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ORIGINAL ARTICLE

Novel isosteric charge-deficient spermine analogue—1,12-diamino-3,6,9-triazadodecane: synthesis, pK_a measurement and biological activity

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Abstract Ionic interactions are essential for the biological functions of the polyamines spermidine and spermine in mammalian physiology. Here, we describe a simple gram scale method to prepare 1,12-diamino-3,6,9-triazadodecane (SpmTrien), an isosteric charge-deficient spermine analogue. The protonation sites of SpmTrien were determined at pH range of 2.2–11.0 using two-dimensional ^{1}H-^{15}N NMR spectroscopy, which proved to be more feasible than conventional methods. The macroscopic pK_a values of SpmTrien (3.3, 6.3, 8.5, 9.5 and 10.3) are significantly lower than those of 1,12-diamino-4,9-diazadodecane (spermine). The effects of SpmTrien and its parent molecule, 1,8-diamino-3,6-diazaoctane (Trien), on cell growth and polyamine metabolism were investigated in DU145 prostate carcinoma cells. SpmTrien downregulated the biosynthetic enzymes ornithine decarboxylase (ODC) and *S*-adenosyl-L-methionine decarboxylase and decreased intracellular polyamine levels, whereas the effects of Trien alone were minor. Interestingly, both SpmTrien and Trien were able to partially overcome growth arrest induced by an ODC inhibitor, α-difluoromethylornithine, indicating that they are able to mimic some functions of the natural polyamines. Thus, SpmTrien is a novel tool to influence polyamine interaction sites at the molecular level and offers a new means to study the contribution of the protonation of spermine amino group(s) in the regulation of polyamine-dependent biological processes.

Keywords Polyamines · Spermine analogues · pK_a · NMR spectroscopy · Cell growth

Abbreviations

AdoMetDC	*S*-adenosyl-*L*-methionine decarboxylase
AG	Aminoguanidine
DENSpm	N^{I},N^{II}-diethylnorspermine
DFMO	α-Difluoromethylornithine (2,5-diamino-2-(difluoromethyl)pentanoic acid)
HMBC	Heteronuclear multiple bond correlation
HSQC	Heteronuclear single quantum correlation
ODC	Ornithine decarboxylase
Pu	Putrescine (1,4-diaminobutane)
Spm	Spermine (1,12-diamino-4,9-diazadodecane)
SpmTrien	(1,12-Diamino-3,6,9-triazadodecane)
Spd	Spermidine (1,8-diamino-4-azaoctane)
SSAT	Spermidine/spermine N^{1}-acetyltransferase
Trien	Triethylenetetramine (1,8-diamino-3,6-diazaoctane)
TSP	Sodium 3-trimethylsilyl[2,2,3,3-d_4]propionate

Introduction

The polyamines spermine (Spm) and spermidine (Spd), and their diamine precursor putrescine (Pu) are present at high concentrations in practically all cell types and are crucial

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for cell growth and viability. The effects are determined by the shape of the molecule providing proper spatial localization of the amino groups. The degree of the protonation of the amino groups along the spatially flexible carbon chain is essential for the molecular recognition of polyamines and their analogues with different cellular constituents (Basu et al. 1990; Ramirez et al. 2002; Bergeron et al. 1995). The investigation of the structure–activity relationships for the Spm and Spd analogues have focused mainly on the contribution of molecular geometry to the biological effects (for the review see Casero and Woster 2001; Wallace and Niiranen 2007; Casero and Marton 2007), while the impact of the protonation of the amino group(s) to the biological activity of polyamines has not been studied in detail. To decrease the pK_a values of the terminal amino groups in terminally *bis*-alkylated derivatives, a family of the analogues bearing piperidine- or pyridine groups has been synthesized together with the terminally *bis*-trifluoroethylated Spm (Bergeron et al. 1988, 1995). Also, a family of 2,2-, 6,6-, and 7,7-difluorospermidines has been designed and investigated as polyamine surrogates (Baillon et al. 1988). However, a rational approach to design abnormally protonated Spm analogues should definitely result in a decrease in the pK_a values of H_2N– or –NH-groups with minimal steric disturbances of the parent polyamine molecule. This seriously limits the possibility of designing isosteric charge-deficient analogues of Spm. One approach is the substitution of terminal H_2NCH_2-group of Spd or Spm with H_2NO-group. The aminooxy analogues of Spd and Spm (Khomutov et al. 1996) have been of some interest to the polyamine field already (Hyvönen et al. 1995; Eloranta et al. 1990; Turchanowa et al. 2002). The oxa-analogues of polyamines containing an –NH–O-group instead of the central –NH–CH_2-group have also been synthesized (Lin et al. 1994; Khomutov et al. 2005), but their biochemistry has not yet been investigated.

