant tumors often have higher polyamine levels.[10] For these reasons, depletion of intracellular polyamine levels is an attractive chemotherapeutic approach for the inhibition of tumor growth.

A number of methods have been used to deplete polyamine levels and thereby inhibit cell growth. Early attempts utilized polyamine synthesis inhibitors that targeted *S*-adenosylmethionine decarboxylase or ornithine decarboxylase.[11–13] Compounds such as methylglyoxal bis(guanylhydrazone) (MGBG) were either too toxic[14] or, for example α-difluoromethylornithine (DFMO), were less effective than expected because the cell compensated for decreased synthesis by enhancing cellular polyamine transport.[8,15] These results led to the hypothesis that a polyamine transport inhibitor, in combination with a synthesis inhibitor, would lead to greater polyamine depletion and cellular growth arrest.

The mammalian polyamine transporter is highly regulated, energy-dependent and able to transport against a large concentration gradient.[8,9,16–19] Little is known about the structure of the mammalian transporter, although polyamine binding proteins have been identified and the *Escherichia coli* transporter has been fully characterized.[6] A number of compounds have been developed in recent years that inhibit polyamine transport. Poulin et al. have synthesized a series of polyamine transport inhibitors beginning with the cross-linked dimeric polyamine analogue 2,2′-dithiobis(*N*-ethyl-spermine-5-carboxamide) (DESC).[20] These researchers have more recently developed a second generation of polyamine dimers that in some instances show substantial

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improvement with respect to polyamine transport inhibition.[21] Several of these analogues have affinities for the polyamine transporter that are nearly equal to or greater than SPM (reported K_m for uptake was 2.7 μM). The polyamine analogue bis(3-aminopropyl)-piperazine (BAP) was found to decrease human peripheral blood monocyte SPM accumulation in a dose dependent fashion with a 50% inhibitory concentration of 7.0 μM.[22] A polymeric spermine-glutaraldehyde analogue also has been described as a competitive transport inhibitor of polyamines with a relatively potent K_i of 10^{-6} M.[23] However, its utility as a transport inhibitor may be limited because of its high toxicity in mammalian cells. A series of N^1-monosubstituted PUT, SPD and SPM derivatives has also been synthesized by a number of laboratories.[24–26] In general, the SPM derivatives are more effective transport inhibitors than the SPD and PUT analogues.

Our laboratory has synthesized a series of primary mono-substituted polyamines.[27,28] A number of these compounds, such as N^1-spermine-L-lysinyl amide **1** (Fig. 1), are very potent competitive inhibitors of polyamine transport with nanomolar K_i values against SPD uptake. Since a number of the N^1-monosubstituted SPM polyamine analogues were found to be such potent inhibitors of polyamine transport, it was reasoned that dimerization of two SPM molecules might lead to substantially more potent inhibition. The molecule could allow for an additional SPM interaction at a peripheral site on the same transporter.[29] Alternatively, binding or interaction of one SPM molecule to a transporter may actually enhance the interaction of a second molecule to another transporter in close proximity. There are numerous compounds with the latter mechanism of action, most notably the bis-intercalators,[30] that exhibit potent anticancer activity. Poulin has also demonstrated that crosslinking of *sym*-norspermidine via its secondary amino groups with compounds such as a planar *p*-xylyl crosslinker were very potent and effective polyamine transport inhibitors.[21]

