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The Use of Novel C-Methylated Spermidine Derivatives To Investigate the Regulation of Polyamine Metabolism

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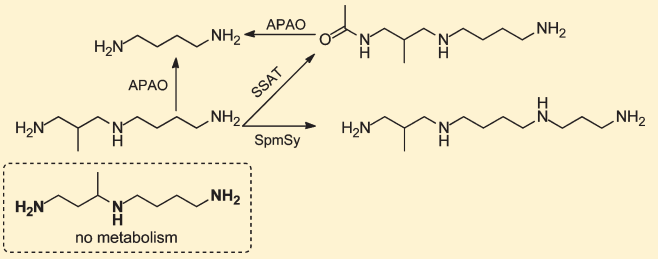
Supporting Information

ABSTRACT: The polyamines are organic polycations present at millimolar concentrations in eukaryotic cells where they participate in the regulation of vital cellular functions including proliferation and differentiation. Biological evaluation of rationally designed polyamine analogs is one of the cornerstones of polyamine research. Here we have synthesized and characterized novel C-methylated spermidine analogs, that is, 2-methylspermidine, 3-methylspermidine, and 8-methylspermidine. 3-Methylspermidine was found to be metabolically stable in DU145 cells, while 8-methylspermidine was a substrate for spermidine/spermine N^1-acetyltransferase (SSAT) and 2-methylspermidine was a substrate for both SSAT and acetylpolyamine oxidase. All the analogs induced the splicing of the productive mRNA splice variant of SSAT, overcame growth arrest induced by 72-h treatment with ornithine decarboxylase (ODC) inhibitor α-difluoromethylornithine, and were transported via the polyamine transporter. Surprisingly, 2-methylspermidine was a weak downregulator of ODC activity in DU145 cells. Our data demonstrates that it is possible to radically alter the biochemical properties of a polyamine analog by changing the position of the methyl group.



INTRODUCTION

The importance of the polyamines putrescine (Put, **1**), spermidine (Spd, **2a**), and spermine (Spm, **3a**) (Figure 1) for diverse physiological functions in living organisms has been elucidated during the past decades, but the distinct roles of **2a** and **3a** are still not completely understood.[1−4] Multilevel regulation of not only activities but also the biosynthesis and degradation of the enzymes of polyamine metabolism, as well as regulation of polyamine uptake and excretion, underline the importance to develop methods either to specifically silence or induce the required regulatory pathway. Mutants and genetically modified microorganisms and cell lines,[1−3] as well as transgenic animals,[5] are widely used for these purposes.[4] However, chemical compounds specifically "switching off" or inducing regulatory pathways of polyamine metabolism are limited, and the most well-known are terminally bis-N-alkylated derivatives of **3a**. Changing the structure of the N-alkyl substituent facilitated the transition from actively transported inducers to superinducers of spermidine/spermine N^1-acetyltransferase (SSAT) that together with efficient downregulation of ornithine decarboxylase (ODC) and *S*-adenosyl-L-methionine decarboxylase (AdoMetDC) resulted in polyamine depletion-induced growth arrest or cytotoxicity. Some of the terminally bis-N-alkylated derivatives of **3a** are at different phases of clinical trials.[4] There is also a growing interest to develop metabolically stable polyamine−drug conjugates, which would deliver the cytotoxic drug into tumor tissue via polyamine transporter.[6] Therefore, the design of novel chemical compounds controlling the metabolism of the enzymes and proteins involved in the regulation of polyamine homeostasis is of importance and may even contribute to practical polyamine-based therapy.

Investigation of the cellular functions of **2a** and **3a** at the molecular level is rather complicated due to their feasible interconversion and their ability to substitute for each other in some functions.[1−3] Considerable original data have been provided with the aid of cells and microorganisms that are deficient in the key enzymes of polyamine metabolism. One of the recent examples is the construction of *E. coli* strains being deficient in all the enzymes of polyamine biosynthesis.[7] The use of specific enzyme inhibitors is an efficient approach *in vitro*, but the necessity to inhibit several enzymatic transformations leads to

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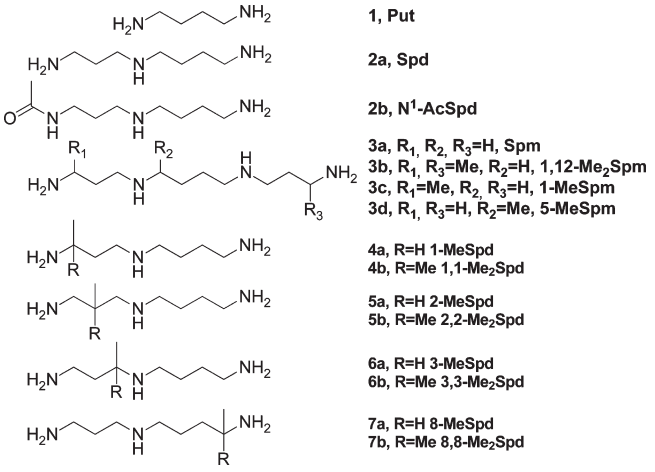


Figure 1. Structures of the compounds.

cumbersome combinations of the inhibitors.[8,9] A relatively simple method to discriminate the cellular effects of **2a** and **3a** is the depletion of the polyamine pool and subsequent application of functionally active metabolically stable mimetics of **2a** and **3a**.