The present paper shows an alternative approach to design an isosteric charge-deficient analogue of Spm consisting of replacement of the Spd fragment of Spm molecule with a triethylenetetramine (Trien) that gives rise to SpmTrien (Table 1). This compound can be considered as an isoster of Spm with non-symmetrical structure. Very limited biochemical data related to the effects on polyamine metabolism is available even for the parent Trien, which in certain cellular processes seems to be capable of mimicking the natural polyamines (Kobayashi et al. 1999). This property is partly interpreted in terms of the protonation status of this Spd analogue. Trien forms a very stable complex with Cu^{2+} [pK_{uns} 20.4 at pH 14 and pK_{uns} 14.2 at pH 7 (Schwarzenbach 1950)] and is used as an alternative treatment of Wilson's disease (hepatolenticular degeneration) if a patient is, or becomes, intolerant to the primary medication with penicillamine (Dixon et al. 1972; Schilsky 2001). Recently, Trien was successfully used to regenerate the diabetic heart by selective copper chelation (Cooper et al. 2004). The ability of SpmTrien to induce pH-dependent condensation of DNA, which can be converted to isotropic state upon the addition of Cu^{2+} ions, is the only known biochemical application of this Spm analogue (Khomutov et al. 2007). Hence, the knowledge of the protonation state of SpmTrien at different pH is of crucial importance for the interpretation of the biological activity of this unusual Spm analogue. Here, we present a convenient, gram scale synthesis of SpmTrien, NMR determination of the pK_a values of SpmTrien amino groups, and biochemical data on the effects of SpmTrien and Trien on cell growth and polyamine metabolism in DU145 prostate cancer cells.

Materials and methods

Acrylonitrile, Adam's catalyst, Celite, and triethylenetetramine (Trien) were purchased from Aldrich (United States); aluminium oxide and the rest of chemicals from Fluka (Switzerland). TLC was carried out on precoated Kieselgel 60 F_{254} plates from Merck (Germany) and SpmTrien was developed with ninhydrin. Melting points were determined in open capillary tubes and are uncorrected.

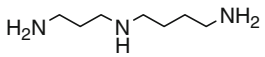
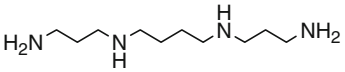
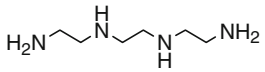
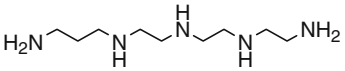
12-Amino-4,7,10-triazadodecanonitrile (TrienC2CN)

A mixture of Trien (5.14 g, 35 mmol) and acrylonitrile (0.627 g, 12 mmol) was stirred at 20°C for 16 h and then all volatile material was evaporated in vacuo that resulted in crude TrienC2CN, which was purified by flash chromatography on aluminium oxide using 1% NH_3 in methanol as an eluent, to give TrienC2CN (1.3 g, 55%) as viscous oil. ^{1}H NMR (D_2O): δ 3.19–3.16 (4H, m), 3.10 (2H, t, *J* 6.1 Hz), 2.99–2.91 (4H, m), 2.69 (2H, t, *J* 6.7 Hz); ^{13}C 20.34, 41.58, 46.47, 46.70, 47.19, 48.09, 49.27, 49.64, 51.77, 123.30.