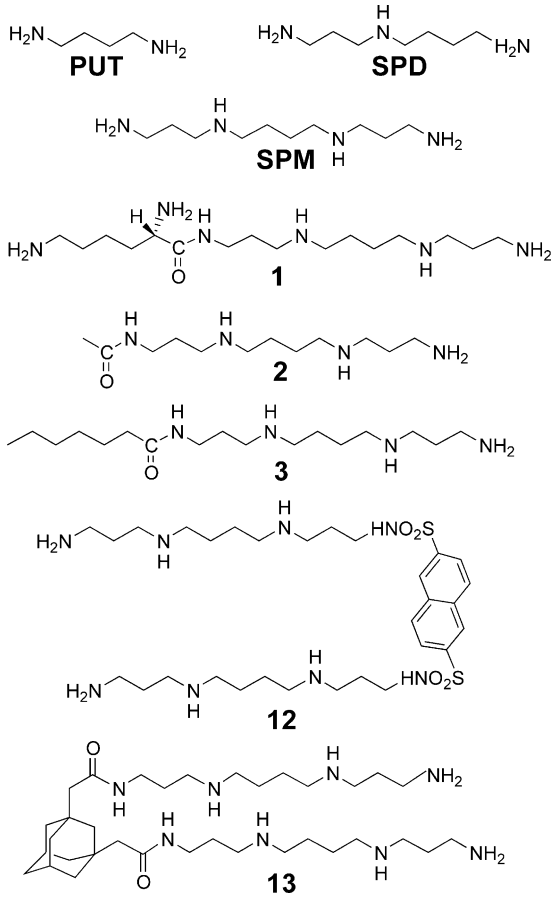


Figure 1. Structures of various polyamine compounds.

Crosslinking at the primary amines of spermine is another approach for the synthesis of polyamine dimers. This strategy has the advantage of allowing the secondary amines to interact unencumbered with the transporter. For this reason, a series of symmetrical SPM dimers was synthesized to explore the relationship between linker structure, length and transport inhibition. The linkers that joined the two SPM molecules consisted of 2,6-naphthalenedisulfonic acid, 1,3-adamantanedicarboxylic acid, and a series of aliphatic dicarboxylic acids that varied by carbon length. These were tested in vitro for their ability to inhibit polyamine transport and growth of an MDA-MB-231 breast carcinoma cell line (MDA)[31] when used in combination with DFMO. DFMO blocks polyamine synthesis by irreversibly inhibiting ornithine decarboxylase.[32]

MDA cells were plated in 96-well plates such that they would be in log growth for the duration of the assay. The day after plating, drugs were added to the cells, cells were grown for six days, and cell growth was measured by MTS/PMS dye assay (Cell Titer 96 Aqueous Non-Radioactive Cell Proliferation Assay; Promega, Madison, WI, USA). The assay was performed in the presence of 0.23–1 mM DFMO, 0.5–1 μM SPD, and 1 mM aminoguanidine (AG) to inhibit serum amine oxidase activity. Initial experiments used lower concentrations of DFMO, but this was later increased to insure complete inhibition of ODC and polyamine synthesis. However, no experimental differences were observed when using concentrations between 0.23 and 1 mM DFMO. DFMO inhibits intracellular polyamine synthesis and induces polyamine transport.[1,8,25,33] As a consequence, the limited quantities of polyamines present in the medium are depleted which can lead to cell growth inhibition. To insure that DFMO alone would not inhibit cell growth, 0.5–1 μM SPD was added to the medium. Under these conditions, polyamine analogues that prevent the uptake of exogenously added SPD inhibit cell growth. Spermidine transport inhibition kinetic data supporting this contention are also presented later in the manuscript. EC_{50} represents the concentration of the SPM dimer that resulted in 50% of maximum growth inhibition. The maximum growth inhibition observed with the combination of the bis-spermine and DFMO ranged between 40 and 80% of control growth as seen in Figure 2A. Therefore, the dual treatment was cytostatic. Most of the compounds tested had no effect on cell growth when tested alone. However, a few compounds were cytotoxic with and without DFMO treatment (Fig. 2B).