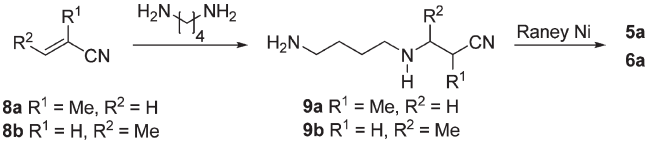
Earlier studies implied that polyamine metabolism could be selectively modulated by the use of 1-methylated polyamine analogs, which can fulfill many cellular functions of polyamines. 1-Methylspermidine (1-MeSpd, **4a**) and 1,12-bis-methylspermine (1,12-Me_2Spm, **3b**) restored early liver regeneration after partial hepatectomy, prevented acute pancreatitis caused by depletion of polyamine pool in SSAT-transgenic rats,[10] and overcame growth inhibition caused by 3-d treatment with α-difluoromethylornithine (DFMO).[11] The use of optical isomers of **4a** revealed that only the (*S*)-isomer was a substrate for deoxyhypusine synthase and capable of supporting the growth of DU145 cells during prolonged polyamine deprivation[12] or growth of *Saccharomyces cerevisiae* polyamine auxotrophs.[13] In addition, the distinct optical isomers of **4a** and **3b** were found to differ in their ability to regulate the biosynthesis of SSAT, AdoMetDC, and antizyme (OAZ), a protein regulating ODC half-life and polyamine transport.[14]

Here, we describe novel possibilities for the regulation of polyamine metabolism using C-methylated derivatives of **2a** (Figure 1). Among these analogs, 2-methylspermidine (2-MeSpd, **5a**) displayed catabolic instability toward SSAT and acetylpolyamine oxidase (APAO), whereas 3-methylspermidine (3-MeSpd, **6a**) was metabolically stable. Our results demonstrate that the biochemical properties of these analogs are determined by the triprotonated backbone and by the position of the methyl substituent.

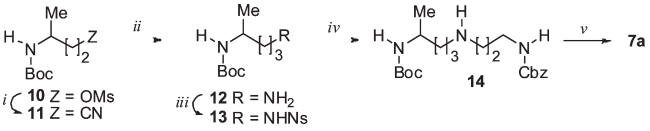
RESULTS AND DISCUSSION

Chemistry. There are various synthetic strategies to build up C−N bonds to polyamines based upon a comprehensive review of this area.[15] In the present paper from the variety of methods, we selected two, the Michael addition of amine to unsaturated nitriles to prepare **5a** and **6a** and alkylation of the *o*-nitrophenyl sulfonates with alkyl halides to prepare **7a**. Hence, the addition of an excess of putrescine to either methacrylonitrile (**8a**) or crotononitrile (**8b**) with subsequent reduction of the intermediate diaminonitriles **9a** and **9b** with hydrogen over Raney-Ni

Scheme 1. Syntheses of Novel Spermidine Derivatives 5a and 6a



Scheme 2. Synthesis of Novel Spermidine Derivative 7a



(i) NaCN/DMSO; (ii) $LiAlH_4/Et_2O/-5$ °C; (iii) NsCl/DCM/Et_3N; (iv) (1) $CbzNH(CH_2)_3Br/DMF/K_2CO_3/45$ °C; (2) PhSH/DMF/K_2CO_3; (v) (1) HCl/EtOH; (2) NaOH/H_2O/DCM; (3) H_2/Pd/AcOH/MeOH; (4) HCl/EtOH.

Table 1. The Analogs as Substrates for Mouse Recombinant SSAT

substrate	K_m (μM)	V_{max} (μmol/(min·mg))
2a	151 ± 15	4.28 ± 0.13
4a	[b]	[b]
5a	132 ± 7	0.77 ± 0.01
6a[a]	[b]	[b]
7a	78 ± 3	7.35 ± 0.10

[a] **6a** K_i = 52 ± 18 μM. [b] Not acetylated.

resulted in target trihydrochlorides **5a** and **6a** in two steps with overall yields of 16% and 32%, respectively (Scheme 1).

The synthesis of **7a** was more complicated and was performed in seven steps (Scheme 2), starting from 3-[*N*-(*tert*-butyloxycarbonyl)]amino-1-butyl methanesulfonate (**10**), which was first converted into nitrile **11**, while subsequent $LiAlH_4$ reduction gave amine **12**. Thus obtained **12** was nosylated, and the resulting sulfonamide **13** was alkylated with 3-(*N*-benzyloxycarbonyl)amino-1-bromopropane that after removal of the nosyl group gave the intermediate Boc-Cbz-triamine **14**. Stepwise removal of Boc and Cbz groups resulted in target **7a** with an overall yield of 31%, as calculated from the starting methanesulfonate **10**.

Catabolic Stability of the Analogs. Nearly 30 years ago, a family of *gem*-dimethylated derivatives was synthesized, and among 1,1-bis-methylspermidine (1,1-Me_2Spd, **4b**), 2,2-bis-methylspermidine (2,2-Me_2Spd, **5b**), 3,3-bis-methylspermidine (3,3-Me_2Spd, **6b**), 8,8-bis-methylspermidine (8,8-Me_2Spd, **7b**), and 5,5-bis-methylspermidine (5,5-Me_2Spd) (Figure 1), only **7b** was a substrate for SSAT.[16] Here we demonstrate that the reduction of the number of substituents dramatically affects the substrate properties of the analogs for SSAT. Like the earlier studied **7b**, 8-methylspermidine (8-MeSpd, **7a**) was a substrate for mouse recombinant SSAT with kinetic parameters reflecting increased affinity and catalytic activity than those observed with **2a** (Table 1). Surprisingly, **5a**, in contrast to **5b**, was a substrate of SSAT, whereas **6a**, like the earlier **4a**,[11] exhibited no substrate properties but was a competitive inhibitor of the enzyme. When

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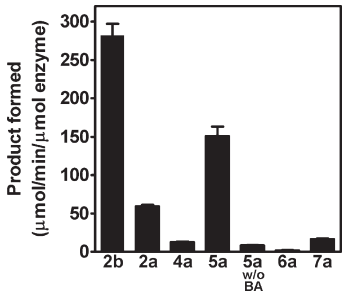

Figure 2. The analogs as substrates for human recombinant APAO with or without (w/o) 5 mM benzaldehyde (BA). Values were determined using 0.1 or 1 μg of enzyme and 0.5 or 1 mM substrate and incubation time of 5−60 min. Data are means ± SD, n = 6.

