Amino Acid/Spermine Conjugates: Polyamine Amides as Potent Spermidine Uptake Inhibitors

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In this paper we describe the synthesis and characterization of a series of simple spermine/amino acid conjugates, some of which potently inhibit the uptake of spermidine into MDA-MB-231 breast cancer cells. The presence of an amide in the functionalized polyamine appeared to add to the affinity for the polyamine transporter. The extensive biological characterization of an especially potent analogue from this series, the Lys-Spm conjugate (**31**), showed this molecule will be an extremely useful tool for use in polyamine research. It was shown that the use of **31** in combination with DFMO led to a cytostatic growth inhibition of a variety of cancer cells, even when used in the presence of an extracellular source of transportable spermidine. It was furthermore shown that this combination effectively reduced the cellular levels of putrescine and spermidine while not affecting the levels of spermine. These facts together with the nontoxic nature of **31** make it a novel lead for further anticancer development.

Introduction

Decades of research on the biological activities that the polyamines putrescine (**1**), spermidine (**2**), and spermine (**3**) play in cellular processes[1] have shown the profound role they play in life. As polycations at physiological pH, they bind tightly to and strongly modulate the biological activities of anionic cellular components.[2−10] Due to these and other findings, many researchers have explored the effect of synthetic modifications of the polyamine structure in the pursuit of chemotherapeutic agents.[11]

Numerous multidisciplinary studies have shown that intracellular concentrations of the polyamines are highly regulated at many steps in their biosynthesis, catabolism, and transport. The fact that the cell contains such a complex apparatus for the tight control of the levels of these molecules indicates that only a very narrow window of concentration is tolerated. Ornithine decarboxylase (ODC), the rate-limiting enzyme in polyamine biosynthesis, catalyzes the production of **1** from its precursor ornithine. An increase in the activity of ODC has been associated with tumor growth.[12−14] Polyamine transport into mammalian cells is energy and temperature dependent, saturable, and carrier mediated and operates against a substantial concentration gradient.[15−17] Ample experimental proof exists that polyamine concentration homeostasis is aided by this transport system. Changes in the requirements for polyamines in response to growth stimulation are reflected by increases in the transport activity. Stimulation of human fibroblasts to proliferate by serum or epidermal growth factor leads to an 18−100-fold increase in the uptake of putrescine.[18,19] Tumors have been shown to have an increased rate of putrescine uptake.[20,21] Inhibition of polyamine biosynthesis in cells in culture by α-(difluoromethyl)ornithine (DFMO), a well-studied mechanism-based inhibitor of ODC,[22] causes a substantial depletion of intracellular **1** and **2** with resultant cell growth inhibition. Upon supplementing the culture medium of these DFMO-treated cells with exogenous polyamines, transport activity rose severalfold.[23,24] The resulting increase in intracellular polyamine levels restored the cells to their original rate of growth.

Several experimental lines of evidence support the conclusion that increased effectiveness of ODC inhibition can be obtained by interfering with the polyamine transport apparatus. A mutant L1210 leukemia cell line was shown to have greatly reduced polyamine transport activity following selection for resistance to methylglyoxal bis(guanylhydrazone) (MGBG), a cytotoxic AdoMetDC inhibitor that is taken up by the same transport system as the polyamines. Mice inoculated with these cells had a much greater response to DFMO treatment (87% increase in median survival time, 13 of 40 mice cured) than mice inoculated with the parental cell line (22% increase in median survival time).[25] A second experimental approach is based on the fact that the microbial flora in the gastrointestinal tract produces a significant source of extracellular polyamines.[26] When this source of polyamines is removed by antibiotic treatment, DFMO's previously moderate growth inhibitory effects on Lewis lung carcinoma or L1210 cells are markedly potentiated.[27] Finally, an additional source of polyamines is from the diet.[28] By feeding a polyamine-free diet to DFMO-treated nude mice, MCF-7 human breast cancer xenografts contained greatly reduced levels of putrescine in comparison to DFMO treatment alone.[29] In additional animal models, complete polyamine deprivation also enhanced DFMO's growth inhibitory effectiveness.[30−32]

Given the importance of extracellular sources of polyamines to the growth of cancer, pharmacological agents that block polyamine transport are desired. In this paper we address these needs by describing the

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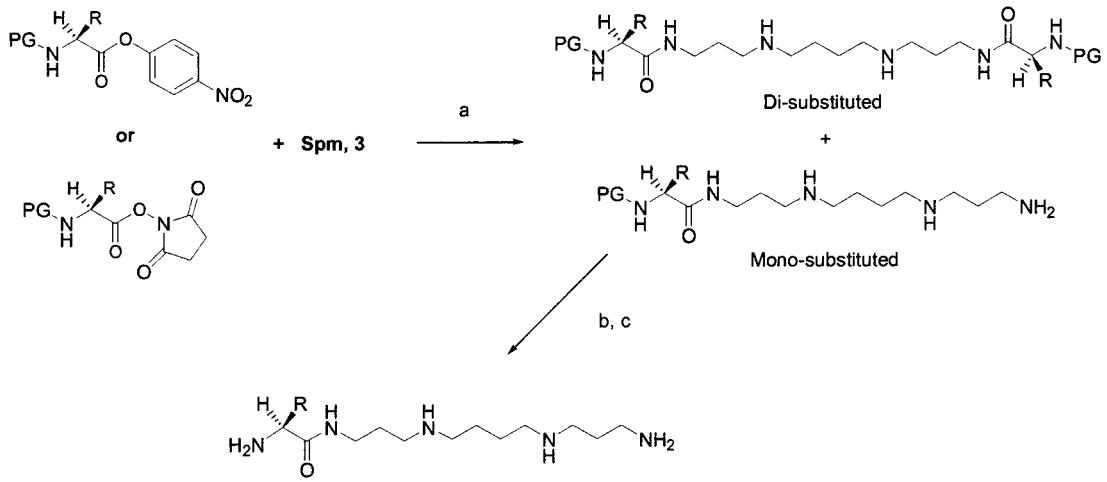
Scheme 1. Synthesis of Amino Acid/Spermine Conjugates[a]



[a] Reagents and conditions: (a) MeOH (for 4-NO_2Ph esters) or CH_2Cl_2 (for *N*-hydroxysuccinimide esters); (b) BioRex 70 (NH_4^+ form) cation-exchange chromatography; (c) 3 N HCl in MeOH (protecting group (PG) = ^{t}Boc) or H_2, $Pd(OH)_2$, EtOH/H_2O (PG = Cbz).

design, synthesis, and biological evaluation of a series of simple amino acid/spermine conjugates that act as potent polyamine transport inhibitors in the MDA-MB-231 human breast cancer cell line. These compounds were evaluated on the basis of their (1) ability to inhibit the uptake of radiolabeled spermidine into MDA-MB-231 breast cancer cells, (2) their ability to increase the growth inhibitory effects of DFMO on MDA-MB-231 cells in culture even in the presence of 1 μM extracellular spermidine, (3) their inability to rescue cells from the growth inhibitory effects of DFMO in the absence of extracellular polyamines, and (4) their ability to deplete the intracellular levels of polyamines after combination treatment with DFMO. Finally, these compounds have limited cytotoxic properties when used alone, thus increasing the potential of providing tumor selectivity.

Synthetic Chemistry

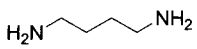
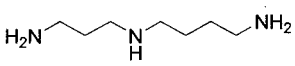
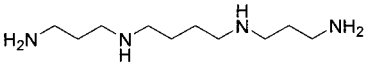
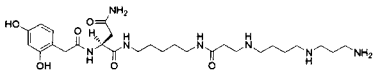
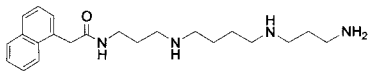
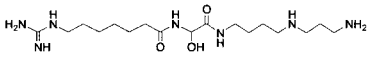
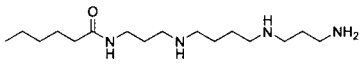
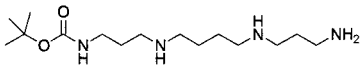
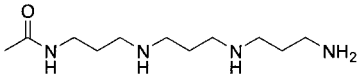
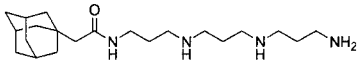
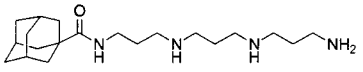
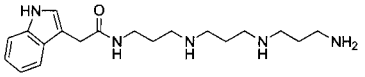
Several reports describe the selective primary monosubstitution of the polyamines.[33,34] Typically, an excess of the polyamine is treated over time with the acylating reagent. Presumably, the higher nucleophilicity of the secondary amines is masked by the greater steric hindrance of these amines, thus giving a higher yield of the monosubstituted primary amide products. 4-Nitrophenyl or *N*-hydroxysuccinyl active esters of the variously protected (*N*-^{t}Boc or *N*-Cbz) amino acids were either purchased from commercial sources or synthesized using standard procedures from the free acid derivatives.[35] The activated amino esters were coupled directly to spermine (1.2 equiv) to yield a mixture of mono- and disubstituted products together with unreacted spermine (Scheme 1). The protected crude mixtures were effectively separated using chromatography over BioRex 70 (NH_4^+ form) cation-exchange resin with a linear gradient of 0−2 N NH_4OH in H_2O.[36] The more lipophilic, protected amino acid derivatives (e.g., **11**, **12**, or **31**) required the inclusion of MeOH (25%) in the eluting buffer to ensure complete solution during chromatography. After this purification, the protecting groups were removed under standard conditions and the desired conjugates were obtained as their hydrochloride salts. All novel analogues gave satisfactory analysis by TLC, ^{1}H and ^{13}C NMR, and HRMS by MALDI-FTMS.