MDA cells were plated in 24-well plates. Three days later, [2,3-^{3}H]PUT (DuPont NEN), [2,9-^{3}H]SPD (DuPont NEN, Boston, MA, USA), or [1,4-^{14}C]SPM (Amersham, Arlington Heights, IL, USA) were added alone or simultaneously with the polyamine transport inhibitors. The cells were incubated at 37 °C for 4 min with SPM and for 15 min with PUT and SPD to determine the initial rate of polyamine uptake. The cells were then washed three times with PBS, lysed with 100 μL of 1% SDS, mixed with 1 mL of scintillation fluid, and the amount of radiolabel incorporated into the cells was determined by liquid scintillation counting. To determine a K_i value, four radioactive substrate concentrations (0.3–3 μM), five inhibitor concentrations (0.01–0.3 μM) and a control (no compound) were tested. The K_i and K_m values were determined using double reciprocal Lineweaver–Burke plot analyses. K_i values were determined from linear equations derived from graphing the slopes of Lineweaver–Burke plot versus inhibitor concentration, with $K_i = y$-intercept/slope. K_m values for PUT, SPD, and SPM were determined directly from the Lineweaver–Burke plot: $K_m = -(1/x\text{-intercept})$.

All chemicals except for 2,6-naphthalenedisulfonyl chloride (The Sigma-Aldrich Library of Rare Chemicals) were purchased from Aldrich Chemical Company (Milwaukee, WI, USA) and used without further purification. The bis(*p*-nitrophenyl) ester linker moieties were prepared by standard procedures giving typical yields of approximately 90%.[34,35] Mono-^{t}Boc-spermine was synthesized via a literature procedure using di-*tert*-butyl dicarbonate.[36] The desired monoprotected spermine was separated from the di-^{t}Boc side-product and unreacted SPM using cation exchange chromatography (Bio-Rex, NH_4^+ salt form) and eluting with a gradient of 0–2 N NH_4OH. The average yield after purification was 30%.

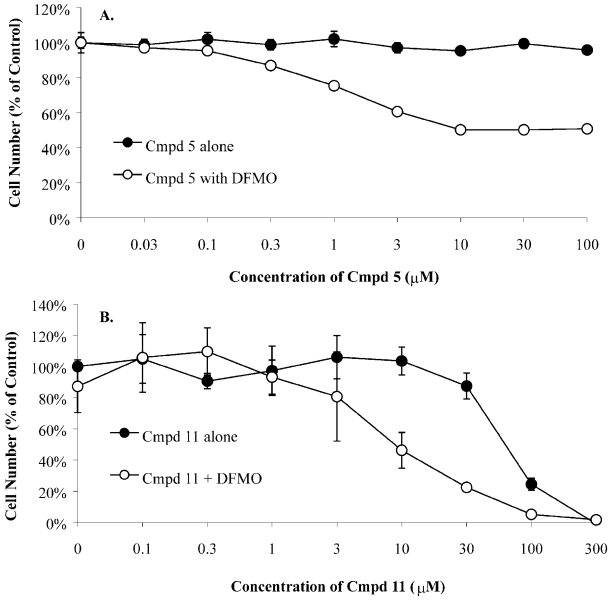


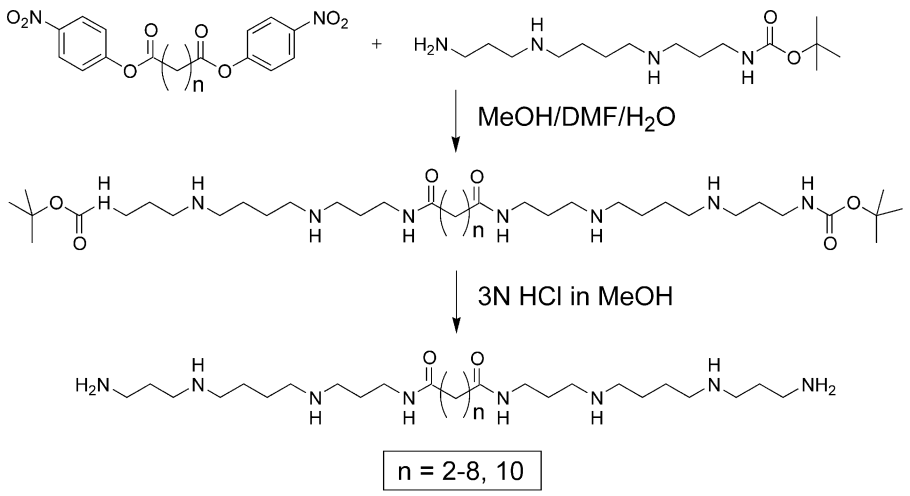
Figure 2. Growth inhibition of MDA cells with **5** (A) or **11** (B) with and without DFMO. MDA cells were incubated with 1 μM SPD, 1 mM AG, and various concentrations of **5** or **11** with 0.23 μM (A) or 1 mM (B) DFMO during a 6-day growth assay. Cell number was determined by MTS/PMS assay from triplicate wells. Bars, SD.