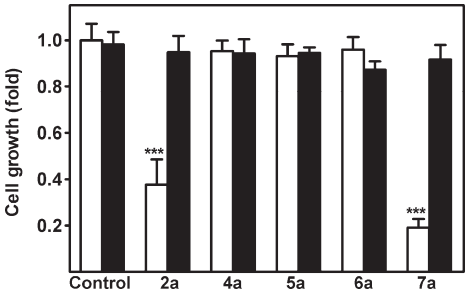

Figure 3. Cytotoxicity of the degradation products of the analogs produced by the action of bovine serum amine oxidases. MTT assay of DU145 cells grown for 48 h with 100 μM analogs, with (black bars) or without (white bars) 1 mM AG. Results are means ± SD, n = 8; *** indicates statistical significance of $p < 0.001$ compared with "w AG".

SSAT was induced in DU145 cells by incubating the analog-preloaded cells with N^1,N^{11}-diethylnorspermine (DENSpm), acetylated derivatives of **5a** and **7a** were found intracellularly (Table S1, Supporting Information). In addition, all analogs were excreted into the media and **7a** was also in an acetylated form, even without DENSpm treatment.

A recent X-ray study of the SSAT complex with **3a** showed that a methyl group at the third position may affect hydrophobic contacts of the analog with the side chains of Leu128 and Trp154, which are positioned close to the secondary (N-4) amino group of substrate.[17] In addition, this methyl group may influence the structure of a "proton wire", which is formed by water molecules coordinated by the substrate amino groups and the side chains of Asp93 and Glu92, which cover not only the acetylated amino group, but also the neighboring N-4 amino group. These properties apparently restrict the N^1-acetylation of **6a**, making it a novel catabolically stable derivative of **2a**.

Earlier studies showed that a chemically synthesized mono-acetylated derivative of **4a** was a good substrate of APAO.[18] In the presence of aromatic aldehydes, APAO degraded not only **2a** and **3a**[19] but also **4a**.[18] Here, we show that benzaldehyde efficiently stimulated APAO-dependent degradation of all the analogs tested, with the exception of **6a** (Figure 2). Methyl group at the third position of **2a** may either restrict the splitting of the proton from third carbon atom, which is required to form the intermediate Schiff base, or interfere with the proper binding of the analog, which may explain the stability of **6a** toward APAO in the presence of benzaldehyde. Surprisingly, **5a** was degraded by APAO even in the absence of benzaldehyde, which is atypical for

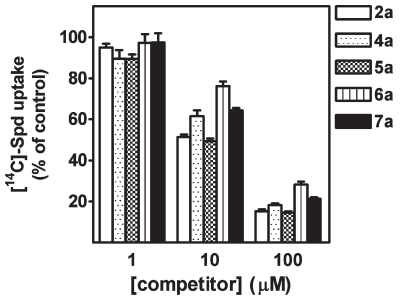

Figure 4. Uptake competition with [^{14}C]-labeled **2a**. DU145 cells were incubated in serum-free medium for 10 min in the presence of 10 μM [^{14}C]-**2a** and 1, 10, or 100 μM analogs as competitors. Data are means ± SD, n = 3.

the substrate specificity of this enzyme, while in the presence of benzaldehyde, **5a** was even a better substrate of APAO than **2a** (Figure 2). Thus, our data reveal two structurally related classes of analogs, one class demonstrating increased catabolic instability (**5a**) and the other considerable catabolic stability (**6a**) toward polyamine-metabolizing enzymes.

Serum amine oxidases degrade extracellular polyamines and many of their analogs to toxic metabolites. The study of the stability of C-methylated analogs of **2a** in cell culture medium showed that at 10 μM none of the analogs was toxic to DU145 cells (data not shown) and at 100 μM concentration only **2a** and **7a** produced toxic metabolites (Figure 3). Since we preferred to use higher concentrations of the analogs in order to efficiently replace the natural polyamines, cells were cultured in the presence of 1 mM aminoguanidine (AG), an inhibitor of serum amine oxidases.