Results

***K*$_i$ Determination.** Each compound was evaluated for its ability to inhibit the uptake of ^{3}H-radiolabeled spermidine into MDA-MB-231 cells. K_i values are shown in Tables 1 and 2. All compounds described in this paper displayed competitive kinetics versus spermidine uptake. The K_m values for putrescine, spermidine, and spermine are included in Table 1 for comparative purposes. On the basis of biological screening of a variety of synthetic and natural products, the Joro spider toxin JSTx-3 (**4**)[37] and a simplified synthetic analogue, (1-naphthylacetyl)spermine (NpAc-Spm, **5**),[38] (Table 1) were both discovered to be effective transport inhibitors with K_i values of 190 and 178 nM, respectively. An additional, relatively complex polyamine natural product, 15-deoxyspergualin (DSQ, **6**),[39] was also shown to have moderate activity as a polyamine uptake inhibitor in MDA-MB-231 cells (K_i = 81 nM). Comparison of these K_i values with the apparent K_m values determined for **1** (1280 ± 340 nM), **2** (113 ± 30 nM), or **3** (280 ± 60 nM) support the view that these polyamine analogues may bind with equal or greater affinity to the polyamine transport apparatus. It is unclear at present whether this property has any role in the pharmacological activities of these natural products.

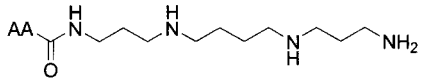
Simple polyamine amides were equally effective at inhibition of spermidine uptake. Nonfunctionalized acyl groups were placed on the N^1 position of spermine to give analogues **7**−**13** (Table 1). An analogue with a low K_i value in this subset was N^1-Boc-spermine (**9**) (K_i = 86 nM), a molecule described in the chemical literature as a protected polyamine intermediate.[40] It is interesting to note that N^1-acetylspermine (**7**) had an activity (K_i = 177 nM) similar to that of the substrate **2** (K_m = 113 ± 30 nM). It appears that the presence of a hydrophobic propylacetyl amide moiety does not harm the affinity compared to the unsubstituted spermidine structure. It is also interesting to note the loss of activity in making the subtle change of removal of one methylene group in the polyamine, exemplified by comparing **7** (K_i = 177

Table 1. K_m or K_i Values for [^{3}H]Spermidine Transport into MDA-MB-231 Cells[a]

Compound	Name	Structure	K_m or K_i (nM)
1	Putrescine		1280 +/- 340
2	Spermidine		113 +/- 30
3	Spermine		280 +/-60
4	JSTx-3		190[b]
5	NpAc-Spm		178
6	DSQ		81[b]
8	N^1-Hep-Spm		284
9	N^1-^{t}Boc-Spm		86
10	N^1-Ac-3,3,3		>1000
11	2-(1-Adam)-Spm		256
12	1-Adam-Spm		672
13	2-(2-indol)-Spm		262

[a] K_i values are the average of two independent determinations using MDA-MB-231 cells unless otherwise noted. Repeat determinations were generally within 25% of each other. See the Experimental Section for the procedure. [b] One K_i determination.

Table 2. K_i and EC_{50} Values for Amino Acid/Spermine Conjugates



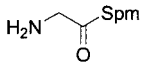
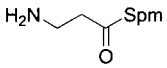
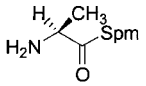
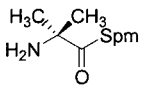
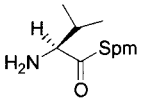
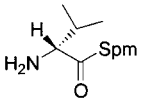
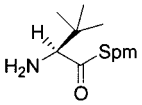
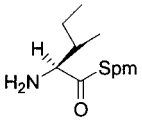
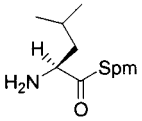
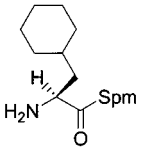
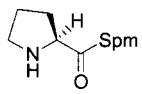
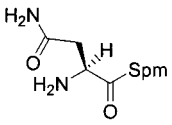
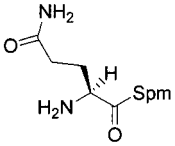
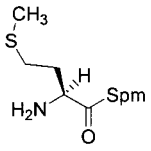
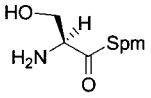
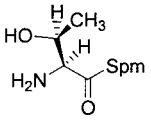
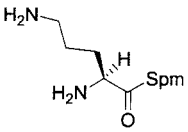
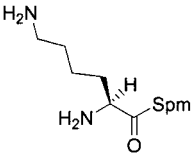
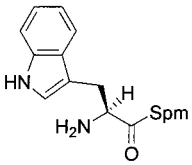
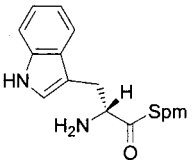
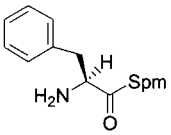
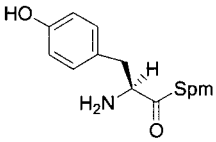
Compound	Name	Structure	K_i[a] (nM)	EC_{50} with DFMO[b] (μM)
14	Gly-Spm		87	>100
15	β-Ala-Spm		65	>300
16	Ala-Spm		144	>300
17	Aib-Spm		191	8.0
18	L-Val-Spm		84	16
19	D-Val-Spm		58	9.0
20	^{t}ButGly-Spm		127	11
21	Ile-Spm		150	9.0
22	Leu-Spm		46	>300
23	Cha-Spm		196	8.0
24	Pro-Spm		38	93
25	Asn-Spm		95	98
26	Gln-Spm		177	>300

Table 2. (Continued)

Compound	Name	Structure	K_i[a] (nM)	EC_{50} with DFMO[b] (μM)
27	**Met-Spm**		**65**	**>300**
28	**Ser-Spm**		**55**	**26**
29	**Thr-Spm**		**213**	**6.0**
30	**Orn-Spm**		**47**	**3.0**
31	**Lys-Spm**		**32**	**5.0**
32	**L-Trp-Spm**		**119**	**15**
33	**D-Trp-Spm**		**63**	**2.5**
34	**Phe-Spm**		**443**	**>100**
35	**Tyr-Spm**		**116**	**95**

[a] K_i values are the average of two independent determinations using MDA-MB-231 cells unless otherwise noted. Repeat determinations were generally within 25% of each other. See the Experimental Section for the procedure. [b] EC_{50} values were determined using a 6 day assay of MDA-MB-231 cells in the presence of 230 μM DFMO, 1 mM AG, and 1.0 μM spermidine.

nM) with N^1-acetylnorspermine (N^1-Ac-3,3,3, **10**) (K_i = >1000 nM). Insights into the steric environment of this binding domain can be seen by comparison of amides **11** and **12**. The bulky adamantyl group is better tolerated if a methylene spacer is included between the polycyclic moiety (**11**, K_i = 256 nM, versus **12**, K_i = 672 nM).

Further increases in transport inhibition potency are obtained by placing an amino group at the end of the acyl group. The glycine (**14**) and β-alanine (**15**) conjugates showed K_i values of 87 and 65 nM, respectively (Table 2). Given the gain in spermidine transport inhibition activity shown by the amino-containing glycine/spermine conjugate (**14**) as compared to **7** (87 nM

versus 177 nM), we next examined the activities of a more extensive series of α-amino acid/spermine conjugates. A series of aliphatic hydrocarbon substituted amino acids was produced. All amino acid derivatives are the L-stereoisomers unless otherwise noted. α-Methyl substitution had a negative impact on the activity (compare Gly-Spm (**14**) (K_i = 87 nM) with α-Ala-Spm (**16**) (K_i = 144 nM) or Aib-Spm (**17**) (K_i = 191 nM)). Comparison of the L-Val-Spm conjugate (**18**) (K_i = 84 nM) with the D-Val-Spm conjugate (**19**) (K_i = 58 nM) showed only a minor stereochemical preference for activity. Notable steric effects were suggested by subtle changes in the placement of alkyl groups on the side chain. Addition of an extra methyl group to the Val side chain reduced the activity (^{t}ButGly-Spm, **20**; K_i = 127 nM). Placement of an extra methyl group at the terminal methyl of the Val conjugate again reduced the activity as a transport inhibitor (Ile-Spm, **21**; K_i = 150 nM). By simply moving this methyl group one position, an improvement in activity was observed (Leu-Spm, **22**; K_i = 46 nM). Increased steric bulk on this derivative again reduced the activity (Cha-Spm, **23**; K_i = 196 nM). Formation of a ring by coupling proline with spermine produced a higher activity analogue (Pro-Spm, **24**; K_i = 38 nM).