1,12-Diamino-3,6,9-triazadodecane pentahydrochloride (SpmTrien)

A mixture of TrienC2CN (0.7 g, 3.5 mmol) and Adams catalyst (0.08 g) in 75% aqueous AcOH (20 ml) was hydrogenated at 3 bar overnight. The catalyst was filtered off through Celite and combined filtrates were evaporated to dryness in vacuo. To the residue 6 M HCl (5 ml) was added and after evaporation to dryness in vacuo the residue was crystallized from minimal volume of 1 M HCl and

Table 1 The structures and the pK_a values of Spd, Spm, and their analogues—Trien and SpmTrien

Abbreviation	Structure	pK_a 1	pK_a 2	pK_a 3	pK_a 4	pK_a 5
Spd		8.24	9.81	10.89		
Spm		7.96	8.85	10.02	10.80	
Trien		3.27	6.56	9.07	9.74	
SpmTrien		3.3	6.3	8.5	9.5	10.3

pK_a Values of Spd, Spm, and Trien are from the review (Bencini et al. 1999)

dried over P_2O_5/KOH in vacuo to give SpmTrien pentachlorohydrate (0.96 g, 71%) as colourless solid, mp 263–265°C (dec.), R_f 0.22 (*n*-butanol–AcOH–pyridine–H_2O; 4:2:1:2). ^{1}H NMR (D_2O, at pH* 4.5): δ 3.375 (2H^6, m, *J* 6.66), 3.363 (2H^1, m, *J* 7.52; 6.31), 3.348 (2H^2, m, *J* 6.31; 7.52), 3.270 (2H^3, m, *J* 6.36), 3.266 (2H^5, m, *J* 6.66), 3.236 (2H^7, m, *J* 5.62; 10.25), 3.209 (2H^4, m, *J* 6.36), 3.133(2H^9, m, *J* 9.81; 5.83), 2.142 (2H^8, m, *J* 5.62; 5,83; 9.81; 10.25); ^{13}C NMR (D_2O, at pH* 4.5): δ 45.72 (C3), 45.20 (C6), 45.04 (C4, C7), 44.68 (C2), 43.87 (C5), 36.74 (C9), 36.48 (C1), 23.84 (C8). $[M + H]^+$ calc for $C_9H_{26}N_5$: 204.2188. Obs: 204.2186.

Determination of pK_a values

Determination of the macroscopic pK_a values of SpmTrien was performed with a Sirius PC-200 automatic titrator according to the manufacturer's protocol. One-dimensional ^{1}H and two-dimensional ^{1}H-^{15}N and ^{1}H-^{13}C NMR spectroscopy were used to discover the protonation order of SpmTrien. NMR samples were prepared by dissolving SpmTrien to H_2O (100 mg/ml) and adjusting pH gradually from 2.1 to 10.9 with NaOH. Samples were taken approximately at intervals of 0.3 pH units. NMR spectra were recorded with a Bruker AVANCE DRX spectrometer operating at 500.13 MHz using a double-tube system facilitating locking and chemical shift referencing. The external reference tube (o.d. 2 mm, supported by a Teflon adapter) containing the reference substance (sodium 3-trimethylsilyl[2,2,3,3-d_4]propionate (TSP) 40 mmol/l, $MnSO_4$ 0.6 mmol/l in 99.8% D_2O) was placed coaxially into the NMR sample tube (o.d. 5 mm) containing 400 µl of each sample. ^{1}H spectra were measured using standard protocol. ^{1}H–^{13}C gradient-enhanced heteronuclear single quantum correlation (HSQC) experiments were carried out in the phase-sensitive mode using the Echo/Antiecho-TPPI gradient selection. The data matrix was 128 × 1 K and the typical spectral width was 4.7 kHz for proton and 15 kHz for carbon. An evolution time of $1/(4J_{CH}) = 1.72$ ms was used. For each FID, 4 transients were accumulated. A pure squared cosine window function was applied in both dimensions prior to Fourier transform. ^{1}H–^{15}N heteronuclear multiple bond correlation (HMBC) experiments were carried out in the magnitude mode. The typical data matrix was 128 × 2 K. The typical spectral width was 3 kHz for proton and 2 kHz for nitrogen. An evolution time of $1/(2J_{NH}) = 71$ ms was used. For each FID, 4–10 transients were accumulated depending on the concentration of the sample. A pure sine window function was applied in both dimensions prior to Fourier transform.