The Analogs Compete with 2a for Uptake. The polyamine transporter allows the uptake of not only the natural polyamines but also many analogs resembling the natural polyamines in structure.[20] We found here that all tested C-methylated analogs of **2a** competed for the uptake with [^{14}C]-**2a** by DU145 cells during 10-min incubation with varying efficiencies (Figure 4). Only **5a** was as efficient as unlabeled **2a** in reducing the accumulation of [^{14}C]-label, and **6a** was clearly the weakest competitor. Thus, the polyamine transport system recognized all tested analogs as polyamines but with different affinities or velocities. However, the C-methylation affected uptake by the polyamine transporter only moderately, compared with the numerous other N-alkylated analogs, such as the N^1-anthracenylmethyl homospermidine derivatives, where the position and the size of the N-substituent had dramatic effects on the polyamine transporter targeting.[6]

Compound 6a Is Not Metabolized to 3-MeSpm. The *gem*-dimethylated derivatives of **2a** (Figure 1) were not substrates of spermine synthase (SpmSy) even at 5 mM concentrations.[16] However, the decrease of the number of the substituents resulted in the biosynthesis of the corresponding C-methylated Spm analogs from **4a**, **5a**, and **7a** in DU145 cells (Table 2). 1-MeSpm (**3c**) and 2-MeSpm accumulated in cells after 24 h incubation in the presence of the corresponding **2a** analog, while 5-MeSpm (**3d**) (according to IUPAC nomenclature this is **3d**, not 8-MeSpm) was detectable only after 48 h (Table 2). The biosynthesis of **3d** was somewhat unexpected, because in chick embryos the formation of only **7a** from 1-methylputrescine was detected.[21] The absence of any 3-MeSpm even after 72 h incubation of DU145 cells with **6a** was very surprising. Our data

Table 2. Effect of the Analogs on Polyamine Content in DU145 Cells after 24, 48, and 72 h of Culture[a]

	intracellular polyamine (pmol/μg DNA)						
treatment	1	2a	3a	2b	analog	MeSpm[e]	analog+ MeSpm
				24 h			
control	11 ± 2	154 ± 7	122 ± 11	[b]			
4a	[b,c]	15 ± 0[c]	62 ± 3[c]	[b]	264 ± 12	37 ± 2	301 ± 14
5a	<5[c]	39 ± 0[c]	67 ± 5[c]	[b]	133 ± 4	67 ± 13	200 ± 17
6a	<5[c]	23 ± 1[c]	92 ± 4[c]	[b]	181 ± 5	[b]	181 ± 5
7a	[b,c]	30 ± 2[c]	83 ± 3[c]	15 ± 2[d]	257 ± 5	[b]	257 ± 5
				48 h			
control	7 ± 1	137 ± 5	133 ± 4	[b]			
4a	n.d[c]	11 ± 1[c]	55 ± 1[c]	[b]	239 ± 6	44 ± 2	283 ± 8
5a	<5[c]	38 ± 5[c]	72 ± 12[c]	[b]	135 ± 12	77 ± 7	202 ± 19
6a	[b,c]	18 ± 1[c]	103 ± 2[c]	[b]	168 ± 6	[b]	168 ± 6
7a	[b,c]	18 ± 1[c]	57 ± 4[c]	7 ± 1[d]	287 ± 16	39 ± 6	326 ± 22
				72 h			
control	6 ± 0	137 ± 3	154 ± 7	[b]			
4a	[b,c]	16 ± 4[c]	74 ± 24[c]	[b]	311 ± 76	60 ± 8	371 ± 84
5a	<5[c]	38 ± 1[c]	75 ± 4[c]	[b]	134 ± 6	80 ± 7	214 ± 13
6a	[b,c]	22 ± 1[c]	115 ± 2[c]	[b]	176 ± 3	[b]	176 ± 3
7a	[b,c]	20 ± 1[c]	70 ± 2[c]	4 ± 1[d]	332 ± 14	43 ± 3	375 ± 17

[a] Cells were cultured in the presence of 100 μM analogs. All plates contained also 1 mM AG. Results are means ± SD, $n = 3$. [b] Not detectable. [c] Statistical significance of $p < 0.001$ compared with AG-treated control group. [d] Ac-8-MeSpd. [e] MeSpm was quantified using **3c** standards.

emphasize the necessity of high complementarity between the central part of the **2a** molecule and the corresponding region of SpmSy responsible for binding of the substrate(s) and their analogs. The lack of such a complementarity renders **6a** metabolically stable.

The Analogs Differ in Their Ability To Downregulate ODC. Incubation of DU145 cells with any of the tested analogs for 24, 48, or 72 h had negligible effects on the activities of AdoMetDC, APAO, and SSAT. Some reduction in SMO activity was observed with **6a** (Table 3). However, the most pronounced effects were observed in the case of ODC, the activity of which was downregulated by all analogs tested, except **5a**. Since OAZ protein is the key regulator of ODC half-life and polyamines are known to stimulate OAZ biosynthesis via promotion of ribosomal frameshifting,[22,23] our data suggests that **5a** may be incapable of inducing OAZ.

We also measured the intracellular levels of *S*-adenosyl-L-methionine (SAM) and decarboxylated *S*-adenosyl-L-methionine (dc-SAM) after 3-d culture with AG and DFMO (Table 4). Increased dc-SAM levels in DFMO-treated cells were reduced by treatment with the analogs, and **5a** was as effective as natural spermidine in reducing dc-SAM level.

The Analogs Modulate Alternative Splicing of SSAT Pre-mRNA. The alternative splicing of SSAT mRNA is regulated by intracellular polyamine level[24] but the molecular mechanisms of this regulatory pathway, as well as the mechanisms of frameshifting of OAZ mRNA, still remain unclear. The alternative splicing of SSAT pre-mRNA yields one variant encoding catalytically active protein (SSAT) and the other containing an additional exon (SSAT-X), targeting the mRNA to rapid degradation via a nonsense-mediated mRNA decay pathway. Our previous studies have shown that many but not all polyamine analogs are able to induce the productive splicing of SSAT.[24,25] Here we found that all tested analogs stimulated the productive splicing of SSAT mRNA (Figure 5), and the efficiency of the analogs correlated with the total intracellular higher polyamine concentration (**2a**+**3a**+analog; $R = 0.90$, $p < 0.01$). Thus, the application of **5a** provides an opportunity to induce the productive splicing of SSAT pre-mRNA without a significant effect on ODC.