We next explored the effects of using functionalized amino acids as conjugates with spermine. The asparagine/spermine (**25**) (K_i = 95 nM) and glutamine/spermine (**26**) (K_i = 177 nM) conjugates were moderately good inhibitors. The Met-Spm (**27**) (K_i = 65 nM), Ser-Spm (**28**) (K_i = 55 nM), Orn-Spm (**30**) (K_i = 47 nM), and Lys-Spm (**31**) (K_i = 32 nM) conjugates were all very good inhibitors and had K_i values substantially below 100 nM. The Thr-Spm conjugate (**29**) was a less effective inhibitor (K_i = 213 nM).

The amino acids containing aromatic groups were examined next. The L-Trp-Spm (**32**) (K_i = 119 nM), D-Trp-Spm (**33**) (K_i = 63 nM), and Tyr-Spm (**35**) (K_i = 116 nM) conjugates all had good activity, whereas the Phe-Spm conjugate (**34**) (K_i = 443 nM) had diminished activity.

Cell Growth Inhibition: EC_{50} in MDA-MB-231 Cells Grown in the Presence of DFMO and Spermidine. A 6-day cell growth assay was used to characterize the ability of the amino acid/spermine amides to work in concert with the ODC inhibitor DFMO in the presence of added spermidine (0.50 or 1.0 μM) and aminoguanidine (1 mM) (Figure 1). In this 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 spermidine added to the culture medium. If the added analogues prevented the uptake of the exogenously added spermidine, growth inhibition due to polyamine depletion was expected. This assay should identify effective transport inhibitors under conditions that mimic in vivo conditions.

Analysis of the data from this assay using the novel polyamine analogues described in Table 2 showed that several of the conjugates were able to effectively inhibit the growth of MDA-MB-231 cancer cells in combination with 230 μM DFMO even in the presence of 1.0 μM added spermidine. The analogues showing the best activity in this assay include **31** (K_i = 32 nM) with an EC_{50} of 5 μM and the analogue **30** (K_i = 47 nM), which

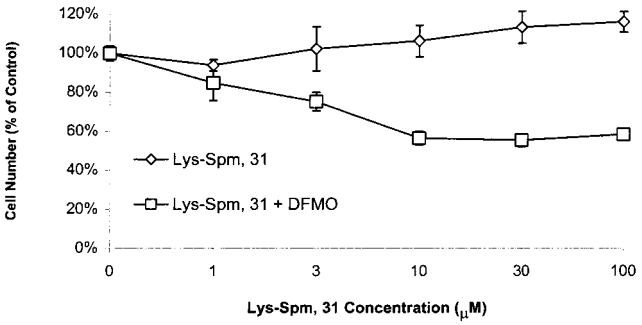


Figure 1. Growth inhibition of MDA-MB-231 cells with **31** and DFMO. MDA-MB-231 cells were incubated with 0.50 μM SPD, 1.0 mM AG, and 0.1−100 μM **31** ± 1.0 mM DFMO during a 6-day growth assay. There was no growth inhibition with 1.0 mM DFMO and 0.50 μM SPD. The cell number was determined by MTS/PMS assay from triplicate wells. The bars represent the standard deviation from triplicate wells.

showed an effective EC_{50} of 3 μM. Other analogues that had notable activity in this assay included the stereochemical pairs of the D- versus L-Trp/spermine and the D- versus L-Val/spermine conjugates. It is interesting to note that **33** gave a better EC_{50} compared with **32**. This slight increase in activity of the D-stereoisomers, as reflected by both the K_i and the EC_{50} values, was repeated with the Val-Spm pairs (**19** more active than **18**).

In several cases there appears to be limited correlation between an analogue's ability to inhibit transport of spermidine and its ability to inhibit cell growth in the presence of DFMO. Some analogues with K_i values below 100 nM failed to give low EC_{50} values in combination with DFMO. Examples of this type of analogue include **14** and **24**. A possible explanation for this observation is that in addition to competitively inhibiting transport of spermidine, these analogues act as substrates for the polyamine transporter, thereby entering and supplying the cells with their necessary polyamines. The ability of analogues **14**, **18**, **24**, **30**, and **31** to rescue the cells from the effect of 230 μM DFMO in the absence of spermidine was tested (data not shown). After 6 days in the presence of 230 μM DFMO, cell growth was inhibited by 60%. **14** and **24** were able to rescue cells from these growth inhibitory effects of DFMO. The analogues **18**, **30**, and **31** did not rescue cells from these growth inhibitory effects of DFMO. These results suggested that analogues **14** and **24** were substrates while analogues **18**, **30**, and **31** were not substrates for the polyamine transporter. Alternatively, if analogues **18**, **30**, and **31** were substrates for the transporter, they could not substitute for the growth functions of the natural polyamines. An additional possibility is that conjugates **14** and **24** are metabolized and the resulting products supply the cells with their required polyamines.

Given the promising results shown by the use of **31** in combination with DFMO in inhibiting the growth of MDA-MB-231 cells, we next explored its growth effect on other tumor cell lines. The results in Table 3 showed that **31** in combination with DFMO demonstrated a good cytostatic effect against 9 out of 10 cell lines tested. For each cell line tested, a DFMO titration curve, without addition of SPD, was used to determine the optimal inhibitory concentration of DFMO (0.23−5.0

Table 3. Growth Inhibition by Polyamine Depletion in Multiple Tumor Types[a]

tumor type	cell line	31 + DFMO EC_{50} (μM)	DFMO concn (mM)
breast carcinoma	MDA-MB-231	4.8	0.23
prostate carcinoma	PC-3	5.3	1.0
	DU 145	5.0	1.0
	LNCaP·FGC	2.6	5.0
bladder carcinoma	T-24	1.6	5.0
melanoma	SK-Mel-5	14	5.0
	SK-Mel-28	3.6	5.0
	A375	1.5	5.0
lung carcinoma	NCI H157	140	0.23
(nonsmall cell)	NCI H226	>300	3.0

[a] **31** EC_{50} values were determined in a 6-day growth assay with the optimal DFMO concentration for each cell line. All assays performed in the presence of 1.0 mM AG and 1.0 μM spermidine.

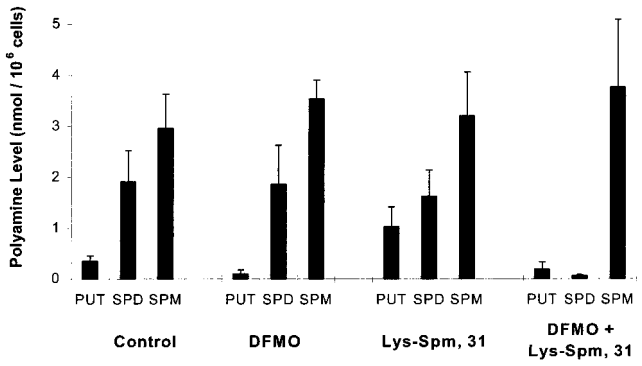


Figure 2. Polyamine levels in MDA-MB-231 cells after 6 days with **31**, DFMO, or the combination of both (all with 1.0 μM spermidine). MDA-MB-231 cells were grown for 6 days (1 week) with 500 μM DFMO or 60 μM **31** or both. All flasks received 1.0 mM AG and 1.0 μM SPD. Cells were counted, washed, lysed in perchloric acid, and dansylated and polyamine levels determined by HPLC. Each point is the mean of three experiments. The bars represent standard deviations.

mM). Again, 1.0 μM SPD was included to ensure that growth inhibition reflected inhibition of transport. Most EC_{50} values were in the low micromolar range (Table 3). The exceptions were the two nonsmall cell lung carcinoma cell lines. Their growth responses may reflect cell-line- or tissue-specific differences in transport or metabolism.