Cell culture

The human prostate carcinoma cell line DU145 (American Type Culture Collection) was cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma) supplemented with 10% heat-inactivated foetal bovine serum (Sigma) and 50 µg/ml gentamycin (Sigma). The cells were incubated in humidified atmosphere +37°C, 10% CO_2. The cells were seeded at density of 2 × 10^6 cells/10 cm plate and let to attach overnight. Fresh medium was then changed, containing 50 µM Trien (Sigma), or SpmTrien with or without 5 mM α-difluoromethylornithine (DFMO, ILEX oncology Inc.) or 1 mM aminoguanidine (AG, Sigma). The cells were detached using solution containing 0.25% trypsin and 1 mM EDTA in phosphate-buffered saline. The cell number was measured electronically with Coulter Counter model Z1. The cells were washed, pelleted and stored at −70°C prior to analysis. The pellets were lysed in lysis buffer [50 mM potassium phosphate buffer pH 7.2, 1 mM

EDTA, 0.1% Triton X-100, 1 mM dithiothreitol, Complete EDTA-free protease inhibitor cocktail (Roche)] and incubated for 20 min on ice. Samples for polyamine measurement were taken and mixed 9:1 with 50% sulphosalicylic acid containing 100 μM diaminoheptane. The rest of the lysate was centrifuged for 12,000×*g* 20 min at +4°C. The supernatant fraction was used for enzymatic assays of spermidine/spermine N^1-acetyltransferase (SSAT), ornithine decarboxylase (ODC), and *S*-adenosyl-L-methionine decarboxylase (AdoMetDC).

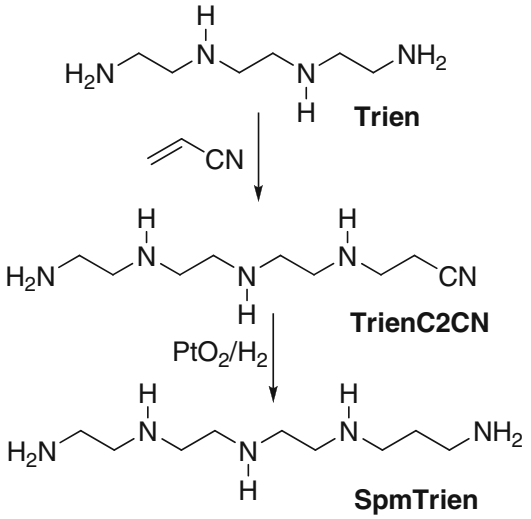
Enzyme activities, polyamines and analogs

Intracellular polyamines were measured with HPLC according to the published method (Hyvönen et al. 1992). ODC, SSAT and AdoMetDC activities were measured as described earlier (Jänne and Williams-Ashman 1971; Bernacki et al. 1992; Hyvönen et al. 2009). ^{14}C-labelled substrates used in the assays were obtained from GE Healthcare.

Results and discussion

Synthesis of SpmTrien

SpmTrien was prepared at gram scale by a cyanoethylation of Trien following a known method (Jones et al. 1995) with a subsequent catalytic reduction of the intermediate nitrile (TrienC2CN) with hydrogen over PtO_2 (Scheme 1). The key point of this synthetic strategy was the purification of the TrienC2CN by flash chromatography on aluminium oxide. Compared to other methods, such as high vacuum distillation, or flash chromatography on silica gel, the use of Al_2O_3 chromatography was superior in separating TrienC2CN from both the remaining starting material and the side-formed *bis*-nitrile derivative. The required (TrienC2CN) was obtained with 98% purity and 50–60% yields. The hydrogenation over Adam's catalyst in 75% aq.AcOH, which dissolves completely the TrienC2CN, afforded target SpmTrien with practically quantitative yield. Subsequent crystallization from 1 M aq.HCl resulted in required SpmTrien pentahydrochloride.



Scheme 1 Synthesis of SpmTrien

NMR determination of pK_a values of SpmTrien

Traditionally, pK_a determination is performed using potentiometric titration. However, the titration method cannot be used to identify individual protonation sites, and therefore a molecular method must be used. With NMR spectroscopy the precise site of the protonation can be determined and ^{1}H, ^{13}C and ^{15}N NMR methods for the determination of pK_a values have been used for various types of molecules ranging from small molecules to large proteins (Szakacs et al. 2004). However, in some cases the overlap of signals in the spectrum prevents the signal assignment, and thus two-dimensional methods must be used. The complexity of the ^{1}H spectrum of SpmTrien at pH 4.5 with somewhat separated proton chemical shifts is shown in Fig. 1. Different NMR methods have been compared for the pK_a determination of Spm, where two-dimensional ^{1}H-^{13}C HMQC gave the most precise values when compared to one-dimensional ^{1}H, ^{13}C or ^{15}N techniques (Frassineti et al. 1995).