The Analogs Support Growth of DFMO-Treated DU145 Cells. Earlier studies indicated that *gem*-dimethylated Spd analogs were able to overcome growth inhibition caused by a 3-d DFMO treatment of SV-3T3 cells.[16] As expected, all tested analogs supported the growth of DFMO-treated DU145 cells and exhibited about the same efficacy as **2a** (Table 4). Thus, among the analogs, **6a** is not only metabolically stable but also a functional mimetic of **2a**, which makes this compound an exceptionally useful instrument to investigate the cellular functions of **2a**.

CONCLUSIONS

In conclusion, our data demonstrates that functional recognition of **2a** analogs is based not only on electrostatic interactions but also on proper spatial organization of the essential recognition part(s) of the **2a** molecule. Therefore, by changing the position of the methyl group in the **2a** backbone, it is possible to design novel compounds that are suitable to study the cellular functions of the individual polyamines. These results also provide considerable insight into the structural limitations that must be met for the design of effective drugs based on the polyamine backbone.

EXPERIMENTAL SECTION

Materials. DFMO was from ILEX Oncology Inc. (U.S.A.), [^{14}C]-labeled compounds from GE Healthcare, and other reagents from Sigma-Aldrich. The synthesis of **4a** is described previously.[18]

Table 3. The Activity of the Enzymes of Polyamine Metabolism after 24, 48, or 72 h Incubation of DU145 Cells with the Analogs[a]

	activity (pmol/h per μg DNA)				
time and treatment	ODC	AdoMetDC	SSAT	SMO	APAO
		24 h			
control	29.3 ± 1.4	61 ± 5	59 ± 1	493 ± 22	540 ± 61
4a	4.3 ± 0.3[b]	64 ± 2	67 ± 3[c]	362 ± 41[c]	545 ± 7
5a	21.7 ± 1.4[b]	67 ± 1	56 ± 3	394 ± 19	562 ± 17
6a	8.8 ± 0.3[b]	53 ± 2	62 ± 2	204 ± 20[b]	528 ± 57
7a	4.7 ± 0.5[b]	53 ± 4	65 ± 3	515 ± 78	562 ± 97
		48 h			
control	17.7 ± 2.6	73 ± 1	58 ± 0	680 ± 29	699 ± 21
4a	4.4 ± 0.3[b]	72 ± 3	61 ± 3	695 ± 44	779 ± 32
5a	16.6 ± 0.7	71 ± 2	59 ± 3	655 ± 39	719 ± 44
6a	9.3 ± 0.8[b]	60 ± 0[b]	59 ± 2	474 ± 29[d]	693 ± 6
7a	5.5 ± 0.6[b]	67 ± 2	62 ± 6	738 ± 89	770 ± 60
		72 h			
control	7.5 ± 0.9	72 ± 6	62 ± 4	742 ± 10	662 ± 48
4a	3.5 ± 0.1[b]	75 ± 5	66 ± 5	754 ± 27	745 ± 43
5a	9.1 ± 0.3[c]	79 ± 3	61 ± 6	608 ± 42	732 ± 79
6a	5.4 ± 0.4[d]	58 ± 1[d]	69 ± 4	571 ± 62[d]	775 ± 73
7a	3.8 ± 0.3[b]	63 ± 1	61 ± 3	832 ± 45	737 ± 99

[a] Cells were cultured in the presence of 100 μM analogs. All plates contained also 1 mM AG. Results are means ± SD, n = 3. [b] Statistical significance of $p < 0.001$ compared with AG-treated control group at each time point. [c] Statistical significance of $p < 0.05$ compared with AG-treated control group at each time point. [d] statistical significance of $p < 0.01$ compared with AG-treated control group at each time point.

Table 4. Effect of the Analogs on Cell Growth and SAM and dc-SAM Levels in DU145 Cells after 72 h of Culture

treatment	growth (%)	SAM (pmol/10^6 cells)	dc-SAM (pmol/10^6 cells)
control	100 ± 4[b]	84 ± 3	<5[b]
DFMO	49 ± 2	80 ± 5	263 ± 14
DFMO+**2a**	89 ± 4[b]	126 ± 5[c]	<5[b]
DFMO+**4a**	84 ± 6[b]	177 ± 25[b]	49 ± 8[b]
DFMO+**5a**	86 ± 2[b]	148 ± 3[b]	<5[b]
DFMO+**6a**	91 ± 5[b]	149 ± 7[b]	109 ± 4[b]
DFMO+**7a**	93 ± 3[b]	138 ± 4[b]	60 ± 3[b]

[a] Cells were cultured in the presence of 100 μM analogs and 5 mM DFMO. All plates contained also 1 mM AG. Results are means ± SD, n = 3. [b] Statistical significance of $p < 0.001$ compared with AG+DFMO-treated group. [c] Statistical significance of $p < 0.01$ compared with AG+DFMO-treated group.