Polyamine Depletion: Ability of Combination Therapy To Deplete Intracellular Polyamines. The intracellular polyamine levels in MDA-MB-231 cells were measured following a 6-day drug treatment period. The polyamine levels were measured by HPLC using fluorescent detection following precolumn derivatization with dansyl chloride.[41] Figure 2 shows the results after various drug treatments. Following a 6-day incubation with DFMO alone (500 μM), **1** and **2** levels were not significantly reduced in MDA-MB-231 cells due to the inclusion of adequate transportable spermidine in the culture medium. Polyamine levels after treatment with 60 μM **31** together with 1.0 μM spermidine raised the levels of **1** in the cells but only had minimal effects on **2** and **3** levels. In contrast, a significant reduction of the intracellular levels of putrescine and spermidine was observed when 60 μM **31** and 500 μM DFMO was used in combination in the presence of 1.0 μM spermidine. These results clearly demonstrate the ability of

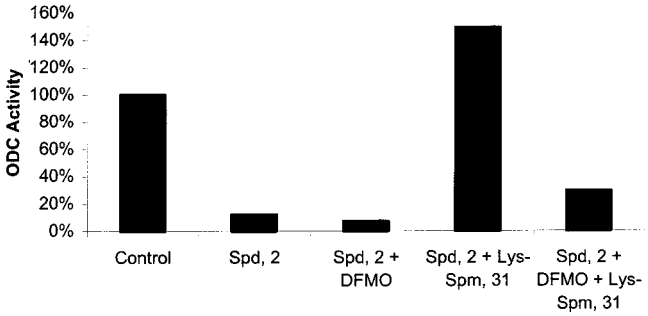


Figure 3. ODC activity in MDA cells. MDA-MB-231 cells were treated for 3 days with SPD (1.0 μM), DFMO (0.50 mM), or Lys-Spm (100 μM) in the combinations shown. Cells were harvested for ODC activity assay while in log growth. The activity was normalized to protein concentration. All experiments contained 1.0 mM AG.

31 to work in combination with DFMO to significantly reduce intracellular putrescine and spermidine levels under conditions that mimic the in vivo tumor environment.

ODC activity was measured in MDA-MB-231 cells following treatment with various drugs (Figure 3). It is theorized that due to the regulated control of ODC activity in the cells the inclusion of transportable spermidine in the culture medium caused a lowering of the measured ODC activity to 12% of the control value seen with no treatment. Treatment with both spermidine and DFMO gave, as expected, a similar lowered value of ODC activity in comparison with control values. When the cells were treated with spermidine and **31**, the ability of the polyamine transport inhibitor to prevent the uptake of spermidine caused the ODC activity of the cells to rise to slightly higher than control levels (149%). This result may explain the observed increased levels of putrescine following **31** treatment (Figure 2). This result shows that treatment with **31** does not inhibit ODC activity of the cells. It also supports the conclusion that **31** prevents the uptake of endogenously supplied spermidine. Finally, Figure 3 shows that treatment with a combination of spermidine, DFMO, and **31** gave the expected result of lowered ODC activity.

Discussion

The genes for the polyamine transporter subunits have been cloned from *Escherichia coli*,[42] and one was recently identified in yeast.[43] The genes for the mammalian transporter await identification. For this reason it is difficult to determine whether different transporters exist for each polyamine. The PotD subunit of the *E. coli* transporter has been crystallized, and its X-ray structure has been determined.[44] Several researchers have studied the ability of polyamine analogues to inhibit the uptake of [^{3}H]spermidine into cells. Bergeron and co-workers studied the effect of the addition of different alkyl group substitutions on the terminal nitrogen atoms of spermidine or spermine analogues.[45] They showed that larger alkyl groups diminished the ability to prevent uptake of radiolabeled spermidine. They later concluded that increases in the number of methylenes between the nitrogen atoms decreased the ability to compete for [^{3}H]spermidine uptake.[46] Of greater importance to the present work was their

conclusion that the polyamine transport apparatus requires only three cationic centers for polyamine recognition and transport.[47]

Several research groups have reported the search for compounds that would prevent the uptake of extracellular polyamines and thus increase the clinical utility of polyamine biosynthesis inhibitors. Poulin and co-workers synthesized 2,2′-dithiobis(*N*-ethylspermine-5-carboxamide) (DESC) and characterized its inhibition of polyamine uptake into ZR-75-1 cells. K_i values of 1.6 ± 5.0, 2.7 ± 1.1, and 5.0 ± 7.0 μM against putrescine, spermidine, and spermine, respectively, were reported.[48] Despite the advantage of it not being a transport substrate, the addition of 0.3 μM spermidine fully restored growth to cells treated with the combination of 1.0 mM DFMO and 50 μM DESC. Analysis of the levels of polyamine in the cells showed that spermidine accumulation was not prevented by large concentrations of this compound. Furthermore, significant cytotoxicity was observed when 200 μM DESC was used alone. Poulin has subsequently published results on a new series of polyamine transport inhibitors.[49] This series of compounds was described as dimers of spermidine or norspermidine and have six ionizable amino functions. Several of these analogues have activities as polyamine transport inhibitors in T-47D human breast cancer cells that are much better than the corresponding apparent affinities of putrescine or spermidine. The best analogues described have affinities nearly equal to that of spermine. Minchin and co-workers described a series of polypyridinium quaternary salts as putrescine uptake inhibitors in B16 melanoma cells.[50] Despite having K_i values in the low micromolar range, combination with DFMO had no effect on the growth inhibitory properties of the described compounds. Again, significant cytotoxicity was observed when the compounds were tested alone. Finally, a macromolecular polymer (~25 kDa) of spermine and glutaraldehyde was shown to inhibit polyamine uptake in the micromolar range, but its cytotoxicity again limits its usefulness.[51] Two groups have analyzed literature examples of numerous polyamine analogues' ability to inhibit [^{3}H]spermidine uptake into L1210 cells by CoMFA[52] and QSAR[53] methods. A recent report explored the effects of acylation of various nitrogen atoms of spermidine or spermine on the inhibition of polyamine uptake together with the resulting effects on polyamine pools in *E. coli* cells.[54]

A great number of polyamine amide natural products have recently been discovered in the venom of arthropods (spider and wasp).[55] These acylpolyamine analogues have been shown to have specific and strong interactions with the neuromuscular junctions of insects.[56] With this capability, these toxins give the insect predators the ability to paralyze or kill their prey. Most of these natural products have the common molecular features of a polyamine moiety (many with structurally diverse polyamine analogues) connected through an amide with an aromatic amino acid structural analogue. Simpler synthetic analogues have been sought that attempt to maximize interactions with either crustacean neuromuscular synapses[38] or mammalian glutamate receptors.[57,58] Given our discovery that the polyamine amide spider toxin **4** and its synthetic analogue **5**, together with the immunosuppressant polyamine natural product **6**, had moderate ability to inhibit the transport of [^{3}H]spermidine in MDA-MB-231 cells, we initiated the synthesis of a series of simple amino acid/spermine amide conjugates (Table 2). These have at least three cationic centers in their polyamine portion and thus satisfy Bergeron's criteria for recognition by the polyamine transporter.[47]

Table 4. Polyamine K_m Values and Inhibition Constants (K_i Values) with **31** on MDA-MB-231 Cells[a]

substrate	K_i(**31**) (nM + SD)	characterization
[^{3}H]putrescine	24 ± 1 n = 7	competitive
[^{3}H]spermidine	32 ± 2 n = 4	competitive
[^{14}C]spermine	84 ± 2 n = 3	competitive

[a] Multiple concentrations of substrate and **31** were used to determine K_i values in 15-min transport assays.

The novel series of amino acid/spermine conjugates described in the present paper included compounds with significant activity as inhibitors of polyamine uptake into MDA-MB-231 cells. A comparison of their K_i values, derived from competition with spermidine, gives a ranking of their potency. It was interesting to note that minor variations in an analogue's structure gave great changes in its relative potency as a transport inhibitor (compare **7** with **10**, 2-(1-Adam)-Spm (**11**) with 1-Adam-Spm (**12**), **14** with **16**, or **22** with **23**). Furthermore, changing the stereochemistry of the α-carbon atom of the amino acid portion of the molecule from the L- to the D-configuration showed the possibility of slight increases in activity (compare **18** with **19** and **32** with **33**). On the basis of this analysis and further biological testing discussed below, **31** was selected as a lead compound for extensive biological testing including in vivo efficacy in combination with DFMO.[59,60] Measurement of the K_i values for **31** versus putrescine, spermidine, and spermine was performed. As shown in Table 4, this molecule showed potent competitive inhibition against each polyamine. Furthermore, this molecule's activity as a transport inhibitor in MDA-MB-231 cells exceeded the apparent affinities of the natural polyamines putrescine, spermidine, and spermine several-fold.