The bis-spermines (Scheme 1) were prepared by adding the appropriate bis(*p*-nitrophenyl) ester to a solution of 2.2 equiv of N^1-^{t}Boc-spermine. The crude di-^{t}Boc-protected SPM dimers were dissolved in 1:1 methanol/water and purified by cation exchange chromatography (Bio-Rex, NH_4^+ salt form) by eluting with 1:1 methanol/water followed by a gradient of 0 to 1 or 2 N NH_4OH in 1:1 methanol/water. The average yield after purification was 20%. Generally, affinity for the column decreased as the linker increased in length. The ^{t}Boc groups were removed by treatment with 3 N HCl in MeOH with a typical yield of 80%. Analogue **1** was synthesized as described previously.[27] All compounds gave spectral data in agreement with the proposed structures.[37]

Compound **1** (Table 1) is one of the first identified polyamine transport inhibitors that competitively inhibits transport of polyamines in the nanomolar range.[27] As shown in Table 1, all of the aliphatic bis-spermine analogues synthesized were potent competitive inhibitors of polyamine transport with K_i values comparable to **1**. All had K_i values lower than the transporter K_m values for the three naturally occurring polyamines (Table 1) suggesting that these analogues have greater affinity for the transporter. To determine if the second SPM moiety contributed to polyamine transport inhibition, N^1-acetyl spermine (**2**) and N^1-heptanyl spermine (**3**) (Fig. 1) were synthesized with a C2 or C7 linker, respectively, but without the second spermine molecule. The K_i values for **2** and **3** were between 2- and 9-fold higher than the K_i for the bis-spermines (Table 1) suggesting that an additional SPM molecule facilitates binding to or interaction with the transporter. An underlying consideration is the difference in charge between the molecules. At physiological pH of 7.2, the vast majority of amino groups will be in their positively charged ammonium state. Compounds **2** and **3** contain 3 positively charged protonated amines but the most potent transport inhibitors were found to have either five or six. This suggests that increasing charge on the SPM analogues enhances polyamine transport inhibition. The enhancement observed with increasing charge appears to plateau at approximately five positive charges. The distance of the additional charge from the amide bond of the derivatized SPM analogues is not critically important since the linker length was varied considerably.

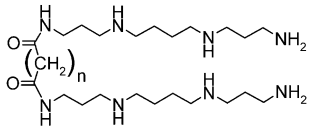
Variation in linker length did not appear to dramatically affect K_i values as would have been predicted if a particular length favored transporter dimerization. These results suggest that, like **1,** the molecules may interact in similar fashion with respect to the transporter and that binding of the ligand with the transporter primarily involves only one SPM molecule. Alternatively, it is possible that a linker longer than 12 carbons is required for efficient dimerization of polyamine transporters or there is a low probability of finding transporters in close proximity on the plasma membrane. There is also the possibility that the transporters have low or hindered mobility within the membrane.

The potent polyamine transport inhibition exhibited by the aliphatic bis-spermine analogues translated to



Scheme 1. Synthesis of aliphatic dicarboxylic acid linked analogues.