In the case of SpmTrien, the protonation pattern could not be studied by these conventional NMR methods due to the seriously overlapping signals. In addition, the effect of pH on the chemical shift differences in ^{15}N NMR spectra was superior compared to ^{1}H NMR spectra. Assignment of (N-II)-(N-III)-(N-IV) was apparent but trials to assign required CH_2 signals unambiguously in ^{1}H NMR spectra were laborious when pH was above 3.5. As an example (see Fig. 1), we analysed the ^{1}H NMR spectrum at pH 4.5 using PERCH NMR software (Laatikainen et al. 1996). At this pH value, the spectral lines were relatively sharp (half width ca. 0.3 Hz) and the chemical shifts of different N–CH_2-protons were resolved. The main problems with these similar N–CH_2-protons are the second order effects which appear when protons having close chemical shifts (here 0.2 ppm or 100 Hz) are coupled to each other. Typically, second order effects give rise to distorted intensities of the chemical shift multiplets and also the number of peaks might increase dramatically. The calculated and observed spectra (Fig. 1) were almost identical and the differences were mostly due to the minor impurities, distinctions in the line widths or long range couplings, which were not included in the system. The calculated vicinal $^3J_{HH}$

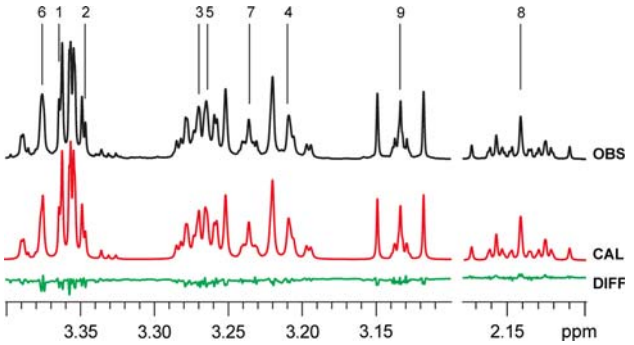


Fig. 1 Observed versus calculated NMR spectra of SpmTrien at pH* 4.5. Figures above signals indicate exact chemical shifts of the assigned $-CH_2-$ protons of SpmTrien

couplings were in the normal range and typically each proton has two different couplings to neighbouring protons due to hindered rotation of C–N- or C–C-bonds in the NMR time scale but germinal $^2J_{HH}$ (H–C–H) coupling constants were not resolved due to identical or almost identical chemical shifts of the CH_2-protons.

Thus, a 2D ^{1}H-^{15}N heteronuclear multiple bond correlation (HMBC) method was used to determine the proton coordination of SpmTrien. Furthermore, the ^{1}H–^{15}N method is an excellent choice for the analysis of protonation of the polyamines, since the binding site of a proton is directly detectable giving more precise way to look at the binding of the proton in detail. Here, we report relatively low pK_a values of the aliphatic amino groups of SpmTrien.

The macroscopic pK_a values of the amino groups of SpmTrien (3.3, 6.3, 8.5, 9.5 and 10.3) determined with potentiometric titration (Fig. 2; Table 1) were comparable to those of similar but symmetric molecules summarized in a review (Bencini et al. 1999). SpmTrien is not a symmetrical molecule and its protonation behaviour differs from other similar, but symmetrical, compounds. The protonation of SpmTrien first occurs at both the ends of the molecule. According to our results, the central N-III amino group started to protonate third, but then the proton migrated from the central N-III amino group to either N-II or N-IV amino groups when pH decreased (Fig. 2). Hence, there was equilibrium in the protonation of the secondary amino groups of SpmTrien (Fig. 2) when the pH value was between 7 and 8. This caused a "wrong way" evolution in the ^{15}N chemical shifts. In general, the microscopic pK_a values of polyprotic acids with more than three binding sites for protons and no symmetry are impossible to obtain due to the proton transfers (Ullmann 2003). Similar behaviour is also seen in diethylenetriaminepentaacetic acid, and is explained by the change in the proton-binding site (Ullmann 2003; Onufriev et al. 2001).