Satisfying one of the major goals of the present research, all of the novel analogues described here had EC_{50} values greater than 300 μM when tested alone in the 6-day growth assay of MDA-MB-231 breast cancer cells. It was only when an effective polyamine transport inhibitor, such as **31**, was used in combination with the polyamine biosynthesis inhibitor DFMO that growth inhibitory effects were observed. As shown in Figure 1, this effect was seen even in the presence of 0.50 μM extracellular spermidine added to the cell culture medium. This amount of spermidine, when added together with DFMO alone, completely abolished the growth inhibitory effects of this polyamine biosynthesis inhibitor (data not shown). The growth inhibition seen with dual-compound therapy was seen in a variety of cell lines representing multiple tumor types (Table 3).

An additional requirement placed on any molecule that would function as a polyamine transport inhibitor in a chemotherapeutic setting would be its lack of polyamine-replacing or -mimicking ability. A test of the

ability of a polyamine analogue's ability to act as a polyamine surrogate, or to be metabolized to the natural polyamines themselves, was its ability to overcome the growth inhibitory effects of DFMO, in the absence of added spermidine. The implication of this test is that either the analogue does not act as a substrate for the polyamine uptake system or it does not function as a polyamine once it enters the cell. With the inability of $<300\ \mu$M **31** to rescue growth from the effects of DFMO treatment, our working assumption is that it does not enter the cell. Experiments are now in progress to determine whether **31** accumulates in cells.

The final, and ultimately the most important, criterion placed on our search for a polyamine transport inhibitor was its ability to deplete intracellular polyamines when used in combination with a biosynthesis inhibitor. The data shown in Figure 2 confirm our design goals by showing that, even in the presence of sufficient spermidine in the culture medium, the combination of **31** and DFMO greatly reduces the intracellular levels of putrescine and spermidine in MDA-MB-231 cells. This reduction in putrescine and spermidine levels is reflected by the reduction in the growth of the MDA-MB-231 cells (60% of control). Even after 2 weeks of growth arrest, the cells will regrow upon removal of the drug (data not shown). Therefore, this is a cytostatic treatment. It is unclear why the spermine levels remain near their control levels. It has been suggested in the literature that the intracellular pool of spermine is completely bound to cellular anionic components.[3] Preliminary in vivo testing showed no gross acute toxicities for **31** that might be expected on the basis of its structural relationship to the acylpolyamine spider toxins.[61] We speculate that the lack of an aromatic moiety common to most, if not all, of the spider toxins limits the neurotoxicity of **31**.

Conclusions

In this paper we describe the synthesis and characterization of a series of simple spermine/amino acid conjugates, some of which potently inhibit the uptake of spermidine into MDA-MB-231 breast cancer cells. The presence of an amide in the functionalized polyamine appeared to add to the affinity for the polyamine transporter. The extensive biological characterization of an especially potent analogue from this series, **31**, showed this molecule will be an extremely useful tool for use in polyamine research. It was shown that the use of **31** in combination with DFMO led to a cytostatic growth inhibition of a variety of cancer cells, even when used in the presence of an extracellular source of polyamines. It was shown that this combination effectively reduced the cellular levels of putrescine and spermidine while not affecting the levels of spermine. Despite the structural similarity between **31** and the acylpolyamine spider toxin natural products, preliminary in vivo data suggest that this compound does not show neurotoxicity to animals at dose levels necessary for transport inhibition.[59,60] These facts together with the nontoxic nature of **31** make it an ideal lead for further anticancer development.

Experimental Section

The Joro spider toxin JSTx-3 was purchased from Calbiochem. (1-Naphthylacetyl)spermine was purchased from RBI. 15-Deoxyspergualin was a generous gift from Dr. Paul Gladstone.

Synthetic Methods. General Information. All chemical reagents and starting materials were of the highest grade available and were used without further purification. Thin-layer chromatography analysis of crude reaction products and column chromatography were performed using Merck F_{254} silica gel plates and Baker 40 μm flash chromatography packing, respectively. TLC analysis used the following solvent system with detection by ninhydrin staining: 2-propanol/pyridine/glacial acetic acid/H_2O, 4:1:1:2. The R_f values were 0.1 for spermine and generally 0.5 for the mono- and 0.7 for the disubstituted products. ^{1}H and ^{13}C NMR spectra were recorded at 200, 300, or 500 MHz and 75.5 or 125.8 MHz, respectively, on a Bruker AC200, AF300, or WM500 spectrometer at the University of Washington, Seattle. Chemical shifts are relative to external 3-(trimethylsilyl)-1-propanesulfonic acid, sodium salt. HRMS was run at The Scripps Research Institute using MALDI FTMS with DHB matrix on an IonSpec HiRes MALDI instrument.

Synthesis of Amino Acid/Spermine Conjugates. The following two procedures are representative of the synthesis of the conjugates. Specific details about each conjugate are included with the spectroscopic characterizations below.

Method A: N^1-Spermine L-*tert*-Butylglycinyl Amide (20). 4-Nitrophenyl *N*-^{t}Boc-L-*tert*-butylglycinate. A heterogeneous solution of 0.463 g (2 mmol) of *N*-^{t}Boc-*tert*-butylglycine (from Novabiochem), 0.334 g (1.2 equiv) of 4-nitrophenol, and 0.495 g (1.2 equiv) of 1,3-dicyclohexylcarbodiimide in 20 mL of dry EtOAc was stirred for 16 h at ambient temperature under argon. The solids were filtered over a pad of Celite, and the pad was washed twice with additional portions of EtOAc. The combined filtrates were evaporated, and the resulting solid was purified by column chromatography over silica gel using 95:5 $CHCl_3$/EtOAc to yield 0.60 g (85%) of clear oil.

***N*^{1}-Spermine *N*-^{t}Boc-L-*tert*-butylglycinyl Amide.** To the clear, homogeneous solution of 136 mg (1.2 equiv) of spermine (free base form) in 15 mL of MeOH was added dropwise through a funnel a solution of 198 mg (0.56 mmol) of 4-nitrophenyl *N*-^{t}Boc-*tert*-butylglycinate in 15 mL of MeOH at ambient temperature. After 2 h, the resulting yellow-green solution was checked by the above TLC method, which showed the expected mixture of di-, mono-, and unsubstituted products had been formed. The solvents were evaporated, and the crude product was suspended in 25 mL of H_2O. Upon adjusting the pH to 4−5 by the addition of 1 N HCl, a homogeneous, clear solution was produced. This was loaded onto a 1 × 30 cm column of BioRex 70 resin that was in the NH_4^+ salt form. The column was washed with 50 mL of H_2O followed by an 800 mL gradient of 0−2 N NH_4OH to elute the products into 80 fractions of 10 mL each. These fractions were analyzed by the above TLC system, and the desired monosubstituted product containing fractions were combined and evaporated to give 105 mg (45%) of the product in its free base form.

***N*^{1}-Spermine L-*tert*-Butylglycinyl Amide (20).** A 6.0 mL portion of 6 N HCl was added to the clear solution of 98 mg (0.24 mmoL) of N^1-spermine *N*-^{t}Boc-L-*tert*-butylglycinyl amide in 6.0 mL of MeOH. The resulting homogeneous colorless solution was stirred at ambient temperature for 3 h, after which the solvent was evaporated to give 110 mg (99%) of desired **20** as its tetrahydrochloride salt. ^{1}H NMR (D_2O, δ): 3.50 (s, 1H), 3.15 (t, 2H), 2.91 (m, 12H), 1.90 (m, 2H), 1.74 (m, 2H). 1.58 (m, 4H), 0.85 (s, 9H). ^{13}C NMR (D_2O, ppm): 171.2, 67.9, 64.4, 49.6, 49.5, 47.8, 47.1, 39.2, 39.0, 35.3, 28.2, 28.0, 26.3, 25.3. HRMS: *m*/*z* calcd for $C_{16}H_{37}N_5O$ (M + H) 316.3076, found 316.3063.

Method B: N^1-Spermine L-Lysinyl Amide (31).

***N*^{1}-Spermine N_α,N_ϵ-Bis(carbobenzyloxy)-L-lysinyl Amide.** A clear solution of 1.02 g (2.0 mmol) of N_α,N_ϵ-bis-(carbobenzyloxy)-L-lysine *N*-hydroxysuccinimide ester (Sigma, St. Louis, MO) in 50 mL of methylene chloride was added dropwise over 15 min at room temperature to a clear, homogeneous solution of 0.486 g (2.4 mmol, 1.2 equiv) of spermine in 50 mL of methylene chloride. A white precipitate formed.

After the resulting solution was stirred for 4 h, TLC analysis showed the expected mixture of di-, mono-, and nonacylated spermine derivatives had formed. The solvent was evaporated to give a white solid. This crude product was suspended in 3:1 H_2O/MeOH (25 mL), and the pH was adjusted to 4−5 using 1 N HCl. The resulting clear, homogeneous solution was applied to a 1 × 30 cm column of BioRex 70 resin in its NH_4^+ form. The column was washed with 50 mL of 4:1 H_2O/MeOH, and then a 800 mL gradient of 0−2 N NH_4OH containing 25% MeOH was used to elute the products into 80 fractions of 10 mL each. These fractions were analyzed by the above TLC system, and the desired monosubstituted product containing fractions were combined and evaporated to give 527 mg (44%) of the product as its free base form.