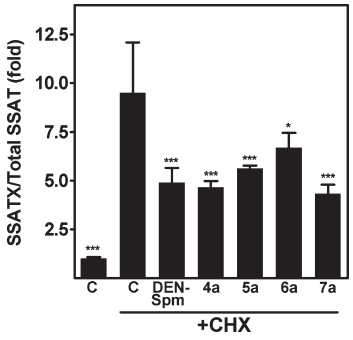

Figure 5. Effect of the analogs on the alternative splicing of SSAT. Mouse primary fetal fibroblasts were treated with CHX (10 μg/mL) for 1 h and then with 100 μM analogs and CHX for 7 h. The ratio of SSAT-X/total SSAT mRNA was measured with quantitative RT-PCR. Data are means ± SD, n = 3. C, control; ∗ and ∗∗∗ indicate statistical significance of $p < 0.05$ and 0.001, respectively, compared with CHX-treated group.

Syntheses. 1,4-Diaminobutane, thiophenol, $LiAlH_4$, NaCN, *o*-nitrophenylsulfonylchloride (NsCl), and anhydrous LiBr were purchased from Aldrich (U.S.A.), and the rest of chemicals were purchased from Fluka (Switzerland). 3-[*N*-(*tert*-Butyloxycarbonyl)]amino-1-butyl methanesulfonate and 3-(*N*-benzyloxycarbonyl)amino-1-propyl methanesulfonate were prepared as recently published.[26] Syntheses of 3-(*N*-benzyloxycarbonyl)amino-1-bromopropane is described in the Supporting Information. TLC was carried out on precoated Kieselgel 60 F_{254} plates, and column chromatography was performed with Kieselgel (40−63 μm, Merck, Germany) using the following elution systems: (A) 8:2 dioxane−25% NH_4OH, (B) 7:3 dioxane−25% NH_4OH, (C) 4:2:1:2 *n*-BuOH−AcOH−Py−H_2O. Flash chromatography was performed on Kieselgel (40−63 μm, Merck, Germany), and systems for elution are indicated in the text. Melting points were determined in open capillary tubes and are uncorrected. ^1H and ^{13}C NMR spectra were measured on a Bruker Avance 500 DRX (Germany) using tetramethylsilane (TMS) in $CDCl_3$ or sodium 3-(trimethylsilyl)-1-propanesulfonate (TSP) in D_2O as internal standards. Chemical shifts are given in ppm, the letter "*J*" indicates normal $^3J_{HH}$ couplings, and all *J* values are given in hertz. All final compounds were ≥99% purity confirmed with analytical HPLC used according to the published method.[27]

*2-Methyl-8-amino-4-azaoctanenitrile (**9a**).* A solution of 1,4-diaminobutane (55.4 g, 0.63 mol) and **8a** (17.16 g, 0.256 mol) in EtOH (8 mL) was stirred for 2 h at 20 °C, 2 h at 60 °C, and 2.5 h at 90 °C. Subsequent distillation of the reaction mixture gave **9a** (18.8 g, 47%). Bp 101 °C/0.5 mmHg; n_D^{20} 1.4646. TLC, NMR, and anal. C, H, N were conducted.

*1,8-Diamino-2-methyl-4-azaoctane trihydrochloride (**5a**), 2-MeSpd.* A suspension of Raney nickel (10 mL) and **9a** (1.5 g, 9.7 mmol) in abs. EtOH (50 mL) was hydrogenated at atmospheric pressure for 24 h. The catalyst was filtered off, the residue was washed with abs. EtOH, and the combined filtrates were evaporated to dryness *in vacuo* to yield crude **5a**, which was purified by column chromatography on a silica gel (130 g) eluting subsequently with solvent mixtures A and B. The crude triamine **5a** was repurified on a silica gel (70 g) eluting as above with solvent mixtures A and B. The free base of **5a** was dissolved in the mixture of EtOH (15 mL) and 5 M HCl (5 mL) and evaporated to dryness *in vacuo*, which after coevaporation with abs. EtOH and crystallization from abs. EtOH gave **5a** (0.69 g, 34%); mp 189−192 °C; R_f 0.22 (C); R_f 0.16 (B). ^1H NMR (D_2O): δ 3.19−2.90 (8H, m, 4*NCH_2); 2.39−2.28 (1H, m, C<u>H</u>); 1.85−1.72 (4H, m, CC<u>H_2</u>C<u>H_2</u>C); 1.16 (3H, d, J = 6.8 Hz, CH_3). ^{13}C NMR (D_2O): δ 53.48, 50.59, 45.23, 41.67, 32.27, 26.79, 25.47, 17.07. Anal. C, H, N.

*3-Methyl-8-amino-4-azaoctanenitrile (**9b**).* Prepared as **9a** starting from 1,4-diaminobutane (58.9 g, 0.67 mol) and **8b** (8.97 g, 0.134 mol) to give after distillation **9b** (17.6 g, 84.7%); bp 102−103 °C/0.75 mmHg; n_D^{20} 1.4688. TLC, NMR, and anal. C, H, N were conducted.

*1,8-Diamino-3-methyl-4-azaoctane trihydrochloride (**6a**), 3-MeSpd.* Following the procedure for **5a** and starting from **9b** (1.7 g, 10.97 mmol) and a suspension of Raney nickel (10 mL) in abs. EtOH (50 mL) provided the target **6a** (1.11 g, 37.8%); mp 231−232 °C; R_f 0.24 (C); R_f 0.10 (B). ^1H NMR ($CDCl_3$): δ 3.48−3.41 (1H, m, CH), 3.23−3.00 (6H, m, 3*NCH_2), 2.25−2.15 (1H, m, CH<u>CH_2</u>), 2.01−1.91 (1H, m, CH<u>CH_2</u>), 1.85−1.75 (4H, m, CC<u>H_2</u><u>CH_2</u>C), 1.36 (3H, d, J = 6.7 Hz, CH_3). ^{13}C NMR ($CDCl_3$): δ 54.88, 47.10, 41.68, 38.75, 33.07, 26.84, 25.83, 17.96. Anal. C, H, N.

