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ORIGINAL ARTICLE

Effects of novel *C*-methylated spermidine analogs on cell growth via hypusination of eukaryotic translation initiation factor 5A

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Abstract The polyamines, putrescine, spermidine, and spermine, are ubiquitous multifunctional cations essential for cellular proliferation. One specific function of spermidine in cell growth is its role as a butylamine donor for hypusine synthesis in the eukaryotic initiation factor 5A (eIF5A). Here, we report the ability of novel *mono*-methylated spermidine analogs (α-MeSpd, *β*-MeSpd, *γ*-MeSpd, and *ω*-MeSpd) to function in the hypusination of eIF5A and in supporting the growth of DFMO-treated DU145 cells. We also tested them as substrates and inhibitors for deoxyhypusine synthase (DHS) in vitro. Of these compounds, α-MeSpd, *β*-MeSpd, and *γ*-MeSpd (but not *ω*-MeSpd) were substrates for DHS in vitro, while they all inhibited the enzyme reaction. As racemic mixtures, only α-MeSpd and *β*-MeSpd supported long-term growth (9–18 days) of spermidine-depleted DU145 cells, whereas *γ*-MeSpd and *ω*-MeSpd did not. The *S*-enantiomer of α-MeSpd, which supported long-term growth, was a good substrate for DHS in vitro, whereas the *R*-isomer was not. The long-term growth of DFMO-treated cells correlated with the hypusine modification of eIF5A by intracellular methylated spermidine analogs. These results underscore the critical requirement for hypusine modification in mammalian cell proliferation and provide new insights into the specificity of the deoxyhypusine synthase reaction.

Keywords Polyamine · Methylated spermidine · Cell growth · Deoxyhypusine synthase · Hypusine · eIF5A

Abbreviations

eIF5A	Eukaryotic translation initiation factor 5A
DHS	Deoxyhypusine synthase
DOHH	Deoxyhypusine hydroxylase
DFMO	α-Difluoromethylornithine
DTT	Dithiothreitol
AG	Aminoguanidine
Spd	Spermidine
Spm	Spermine
Put	Putrescine
MeSpd	Methylated spermidine
MeSpm	Methylated spermine
SSAT1	Spermidine/spermine N^1-acetyltransferase
APAO	Acetylpolyamine oxidase
TCA	Trichloroacetic acid
DENSpm	N^1,N^{11}-Diethylnorspermine

Introduction

The organic cations, putrescine, spermidine, and spermine, are present in almost all living cells up to millimolar

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concentrations and play important roles in many cellular functions, including proliferation, differentiation, and regulation of ion channels (Thomas and Thomas 2001; Pegg and Casero 2011). The polyamines bind to DNA, RNA, acidic proteins, and phospholipids and regulate cellular activities at the transcriptional, translational, and post-translational levels. As expected for molecules with multiple regulatory roles, polyamine levels are tightly controlled through a series of feedback circuits to maintain appropriate intracellular concentrations (Park et al. 2010). Polyamine depletion leads to growth arrest in mammalian cells, while enhanced levels of polyamines can induce apoptosis or cellular transformation (Thomas and Thomas 2001). In addition to their polycationic functions, a small portion of cellular polyamines is used for posttranslational modification through their covalent incorporation into proteins, either by a transglutaminase catalyzed reaction (Lentini et al. 2009) or by the hypusine modification reaction (Park 2006; Wolff et al. 2007; Park et al. 2010).

Hypusine [N^{ε}-(4-amino-2-hydroxybutyl)lysine] is a polyamine-lysine conjugated amino acid occurring in only one cellular protein, eukaryotic translation initiation factor 5A (eIF5A) (see reviews, Chen and Liu 1997; Park 2006; Park et al. 2010). It is synthesized posttranslationally by two consecutive enzymatic steps. First, deoxyhypusine synthase (DHS) catalyzes the transfer of the aminobutyl moiety from spermidine to a specific lysine of the eIF5A precursor to form the deoxyhypusine [N^{ε}-(4-aminobutyl)lysine] intermediate (Joe et al. 1995). This deoxyhypusine residue is hydroxylated by deoxyhypusine hydroxylase (DOHH) (Abbruzzese et al. 1986; Park et al. 2006). Hypusine modification is an irreversible protein modification, and naturally occurring eIF5A exists predominantly as the hypusinated form, containing 1 mol of hypusine per mole of the protein.

eIF5A is an abundant protein with a long half-life. It is highly conserved, and the amino acid sequence surrounding the hypusine modification site is strictly conserved in eukaryotes. Both of the hypusine modification enzymes are also highly conserved and are strictly specific for eIF5A. The requirement of hypusine modification for eIF5A activity was illustrated by comparison of the activities of the eIF5A precursor, the eIF5A intermediate, and hypusine-containing eIF5A in methionyl-puromycin synthesis (Smit-McBride et al. 1989; Park et al. 1991). The essential nature of eIF5A and its hypusine modification was further demonstrated by gene disruption studies in yeast and mouse, in which inactivation of the eIF5A gene(s) (Schnier et al. 1991; Wöhl et al. 1993) or of the deoxyhypusine synthase gene caused loss of viability in *S. cerevisiae* (Sasaki et al. 1996; Park et al. 1998) and in mouse embryos (Nishimura et al. 2011). Hypusine synthesis defines an indispensible function of the polyamine spermidine in eukaryotic cell proliferation (Byers et al. 1994; Chattopadhyay et al. 2008; Pegg 2009).