***N*¹-Spermine L-Lysinyl Amide (31).** A mixture of 500 mg (0.83 mmol) of N^1-spermine N_α,N_ϵ-bis(carbobenzyloxy)-L-lysinyl amide and 300 mg of $Pd(OH)_2$ in 50 mL of 1:1 EtOH/H_2O containing 10 mL of 2 N HCl was stirred under an atmosphere of H_2 gas using a balloon reservoir. After 18 h at ambient temperature the catalyst was filtered off over a pad of Celite, and the pad was washed twice each with EtOH, H_2O, and then 1 N HCl. The combined filtrates were evaporated to give 420 mg (98%) of product **31** as its pentahydrochloride salt. ^{1}H NMR (D_2O, δ): 4.09 (t, 1H), 3.39 (m, 2H), 3.17 (m, 14H), 2.16 (m, 2H), 1.99 (m, 4H), 1.85 (m, 4H), 1.51 (m, 2H). ^{13}C NMR (D_2O, ppm): 173.2, 56.4, 50.3, 50.2, 48.5, 47.9, 42.4, 40.0, 39.8, 33.6, 29.6, 28.8, 27.1, 26.4 (2C), 24.7. HRMS: *m/z* calcd for $C_{16}H_{38}N_6O$ (M + H) 331.3185, found 331.3173.

***N*¹-Spermine 2-(1-Adamantyl)acetamide (11).** The 4-nitrophenyl ester of 2-(1-adamantyl)acetic acid was produced (52% yield) and coupled to spermine via method A described for **20** above. Purification over BioRex 70 resin gave pure **11** as its free base (17% yield). It was converted to its trihydrochloride salt for analysis and biological testing. ^{1}H NMR (D_2O, δ): 2.98 (t, 2H), 2.82 (m, 10H), 1.80 (m, 2H), 1.76 (s, 3H), 1.63 (m, 6H), 1.48 (m, 4H), 1.36 (m, 4H), 1.22 (s, 6H). HRMS: *m/z* calcd for $C_{22}H_{42}N_4O$ (M + H) 379.3437, found 379.3443.

***N*¹-Spermine 1-Adamantyl Amide (12).** A CH_2Cl_2 solution of spermine and triethylamine was treated with a CH_2Cl_2 solution of 1-adamantanecarbonyl chloride. After 2 h, the solvents were evaporated and the residue was purified via method A above using BioRex 70 (NH_4^+ form) resin. Pure **12** in its free base form was obtained (19% yield) and converted to its trihydrochloride salt for analysis and biological testing. ^{1}H NMR (D_2O, δ): 3.24 (t, 2H), 3.00 (m, 10H), 1.95 (m, 4H), 1.74 (m, 10H), 1.62 (m, 9H). HRMS: *m/z* calcd for $C_{21}H_{40}N_4O$ (M + Na) 387.3100, found 387.3085.

***N*¹-Spermine 2-(2-Indolyl)acetamide (13).** The 4-nitrophenyl ester of 2-(2-indolyl)acetic acid was produced (77% yield) and coupled to spermine via method A described for **20** above. Purification over BioRex 70 resin gave pure **13** as its free base (48% yield). It was converted to its trihydrochloride salt for analysis and biological testing. ^{1}H NMR (D_2O, δ): 7.43 (d, 1H), 7.38 (d, 1H), 7.17 (s, 1H), 7.11 (t, 1H), 7.02 (t, 1H), 3.56 (s, 2H), 3.08 (t, 2H), 1.93 (m, 4H), 2.84 (t, 2H), 2.61 (m, 4H), 1.94 (m, 2H), 1.62 (m, 2H), 1.48 (m, 4H). ^{13}C NMR (D_2O, ppm): 178.3, 138.7, 129.0, 127.6, 124.6, 122.1, 120.8, 114.6, 110.1, 49.4, 49.2, 47.3, 47.0, 39.1, 38.4, 34.9, 28.0, 26.3, 25.2, 25.0. HRMS: *m/z* calcd for $C_{20}H_{33}N_5O$ (M + H) 360.2763, found 360.2773.

***N*¹-Spermine Glycinyl Amide (14).** 4-Nitrophenyl *N*-^{t}Boc-glycinate was obtained from Novabiochem and directly coupled to spermine in 43% yield using method A above. Deprotection in 3 N HCl gave the desired **14** as its tetrahydrochloride salt in 93% yield. ^{1}H NMR (D_2O, δ): 3.91 (s, 2H), 3.43 (t, 2H), 3.20 (m, 10H), 2.21 (m, 2H), 2.18 (m, 2H), 1.87 (m, 4H). ^{13}C NMR (D_2O, ppm): 175.4, 47.8, 47.7, 46.0, 45.3, 41.2, 37.4, 37.1, 26.2, 24.5, 23.6 (2C). HRMS: *m/z* calcd for $C_{12}H_{29}N_5O$ (M + H) 260.2450, found 260.2442.

***N*¹-Spermine *β*-Alanyl Amide (15).** 4-Nitrophenyl ^{t}Boc-*β*-alanine ester was obtained from Sigma and coupled to spermine in 39% yield via method A above. Deprotection gave 98% of **15** as its tetrahydrochloride salt. ^{1}H NMR (D_2O, δ): 3.29 (m, 4H), 3.10 (m, 10H), 2.68 (t, 2H), 2.09 (m, 2H), 1.90 (m, 2H), 1.78 (m, 4H). ^{13}C NMR (D_2O, ppm): 175.0, 49.6, 49.5, 47.8, 47.1, 39.2, 38.8, 38.3, 34.5, 28.1, 26.3, 25.4 (2C). HRMS: *m/z* calcd for $C_{13}H_{31}N_5O$ (M + H) 274.2607, found 274.2604.

***N*¹-Spermine L-Alanyl Amide (16).** 4-Nitrophenyl ^{t}Boc-L-alanine ester was obtained from Novabiochem and coupled to spermine in 56% yield. The ^{t}Boc protecting group was removed through acid treatment as in method A to give **16** as its tetrahydrochloride salt in 98% yield. ^{1}H NMR (D_2O, δ): 4.08 (quartet, 1H), 3.19 (t, 2H), 3.08 (m, 10H), 2.13 (m, 2H), 1.96 (m, 2H), 1.79 (m, 4H), 1.52 (d, 3H). ^{13}C NMR (D_2O, ppm): 173.6, 51.7, 49.6, 49.5, 47.8, 47.2, 39.2, 40.0, 28.1, 26.4, 25.4 (2C), 19.2. HRMS: *m/z* calcd for $C_{13}H_{31}N_5O$ (M + H) 274.2607, found 274.2604.

***N*¹-Spermine α-Aminoisobutyryl Amide (17).** *N*-^{t}Boc-α-aminoisobutyric acid was obtained from Sigma and was converted to its 4-nitrophenyl active ester as in method A above. Coupling to spermine gave 57% of the protected conjugate as its free base. Deprotection via acid treatment gave **17** as its tetrahydrochloride salt in 92% yield. ^{1}H NMR (D_2O, δ): 3.37 (t, 2H), 3.14 (m, 10H), 2.13 (m, 2H), 1.96 (m, 2H), 1.82 (m, 4H), 1.64 (s, 6H). ^{13}C NMR (D_2O, ppm): 173.5, 57.6, 47.7, 47.6, 45.9, 45.3, 37.3 (2C), 26.2, 24.4, 24.0 (2C), 23.5 (2C). HRMS: *m/z* calcd for $C_{14}H_{33}N_5O$ (M + H) 288.2763, found 288.2762.

***N*¹-Spermine L-Valinyl Amide (18).** *N*-^{t}Boc-L-valine *N*-hydroxysuccinimide ester was obtained from NovaBiochem and coupled to spermine using method B (48% yield as free base). Deprotection by acid treatment gave **18** as its tetrahydrochloride salt in 99% yield. ^{1}H NMR (D_2O, δ): 3.36 (d, 1H), 3.18 (m, 2H), 3.11 (m, 10H), 2.02 (m, 3H), 1.82 (m, 2H), 1.54 (m, 4H), 0.90 (d, 6H). ^{13}C NMR (D_2O, ppm): 172.1, 61.3, 49.6, 49.5, 47.9, 47.2, 39.2, 39.1, 32.4, 28.1, 26.4, 25.4 (2C), 20.3, 19.7. MS (ESI$^+$): 302 (M + H). HRMS: *m/z* calcd for $C_{15}H_{35}N_5O$ (M + H) 302.2920, found 302.2914.