*4-[N-(tert-Butyloxycarbonyl)]-aminovaleronitrile (**11**).* Finely pulverized NaCN (4.5 g, 92 mmol) was added in small portions to a stirred solution of 3-[*N*-(*tert*-butyloxycarbonyl)]amino-1-butyl methanesulfonate (**10**) (4.8 g, 18 mmol) in abs. DMSO (20 mL) and stirring was continued for 8 h at 45 °C. The reaction mixture was cooled to 0 °C, then 2 M NaOH (75 mL) was added, and the resulting mixture was extracted with Et_2O (4 × 20 mL). Combined Et_2O extracts were washed with brine (15 mL), dried ($MgSO_4$), filtered, and concentrated *in vacuo*. The residue was dried at 0.5 mmHg at 30 °C to afford **11** as an oil (4.73 g, 99%), which self-solidified upon the addition of a few crystals of **11**. Treatment with hexane gave **11** (3.39 g, 95.2%) as a low melting solid (45 °C). TLC, NMR, and anal. C, H, N were conducted.

*4-[N-(tert-Butyloxycarbonyl)]-1,4-diaminopentane (**12**).* A solution of **11** (4.46 g, 22.5 mmol) in Et_2O (35 mL) was added with mechanic stirring for 20 min to a cooled (−10 °C) suspension of $LiAlH_4$ (2.33 g, 61 mmol) in Et_2O (50 mL) and stirring was continued for 1 h at ca. −5 °C. The reaction mixture was cooled to −10 °C and carefully quenched subsequently with H_2O (3.3 mL), 20% aq NaOH (3.1 mL), H_2O (8.5 mL), and 40% aq NaOH (10.0 mL), maintaining the temperature below −5 °C. On warming to 20 °C, the organic layer was separated, and the residue was treated with Et_2O (3 × 30 mL). The combined Et_2O extracts were washed with 1 M $NaHCO_3$ (15 mL), H_2O (5 mL), and brine (15 mL) and dried (K_2CO_3). Solvent was distilled off *in vacuo*, and the residue was dried *in vacuo* over P_2O_5/KOH to give **12** (3.33 g, 75%) as a colorless oil. TLC, NMR, and anal. C, H, N were conducted.

*1-(N-o-Nitrophenylsulfonyl)amino-4-[N-(tert-butyloxycarbonyl)]-aminopentane (**13**).* A solution of NsCl (1.6 g, 7.3 mmol) in DCM (10 mL) was added over 30 min with stirring to a cooled (0 °C) solution of **12** (1.54 g, 7.6 mmol) and Et_3N (1.23 mL, 8.8 mmol) in DCM (20 mL), and stirring was continued for 1 h at 0 °C and 3 h at 20 °C. The precipitate was filtered off, the filtrate was washed with 1 M $NaHCO_3$ (3 × 10 mL), H_2O (5 mL), 10% citric acid (4 × 10 mL), H_2O (5 mL), and brine (5 mL), dried ($MgSO_4$), and filtered. The solvent was distilled off *in vacuo*, and the residual oil was dried *in vacuo* over P_2O_5 at 30 °C. This oil self-solidified on storage at +4 °C and was recrystallized from EtOAc/hexane to give **13** (2.61 g, 89%) as small colorless cubic crystals; mp 80−81 °C. TLC, NMR, and anal. C, H, N were conducted.

*1-(N-Benzyloxycarbonyl)amino-8-[N-(tert-butyloxycarbonyl)]amino-4-azanonane (**14**).* A mixture of **13** (1.94 g, 5 mmol), 3-(*N*-benzyloxycarbonyl)amino-1-bromopropane (1.63 g, 6 mmol), and K_2CO_3 (2.07 g, 15 mmol) was stirred in DMF (20 mL) for 16 h at 45 °C followed by addition of K_2CO_3 (1.38 g, 10 mmol) and PhSH (0.88 g, 8 mmol). After stirring for additional 6 h at 20 °C, the salts were separated and washed with DMF, and combined DMF filtrates were evaporated to dryness *in vacuo*. The residue was treated with a mixture of EtOAc (25 mL) and H_2O (15 mL), the water phase was extracted with EtOAc (3 × 5 mL), and combined EtOAc extracts were washed with H_2O (5 mL) and brine (10 mL). Solvent was distilled off *in vacuo*, and the residue was purified on a silica gel column (160 g) eluting subsequently with $CHCl_3$, $CHCl_3$−MeOH (100:1), and $CHCl_3$−MeOH (95:5), which after drying *in vacuo* over P_2O_5 at 30 °C gave **14** (1.35 g, 69%) as a colorless oil. TLC, NMR, and anal. C, H, N were conducted.