Table 1. EC_{50}, K_i or K_m values of polyamine compounds determined with MDA cells



Compd	*n* (Linker length)	K_m[a] (μM)	K_i[a] (μM)	EC_{50} with DFMO[a] (μM)
PUT	See Fig. 1	1.28±0.34	—	—
SPD	See Fig. 1	0.11±0.03	—	—
SPM	See Fig. 1	2.0±0.9	—	—
1	See Fig. 1	—	0.03±0.02	7±4
2	See Fig. 1	—	0.18[b]	>1000
3	See Fig. 1	—	0.28[b]	63[c]
4	2	—	0.03±0.02	2±2
5	3	—	0.06±0.02	1.6±0.6
6	4	—	0.07±0.01	2.2±0.2
7	5	—	0.04±0.03	6±3
8	6	—	0.06±0.03	3.7±0.9
9	7	—	0.07±0.05	3±1
10	8	—	0.08±0.04	9±1[d]
11	10	—	0.03±0.02	8±2[e]
12	See Fig. 1	—	0.06±0.03	230±60
13	See Fig. 1	—	0.06[f]	>300

[a]Unless otherwise noted, K_m, K_i and EC_{50} values were averaged from at least three independent determinations. Data are presented as the mean±SD. Radiolabled PUT, SPD, or SPM were used to determine, respectively, the K_m values of PUT, SPD, and SPM. K_i values were determined by measuring the ability of the compounds to inhibit uptake of radiolabeled SPD. The concentration of DFMO used to determined EC_{50} ranged between 0.23 and 1 mM.
[b]K_i and EC_{50} values were averaged from two independent determinations. Repeat determinations were generally within 25% of each other.
[c]Compound **3** was cytotoxic. Its EC_{50} value was the same with or without DFMO.
[d]Compound **10** was cytotoxic without DFMO with an EC_{50} of 240±59 μM.
[e]Compound **11** was cytotoxic without DFMO with an EC_{50} of 53±23 μM.
[f]This value was an average of two independent experiments that were within 10% of each other.

efficient growth inhibition of MDA cells when used in combination with the ODC inhibitor DFMO. A number of these analogues gave similar growth inhibition results when tested in identical fashion with the PC-3 prostate cell line (data not shown). In a 6-day assay, no growth inhibition was observed with DFMO alone due to the ability of the cells to meet their polyamine needs by the uptake of the exogenous SPD added to the extracellular medium. In combination with DFMO, analogues that prevent transport of exogenous polyamine into the cell were expected to inhibit cell growth. Like **1**, analogues **4**–**9** of the bis-spermine analogues were cytostatic, inhibiting MDA cell growth in the low μM range with DFMO present (exemplified in Fig. 2A). As a result, maximum growth inhibition reached a plateau between 40–80% of control growth. Compounds **4**–**9** had no effect on cell growth when tested alone. Although analogues **10** and **11** were growth inhibitory in the presence of DFMO, these also exhibited cellular toxicity with or without DFMO present (Fig. 2B, Table 2). N^1-heptanyl spermine (**3**) was similarly toxic (Table 1). It is possible that the increased hydrophobicity of these analogues may disrupt cell membranes or interact unfavorably with cellular structures.

Compounds **12** and **13** (Fig. 1) linked the two spermines with 2,6-naphthalene disulfonic acid, or 1,3-adamantanedicarboxylic acid, respectively. Both **12** and **13** were potent inhibitors of polyamine transport with K_i values below 100 nM. However, in the presence of DFMO, **12** was a very poor inhibitor of MDA cell growth and **13** showed no growth inhibition at the highest concentration tested (300 μM). It is possible that **12** and **13** were substrates for the transporter and could enter the cell. Once these molecules entered the cell, they may have either substituted for the normal polyamines or were degraded into SPM inside the cell, thus supplying the cell with polyamine. The other bis-spermine transport inhibitors are presumably cell-impermeant. Thus, they do not enter the cell but prevent transport of exogenous polyamines into the cell and deprive the cell of an extracellular polyamine source.