***N*¹-Spermine D-Valinyl Amide (19).** The 4-nitrophenyl ester of *N*-^{t}Boc-D-valine (from Sigma) was synthesized via method A (66% yield). Coupling to spermine and purification gave 48% of the ^{t}Boc-protected conjugate. Deprotection using acid gave **19** as its tetrahydrochloride salt in 99% yield. ^{1}H NMR and ^{13}C NMR were the same as for **18**.

***N*¹-Spermine L-Isoleucinyl Amide (21).** *N*-^{t}Boc-L-isoleucine 4-nitrophenyl ester was obtained from Sigma and coupled to spermine via method A (60% yield). Deprotection by acid treatment gave **21** as its tetrahydrochloride salt in 99% yield. ^{1}H NMR (D_2O, δ): 3.84 (d, 1H), 3.37 (t, 2H), 3.11 (m, 10H), 2.09 (m, 2H), 1.96 (m, 3H), 1.78 (m, 4 H), 1.50 (m, 1H), 1.26 (m, 1H), 0.99 (d, 3H), 0.93 (t, 3H). ^{13}C NMR (D_2O, ppm): 172.1, 60.4, 49.5, 47.8, 47.1, 39.2, 39.0, 38.8, 28.0, 26.8, 26.3, 25.4 (2C), 25.2, 16.7, 13.1. HRMS: *m/z* calcd for $C_{16}H_{37}N_5O$ (M + H) 316.3076, found 316.3064.

***N*¹-Spermine L-Leucinyl Amide (22).** *N*-^{t}Boc-L-leucine 4-nitrophenyl ester was obtained from Sigma and coupled to spermine via method A (40% yield). Deprotection by acid treatment gave **22** as its tetrahydrochloride salt in 99% yield. ^{1}H NMR (D_2O, δ): 3.98 (t, 1H), 3.33 (m, 2H), 3.09 (m, 10 H), 2.09 (m, 2H), 1.91 (m, 2H), 1.74 (m, 7H), 0.93 (dd, 6H). ^{13}C NMR (D_2O, ppm): 173.2, 54.6, 49.6, 47.8, 47.2, 42.4, 39.2, 39.0, 28.0, 27.9, 26.5, 26.3, 25.4 (2C), 24.4, 23.8. HRMS: *m/z* calcd for $C_{16}H_{37}N_5O$ (M + H) 316.3076, found 316.3073.

***N*¹-Spermine L-Cyclohexylalanyl Amide (23).** The 4-nitrophenyl ester of *N*-^{t}Boc-L-cyclohexylalanine (from Sigma) was synthesized via method A (82% yield). Coupling to spermine and purification gave 59% of the ^{t}Boc-protected conjugate. Deprotection using acid gave **23** as its tetrahydrochloride salt in 98% yield. ^{1}H NMR (D_2O, δ): 4.03 (t, 1H), 3.36 (m, 2H), 3.12 (m, 10H), 2.11 (m, 2H), 1.95 (m, 2H), 1.77 (m, 12H), 1.11 (m, 5H). ^{13}C NMR (D_2O, ppm): 173.4, 54.0, 49.6, 49.5, 47.8, 47.1, 41.0, 39.2, 39.0, 35.7, 35.2, 34.6, 28.3, 28.2, 28.0, 26.3, 25.4. HRMS: *m/z* calcd for $C_{19}H_{41}N_5O$ (M + H) 356.3389, found 356.3375.

***N*¹-Spermine L-Prolinyl Amide (24).** *N*-^{t}Boc-L-proline *N*-hydroxysuccinimide ester was obtained from Sigma and coupled to spermine using method B (56% yield as free base). Deprotection by acid treatment gave **24** as its tetrahydrochlo-

ride salt in 94% yield. ^{1}H NMR (D_2O, δ): 4.45 (t, 1H), 3.53 (m, 2H), 3.50 (t, 2H), 3.18 (m, 14H), 2.19 (m, 4H), 2.01 (m, 2H), 1.86 (m, 2H). ^{13}C NMR (D_2O, ppm): 172.9, 63.2, 57.1, 50.4, 50.3, 49.8, 48.6, 47.9, 40.1, 40.0, 33.1, 28.8, 27.3, 27.2, 26.3. HRMS: *m/z* calcd for $C_{15}H_{33}N_5O$ (M + H) 300.2763, found 300.2762.

***N*^{1}-Spermine L-Asparaginyl Amide (25).** *N*-^{t}Boc-L-asparagine 4-nitrophenyl ester was obtained from Sigma and coupled to spermine via method A (44% yield). Deprotection by acid treatment gave **25** as its tetrahydrochloride salt in 99% yield. ^{1}H NMR (D_2O, δ): 4.33 (m, 1H), 3.30 (m, 2H), 3.10 (m, 12H), 2.09 (m, 2H), 1.92 (m, 2H), 1.78 (m, 4H). ^{13}C NMR (D_2O, ppm): 173.8, 173.7, 52.3, 52.1, 49.6, 47.7, 47.1, 39.1, 38.7, 37.4, 37.0, 28.1, 26.3, 25.4. HRMS: *m/z* calcd for $C_{14}H_{32}N_6O_2$ (M + H) 317.2665, found 317.2662.

***N*^{1}-Spermine L-Glutaminyl Amide (26).** *N*-^{t}Boc-l-glutamine 4-nitrophenyl ester was obtained from Sigma and coupled to spermine via method A (57% yield). Deprotection by acid treatment gave **26** as its tetrahydrochloride salt in 99% yield. ^{1}H NMR (D_2O, δ): 4.05 (m, 1H), 3.34 (t, 2H), 3.13 (m, 10H), 2.48 (m, 2H), 2.18 (m, 4H), 1.93 (m, 2H), 1.79 (m, 4H). ^{13}C NMR (D_2O, ppm): 176.1, 170.1, 57.7, 53.2, 47.7, 45.8, 45.2, 37.3, 36.8, 29.9, 26.3, 26.0, 24.4, 23.5 (2C). HRMS: *m/z* calcd for $C_{15}H_{34}N_6O_2$ (M + H) 331.2821, found 331.2826.

***N*^{1}-Spermine L-Methionyl Amide (27).** *N*-^{t}Boc-L-methionine 4-nitrophenyl ester was obtained from Sigma and coupled to spermine via method A (45% yield). Deprotection by acid treatment gave **22** as its tetrahydrochloride salt in 92% yield. ^{1}H NMR (D_2O, δ): 4.10 (t, 1H), 3.34 (m, 2H), 3.10 (m, 12H), 2.60 (t, 2H), 2.18 (m, 2H), 2.11 (s, 3H), 2.07 (m, 2H), 1.78 (m, 4H). ^{13}C NMR (D_2O, ppm): 172.1, 55.0, 49.6, 49.5, 47.8, 47.1, 39.1, 32.4, 30.8, 28.0, 26.3, 25.4 (2C), 16.6. MS (ESI$^+$): 334 (M + H). HRMS: *m/z* calcd for $C_{15}H_{35}N_5OS$ (M + H) 334.2640, found 334.2643.

***N*^{1}-Spermine L-Serinyl Amide (28).** *N*-^{t}Boc-*O*-benzyl-L-serine *N*-hydroxysuccinimide ester was obtained from Sigma and coupled to spermine using method B (48% yield as free base). The *O*-benzyl protecting group was removed via the hydrogenation procedure in method B. Deprotection by acid treatment gave **28** as its tetrahydrochloride salt in 94% yield. ^{1}H NMR (D_2O, δ): 4.13 (t, 1H), 4.00 (m, 2H), 3.36 (t, 2H), 3.12 (m, 10H), 2.13 (m, 2H), 2.09 (m, 2H), 1.93 (m, 4H). ^{13}C NMR (D_2O, ppm): 168.7, 61.0, 55.4, 47.9, 47.8, 46.0, 45.4, 37.5, 37.4, 36.4, 24.6, 23.7 (2C). HRMS: *m/z* calcd for $C_{13}H_{31}N_5O_2$ (M + H) 290.2556, found 290.2547.

***N*^{1}-Spermine L-Threoninyl Amide (29).** *N*-^{t}Boc-*O*-benzyl-L-threonine *N*-hydroxysuccinimide ester was obtained from Sigma and coupled to spermine using method B (56% yield as free base). The *O*-benzyl protecting group was removed via the hydrogenation procedure in method B. Deprotection by acid treatment gave **28** as its tetrahydrochloride salt in 90% yield. ^{1}H NMR (D_2O, δ): 4.04 (m, 1H), 3.72 (d, 1H), 3.23 (t, 2H), 2.97 (m, 12H), 1.96 (m, 2H), 1.81 (m, 2H), 1.65 (m, 4H), 1.16 (d, 3H). ^{13}C NMR (D_2O, ppm): 169.8, 67.2, 58.4, 57.7, 47.4, 46.3, 45.4, 37.6, 37.5, 37.1, 25.3, 24.2 (2C), 20.1. HRMS: *m/z* calcd for $C_{14}H_{33}N_5O_2$ (M + H) 304.2712, found 304.2715.