*1,8-Diamino-4-azanonane Trihydrochloride, 8-MeSpd (**7a**).* To a cooled (0 °C) solution of **14** (1.1 g, 2.8 mmol) in MeOH (7 mL) was added HCl/MeOH (1.2 mL, 10 M), and the mixture was stirred for 30 min at 20 °C followed by evaporation to dryness *in vacuo* (bath temperature 20 °C). The residue was dissolved in EtOH (2 mL) and precipitated with an excess of Et_2O, which after drying *in vacuo* over P_2O_5/KOH resulted in 1-(*N*-benzyloxycarbonyl)amino-4-aza-8-aminononane dihydrochloride (0.88 g, 85%); mp 134−136 °C. TLC, NMR, and anal. C, H, N were conducted. Thus obtained 1-(*N*-benzyloxycarbonyl)amino-4-aza-8-aminononane dihydrochloride (0.85 g, 2.3 mmol) was dissolved in 2 M NaOH (5 mL) and extracted with DCM (3 × 5 mL). Combined DCM extracts were washed with 1 M $NaHCO_3$ (5 mL), H_2O (3 mL), and brine (5 mL), dried ($MgSO_4$), filtered, and concentrated *in vacuo*. The residue was dried *in vacuo* over P_2O_5 to give 1-(*N*-benzyloxycarbonyl)amino-4-aza-8-aminononane free amine (0.62 g, 91%) as a viscous oil. Pd black in MeOH (∼0.5 mL) was added to a solution of the above free amine (0.52 g, 1.77 mmol) in a mixture of AcOH−MeOH (1:1, 6 mL total), and hydrogenation was carried out at atmospheric pressure. The catalyst was filtered off, the residue was washed with MeOH, and the combined filtrates were evaporated to dryness *in vacuo*. The residue was dissolved in EtOH and upon addition of HCl/EtOH (0.8 mL, 7 M) was evaporated to dryness *in vacuo*, and the residue was recrystallized from MeOH/EtOH mixture to give **7a** (0.4 g, 84.2%) as colorless crystals; mp 195−196 °C; R_f 0.24 (C); R_f 0.12 (B). ^{1}H NMR (D_2O): δ 3.40 (1H, qdd, J = 8.6, 6.6, 4.9 Hz, CH); 3.20−3.07 (6H, m, 3*NCH_2); 2.15−2.06 (2H, m, $(NCH_2)_2CH_2$); 1.87−1.61 (4H, m, $CHCH_2CH_2$); 1.32 (3H, d, J = 6.6 Hz, CH_3). ^{13}C NMR (D_2O): δ 50.02, 49.93, 47.28, 39.33, 33.57, 26.46, 24.59, 20.04. Anal. C, H, N.

Cell Culture. The DU145 cell line was obtained from American Type Culture Collection, USA. Mouse primary fetal fibroblasts were obtained as described earlier.[28] Cells were cultured in humidified atmosphere at +37 °C, 10% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine and 50 μg/mL gentamycin. The cells were lysed in a buffer containing 50 mM potassium phosphate buffer, pH 7.2, 0.1 mM EDTA, 0.1% Triton X-100, 0.1 mM dithiothreitol, and Complete EDTA-free protease inhibitor (Roche Diagnostics). Aliquots of the lysate were taken for polyamine measurement, and the rest was centrifuged at 13 000 rpm for 20 min at +4 °C. The supernatant was used for enzyme assays. Uptake competition experiments were performed as described earlier.[25]

Cytotoxicity Assay. DU145 cells were grown on 96-well plates with 10 or 100 μM analogs in the presence or absence of 1 mM AG. After 72 h, 10 μL of (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (5 mg/mL in PBS) was added, and cells were incubated for 4 h before extracting the stain with a solution containing 4 mM HCl, 0.1% Triton X-100, and 80% isopropanol. Absorbances were measured at 595 nm using a microplate reader.

Recombinant Enzyme Assays. Plasmids coding human SMO or APAO were a kind gift from Dr. C. Porter, Roswell Park Cancer Institute, New York, USA. Cloning of mouse recombinant SSAT was described earlier.[29] Recombinant proteins were produced and assays were carried out as published previously.[30]

Analytical Methods. Alternative splicing of SSAT was analyzed as described earlier.[24] Polyamines were measured with HPLC according to the published method.[27] Acid-precipitated pellets were dissolved in 0.1 M NaOH, and the amount of DNA was measured using the PicoGreen reagent (Invitrogen) according to manufacturer's instructions. Enzyme activities were measured from the cytosolic fractions as described earlier.[14,25,31,32]

Statistical Analysis. One-way analysis of variance with Tuckey's *post-hoc* test was used for multiple comparisons with the aid of GraphPad Prism 4.03 (GraphPad Software Inc.).

■ ASSOCIATED CONTENT

Supporting Information. Synthesis and spectroscopic details for 3-(*N*-benzyloxycarbonyl)amino-1-bromopropane, physicochemical data for compounds **9a**, **9b**, **11**, **12**, **13**, **14**, and 1-(*N*-benzyloxycarbonyl)amino-4-aza-8-aminononane dihydrochloride, elementary analysis results of target compounds and table of metabolism and efflux of methylated analogs in DU145 cells. This material is available free of charge via the Internet at http://pubs.acs.org.

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ABBREVIATIONS

AdoMetDC, *S*-adenosyl-L-methionine decarboxylase; APAO, acetylpolyamine oxidase; dc-SAM, decarboxylated *S*-adenosyl-L-methionine; DFMO, α-difluoromethylornithine (2,5-diamino-2-(difluoromethyl)pentanoic acid); DENSpm, N^1,N^{11}-diethylnorspermine; N^1-AcSpd, N^1-acetylspermidine; N^1-AcSpm, N^1-acetylspermine; NMD, nonsense-mediated mRNA decay; OAZ, antizyme; ODC, ornithine decarboxylase; Put, putrescine (1,4-diaminobutane); SAM, *S*-adenosyl-L-methionine; Spd, spermidine (1,8-diamino-4-azaoctane); Spm, spermine (1,12-diamino-4,9-diazadodecane); SMO, spermine oxidase; SSAT, spermidine/spermine N^1-acetyltransferase

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