The results from this study indicate that N^1-linked bis-spermine polyamines are potent polyamine transport inhibitors. Although the length and the structure of the linker connecting the two SPM molecules were varied considerably, all of the bis-spermines inhibited poly-

amine transport nearly equally. These results suggest that all of the molecules interacted with the transporter in a similar manner. It is also highly likely that charge plays some role in this interaction since the mono-spermine analogues **2** and **3**, which carried fewer charges, were not as effective polyamine transport inhibitors. Most but not all of the analogues were similarly effective growth inhibitors in the presence of the polyamine synthesis inhibitor DFMO. In those instances where the bis-spermines were poor growth inhibitors, it is possible that they were also substrates and transported into the cell. The relationship between a molecule's ability to inhibit polyamine transport versus growth inhibition in the presence of DFMO is not directly comparable. In the first instance, K_i measurements are determined over a short time frame whereas growth inhibition is determined over the course of 6 days. There are a large number of potential biochemical effects that may be altered over this extended time frame. It was previously shown that **1**, in the presence of DFMO, reduced intracellular polyamines levels after 6 days.[27] Presumably, the growth inhibition observed with the bis-spermines in combination with DFMO was also a result of reduced polyamine levels. Although not in the scope of this paper, measuring intracellular polyamine levels after long-term incubation with DFMO and the bis-spermines would be insightful.

There are a number of potential mechanisms by which the polyamine analogues could inhibit polyamine transport. These molecules may bind to the transporter and physically block the entry of exogenous polyamines into the cell. The interaction could also be indirect such that binding of the polyamine analogue to the transporter induces a conformational change. The conformational change would prevent transport of exogenous polyamines into the cell. The structural and molecular determinants responsible for this inhibition are currently not well understood. Advancement in this area awaits the identification of the protein and solving the crystal structure of the polyamine transporter. Until that time, future work will continue to focus on developing more potent polyamine transport inhibitors to gain insights into the structure–function relationships leading to potent polyamine transport inhibition.

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31. The human breast carcinoma cell line MDA-MB-231 will be referred to as MDA. It was obtained from ATCC (Rockville, MD, USA) and cultured in DMEM, 10% fetal bovine serum, and 10% CO_2. Medium was obtained from Mediatech, Inc. (Herndon, VA, USA) and serum from Gibco BRL (Gaithersburg, MD, USA). 50 U/mL penicillin, 50 μg/mL streptomycin and 2 mM L-glutamine (all from BioWhittaker, Walkersville, MD, USA) were included in all cultures. DFMO was obtained from Marion Merrell Dow (Cincinnati, OH, USA). When cells were cultured with polyamines or bis-spermines, 1 mM AG (Sigma) was included to inhibit serum amine oxidase activity.
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37. Data for **5** (·6 HCl) as an example. HRMS were run at The Scripps Research Institute using MALDI FTMS with DHB matrix on an IonSpec HiRes MALDI instrument. NMR spectra were recorded with a Bruker WM 500 spectrophotometer. ^{1}H NMR were recorded at 500 MHz and ^{13}C NMR were recorded at 125.8 MHz. Chemical shifts were set to residual HOD peak in sample (4.80 ppm). We suspect the multiplets in the ^{13}C spectra are due to rotamers about the amide functionalities (1:3:1 rotamer ratio, * denotes minor rotamer peaks). ^{1}H NMR δ: 1.82 (m, 8H), 1.96 (m, 6H), 2.16 (m, 4H), 2.35 (t, 4H), 3.15 (m, 20H), 3.34 (t, 4H). ^{13}C NMR δ: 25.08, 26.17, 27.1, 28.96, 38.26, 39.24*, 39.44, 39.66*, 39.78*, 39.96, 40.15*, 47.71*, 47.9, 48.08*, 48.34*, 48.5, 48.68*, 50.05*, 50.18*, 50.23, 50.36, 50.41*, 50.55*, 179.4. HRMS m/z calcd for $C_{25}H_{56}N_8O_2$ $(M+Na)^+$ 523.4424, found 523.4419.