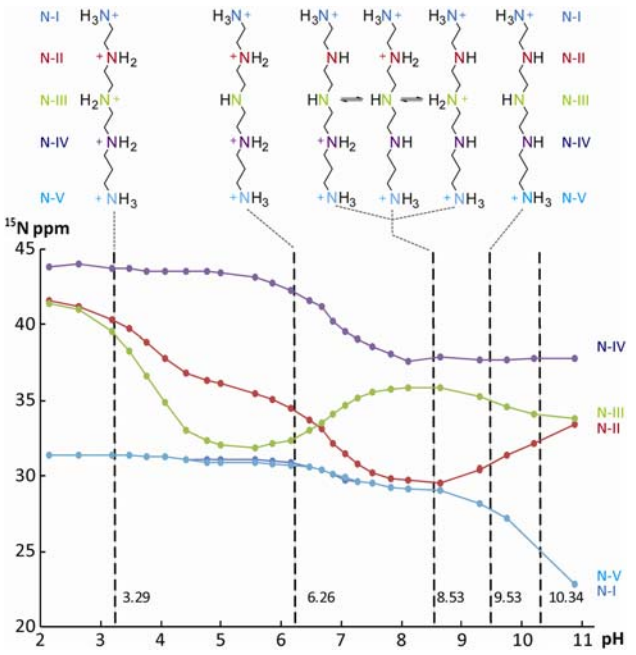


Fig. 2 ^{15}N Chemical shifts (ppm) as a function of pH. The *dashed lines* illustrate the titrated pK_a values of SpmTrien. The chemical shifts evidence that the charge is delocalized into the different amines and the protonation does not occur in classical way

Effects of SpmTrien on cell growth and polyamine metabolism in prostate carcinoma cells

DU145 cells could be cultured with Trien and SpmTrien (50 μM) in the absence of aminoguanidine (AG), an inhibitor of amine oxidases present in culture media serum (Fig. 3). By contrast, under the same conditions the natural polyamines Spd and Spm were toxic to the cells (data not shown). Thus, Trien and SpmTrien seemed to be resistant to serum amine oxidases. This may be partly due to their copper chelating properties, since many serum amine oxidases are Cu^{2+} dependent (Agostinelli et al. 1998; Nocera et al. 2003). Cells treated with Trien for 72 h grew as well as control cells, whereas SpmTrien exposure resulted in a slightly cytostatic effect (Fig. 3). This difference is likely to be attributable to their effects on intracellular polyamine pools (Table 2). After 72 h incubation, Trien exerted only minor effects on the activities of the polyamine-metabolizing enzymes. By contrast, treatment with SpmTrien inhibited ODC activity more efficiently than DFMO (Table 2), the commonly used irreversible inhibitor of ODC (Wallace and Fraser 2004). SpmTrien also inhibited the activity of another important biosynthetic enzyme, AdoMetDC, and slightly induced the catabolic enzyme SSAT. However, the extent of SSAT induction was low compared to the superinducers, such as N^1,N^{11}-diethylnorspermine (DENSpm), which can induce SSAT activity several 1,000-fold

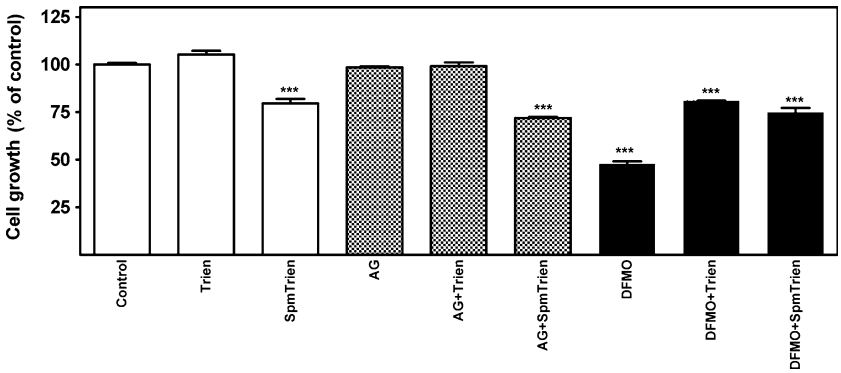


Fig. 3 DU145 cell growth (72 h) with 50 μM analogs with or without 5 mM DFMO, or with or without 1 mM AG. Results are mean ± SD ($n = 3$). ***P values of <0.001 as analysed by one-way analysis of variance with Tuckett's post hoc test