***N*^{1}-Spermine L-Ornithyl Amide (30).** The 4-nitrophenyl ester of N_α-^{t}Boc-N_δ-Cbz-L-ornithine (from Sigma) was synthesized via method A (65% yield). Coupling to spermine and purification gave 69% of the protected conjugate. Deprotection using hydrogenation (method B) and then acid treatment (method A) gave **30** as its pentahydrochloride salt in 98% yield. ^{1}H NMR (D_2O, δ): 4.04 (t, 1H), 3.38 (m, 2H), 3.12 (m, 14H), 2.11 (m, 2H), 1.96 (m, 4H), 1.80 (m, 6H). ^{13}C NMR (D_2O, ppm): 170.0, 53.6, 47.8, 47.7, 46.1, 45.4, 39.6, 37.4, 28.7, 26.3, 24.6, 23.7, 23.6, 23.4. HRMS: *m/z* calcd for $C_{15}H_{36}N_6O$ (M + Na) 339.2848, found 339.2841.

***N*^{1}-Spermine L-Tryptophanyl Amide (32).** *N*-^{t}Boc-L-tryptophan 4-nitrophenyl ester was obtained from Sigma and coupled to spermine via method A (39% yield). Deprotection by acid treatment gave **32** as its tetrahydrochloride salt in 92% yield. ^{1}H NMR (D_2O, δ): 7.67 (d, 1H), 7.58 (d, 1H), 7.37 (s, 1H), 7.32 (t, 1H), 7.23 (t, 1H), 4.27 (t, 1H), 3.37 (m, 2H), 3.19 (m, 8H), 2.89 (t, 2H), 2.53 (m, 2H), 2.15 (m, 2H), 1.73 (m, 4H), 1.59 (m, 2H). ^{13}C NMR (D_2O, ppm): 170.5, 136.6, 127.3, 126.0, 123.0, 120.2, 118.6, 112.7, 108.8, 54.6, 47.7, 47.4, 45.6, 45.5, 37.2, 37.1, 27.7, 25.4, 24.6, 23.9 (2C). MS (ESI$^+$): 389 (M + H). HRMS: *m/z* calcd for $C_{21}H_{36}N_6O$ (M + Na) 411.2848, found 411.2835.

***N*^{1}-Spermine D-Tryptophanyl Amide (33).** The 4-nitrophenyl ester of *N*-^{t}Boc-D-tryptophan (from Sigma) was synthesized via method A (85% yield). Coupling to spermine and purification gave 54% of the ^{t}Boc-protected conjugate. Deprotection using acid gave **33** as its tetrahydrochloride salt in 94% yield. ^{1}H NMR and ^{13}C NMR were the same as for **32**.

***N*^{1}-Spermine L-Phenylalanyl Amide (34).** *N*-^{t}Boc-L-phenylalanine 4-nitrophenyl ester was obtained from Sigma and coupled to spermine via method A (52% yield). Deprotection by acid treatment gave **34** as its tetrahydrochloride salt in 97% yield. ^{1}H NMR (D_2O, δ): 7.46 (m, 5H), 4.28 (dd, 1H), 3.25 (m, 12H), 2.85 (t, 2H), 2.19 (m, 2H), 1.86 (m, 6H). ^{13}C NMR (D_2O, ppm): 171.7, 136.7, 132.1, 131.8, 130.6, 83.8, 57.2, 49.7, 49.6, 47.7, 47.2, 39.5, 39.3, 39.0, 27.7, 26.4, 25.4. HRMS: *m/z* calcd for $C_{19}H_{35}N_5O$ (M + H) 350.2920, found 350.2904.

***N*^{1}-Spermine L-Tyrosinyl Amide (35).** *N*-^{t}Boc-*O*-benzyl-L-tyrosine *N*-hydroxysuccinimide ester was obtained from Novabiochem and coupled to spermine using method B (50% yield as free base). The *O*-benzyl protecting group was removed via the hydrogenation procedure in method B. Deprotection by acid treatment gave **35** as its tetrahydrochloride salt in 90% yield. ^{1}H NMR (D_2O, δ): 7.20 (d, 2H), 6.96 (d, 2H), 4.15 (t, 1H), 3.36 (m, 2H), 3.18 (m, 10H), 2.77 (t, 2H), 2.14 (m, 2H), 1.82 (m, 6H). HRMS: *m/z* calcd for $C_{19}H_{35}N_5O_2$ (M + H) 366.2869, found 366.2852.

Biological Methods. Cell Culture and Reagents. The human carcinoma cell lines were obtained from ATCC (Rockville, MD) and cultured in the recommended medium, serum, and CO_2 concentration. The medium was obtained from Mediatech, Inc. (Herndon, VA) and the serum from Gibco BRL (Gaithersburg, MD). Penicillin (50 U/mL), streptomycin (50 μg/mL), and l-glutamine (2 mM) (all from BioWhittaker, Walkersville, MD) were included in all cultures. DFMO was obtained from Marion Merrell Dow (Cinncinati, OH). When cells were cultured with polyamines or any of the novel analogues, 1 mM aminoguanidine (AG; Sigma) was included to inhibit serum amine oxidase activity.

Polyamine Transport and K_i Assays. To MDA-MB-231 cells in log growth in 24-well plates was added [2,3-$^3H_2$]PUT (DuPont NEN), [2,9-$^3H_2$]SPD (DuPont NEN, Boston, MA), or [1,4-$^{14}C_2$]SPM (Amersham, Arlington Heights, IL) alone or simultaneously with the polyamine analogue. The cells were incubated at 37 °C for 15 min, which had previously been shown to be within the linear range for polyamine uptake. The cells were then washed three times with PBS and lysed with 0.1% SDS, and the amount of radiolabeled polyamine incorporation into the cells was determined by scintillation counting of the cell lysates. To determine a K_i, four polyamine substrate concentrations and five inhibitor concentrations together with a control were tested. The K_i and K_m values were calculated using a Lineweaver−Burke analysis.

Growth Inhibition Assay. MDA-MB-231 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 6 days, and cell growth was measured by MTS/PMS dye assay (Cell Titer 96 aqueous nonradioactive cell proliferation assay; Promega, Madison, WI). The assay was done in the presence of 1.0 mM AG and 1.0 μM SPD to ensure that any growth inhibition was not the result of depletion of external polyamines in the medium. EC_{50} values represent the concentration of analogue that resulted in 50% of the maximum growth inhibition achievable in the presence of both DFMO and analogue.

Polyamine Analysis. MDA-MB-231 cells were seeded in flasks such that they would be in log growth for 7 days. All flasks received AG (1.0 mM), SPD (1.0 μM), and the appropriate compound the day after seeding. After 6 days, cells were harvested, washed, counted, and lysed in 0.40 M perchloric acid. The HPLC method for the fluorometric detection of

dansylated polyamines from the lysates was based on the procedure by P. M. Kabra et al.[41] A detection level of 1.0 pmol was achievable.

ODC Activity Assay. This assay was slightly modified from one previously described.[62] MDA-MB-231 cells were seeded to be in log growth for the duration of the experiment. The day after seeding, compounds were added to the appropriate flasks: 1 mM AG, 1.0 μM SPD, 100 μM Lys-Spm, **31**, and 0.5 mM DFMO. After 3 days with the drugs the cells were harvested by trypsinization, counted, and washed with PBS prior to freezing. At the time of the assay, cells were thawed in cold breaking buffer (25 mM Tris, 0.10 mM EDTA, 2.5 mM DTT, at pH 7.5), homogenized, and frozen/thawed twice and aliquots removed for protein determinations (Pierce Coomassie protein assay). The homogenates were centrifuged at 10000 rpm for 10 min. A 50 μL sample of the supernatant was combined with 200 μL of the reaction buffer containing 5 mM Tris at pH 7.4, 0.4 mM L-ornithine, 40 μM pyridoxal-5-phosphate, 5 mM DTT, and 1 μCi of l-[1-^{14}C]ornithine (American Radiolabeled Chemicals, Inc., 55 mCi/mmol). The assay mixture was incubated at 37 °C for 2 h in an Eppendorf tube that was contained in a tightly capped 4 mL glass vial containing 400 μL of 0.1 N NaOH. The reaction was stopped by the addition of 125 μL of 10 M H_2SO_4. The 0.1 N NaOH in the vial was counted by scintillation for radioactivity from absorbed $^{14}CO_2$ that was released from ornithine substrate. Cell lysates were assayed in duplicate and were normalized to protein concentration.

Supporting Information Available: ^{1}H and ^{13}C NMR spectra of novel compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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