Table 2 Effect of 50 μM Trien and SpmTrien (72 h) on polyamine metabolism in DU145 cells

Treatment	ODC (pmol/h per 10^6 cells)	AdoMetDC (pmol/h per 10^6 cells)	SSAT (pmol/h per 10^6 cells)	Pu (pmol/h per 10^6 cells)	Spd (pmol/h per 10^6 cells)	Spm (pmol/h per 10^6 cells)	Analogue (pmol/h per 10^6 cells)	Total PA (pmol/h per 10^6 cells)
Control	1,401 ± 193	71 ± 3	756 ± 12	164 ± 5	1,231 ± 54	1,709 ± 61		2,940
Trien	772 ± 65	68 ± 4	720 ± 18	165 ± 9	958 ± 54	1,730 ± 26	481 ± 12	3,169
SpmTrien	43 ± 5	23 ± 2	1,128 ± 42	n.d.	522 ± 27	1,683 ± 140[a]	[a]	2,205
AG	1,001 ± 75	89 ± 7	720 ± 24	100 ± 17	1,195 ± 103	1,680 ± 52		2,875
AG + Trien	1,004 ± 50	85 ± 5	744 ± 12	132 ± 9	777 ± 16	1,688 ± 57	503 ± 8	2,968
AG + SpmTrien	73 ± 8	30 ± 2	1,116 ± 36	n.d.	424 ± 46	1,668 ± 83¤	[a]	2,092
DFMO	57 ± 8	682 ± 49	630 ± 12	n.d.	<10	1,125 ± 89		1,135
DFMO + Trien	46 ± 9	304 ± 19	666 ± 24	n.d.	<10	1,120 ± 78	1,572 ± 67	2,702
DFMO + SpmTrien	44 ± 8	22 ± 1	1,152 ± 66	n.d.	192 ± 7	1,328 ± 133[a]	[a]	1,520

The cells were cultured with 5 mM DFMO or 1 mM AG and 50 μM analogs for 72 h. "Total PA" indicates the total amount of Spd, Spm and analogue. Data are mean ± SD ($n = 3$). Two different unidentified metabolic products of SpmTrien were also found (not shown)

n.d. not detectable

[a] SpmTrien is not separated from Spm by our HPLC system, thus it is included in SPM value

(Seiler 2004). The SpmTrien-induced changes in enzyme activities were reflected in intracellular polyamine pools, which were decreased markedly, compared to control cells or cells treated with Trien (Table 2). No additional metabolic products for Trien were detectable by our HPLC system, while two different unidentified products were observed for SpmTrien (data not shown). The only metabolites characterized for Trien were terminally *mono*- and *bis*-acetylated Trien's, thus two different metabolites originating from SpmTrien are most likely terminally *mono*-acetylated SpmTriens, since *o*-phthalaldehyde derivatization requires primary amino groups to be present (Lu et al. 2007). However, the true nature of the metabolites should be verified with chemically synthesized reference compounds.

When cytostasis was induced by incubating the DU145 cells for 3 days with DFMO and the analogues, both Trien and SpmTrien were able to partially restore cell growth (Fig. 3), indicating that these compounds could at least partially substitute for natural polyamines in their growth-supporting functions.

Conclusion

In living cells, pH varies between different cellular organelles, thus one could expect SpmTrien to have unique biological properties in comparison to Spm. The protonation of SpmTrien at physiological pH may allow interaction with some Spm binding sites due to the proton migration in-between the (N-II)-(N-III)-(N-IV) secondary amino groups. This unique property of SpmTrien may be useful in the field of Spm biochemistry, since it may facilitate the discrimination of, at least some of the diverse cellular targets and functions of Spm. The present paper demonstrates that despite the difference in protonation of SpmTrien, as compared to Spm, it is recognized as a polyamine-related regulator of such enzymes as ODC, AdoMetDC and SSAT as well as a growth-supporting agent. Thus, the knowledge of the pK_a and the protonation sites of SpmTrien molecule can give more precise answers to questions arising from the enzymatic and cellular experiments with SpmTrien and may be helpful in understanding some aspects of the Spm biochemistry.

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