The essential functions of polyamines in mammalian cell proliferation have been extensively investigated using a variety of inhibitors of polyamine biosynthesis, inducers of polyamine catabolism, or polyamine mimetics. The most widely used inhibitor is α-difluoromethylornithine (DFMO) (Metcalf et al. 1978), an irreversible inhibitor of ornithine decarboxylase (ODC), the rate-limiting enzyme of polyamine biosynthesis. Although some positive clinical results are emerging with DFMO in cancer prevention/therapy, in many cases, tumor cells can compensate for the lack of polyamines by down-regulating catabolic enzymes and by enhancing the uptake system. During the past decades, synthetic polyamine mimetics (antimetabolites), such as the *N*-alkylated spermine derivative N^1,N^{11}-diethylnorspermine (DENSpm), have received much attention due to their ability to inhibit cell growth by depleting natural polyamines via inducing polyamine catabolism and efflux and inhibiting the uptake system (Bernacki et al. 1992; Alhonen et al. 1998).

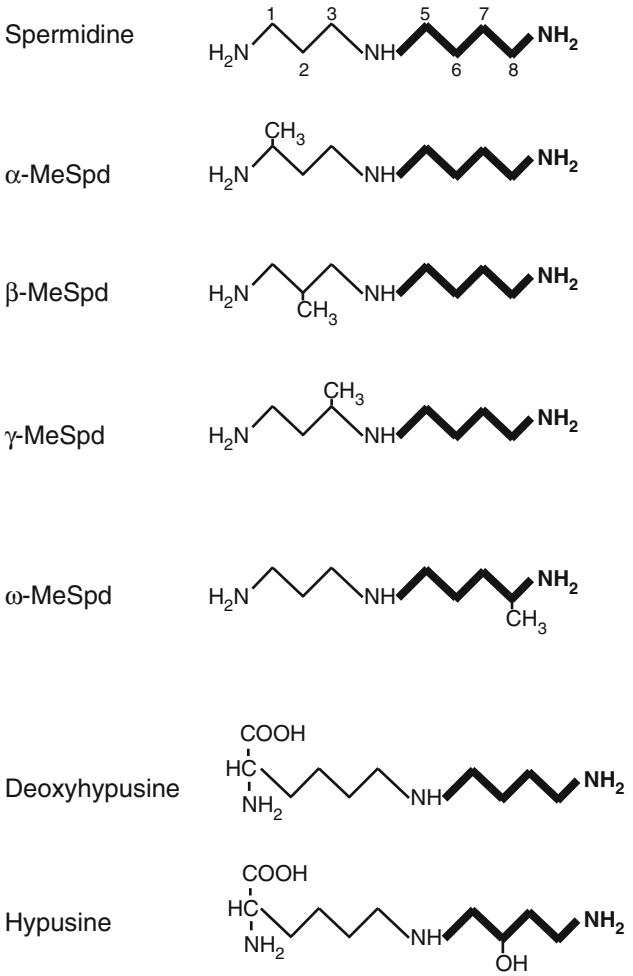
One interesting group of polyamine analogs is the *C*-substituted compounds, of which α-methylated spermidine (α-MeSpd) and spermine (α, ω-Me_2Spm) are metabolically stable, functional mimetics of natural polyamines (Lakanen et al. 1992). In contrast to DENSpm and many other terminally bis-*N*-alkylated analogs, α-MeSpd is growth supporting and mimics many other cellular functions of natural polyamines. We and others have shown that polyamine depletion induces cessation of cell growth in two different phases; the first phase occurs within few days after treatment with DFMO in cell culture, and in this phase, both spermidine, spermine, or even some other unnatural polyamine analogs can support cell growth (Nagarajan et al. 1988; Byers et al. 1994; Nishimura et al. 2005; Hyvönen et al. 2007). In contrast, the late phase of cytostasis, occurring after a week's treatment with DFMO in cell culture, is specific to the depletion of intracellular spermidine, which serves as the sole natural substrate for hypusine synthesis in eIF5A (Park et al. 1981). These late phase cells cannot be rescued by all polyamine mimetics, but only by those few that fulfill the function of eIF5A activation by acting as substrates for deoxyhypusine synthase (Byers et al. 1992, 1994; Hyvönen et al. 2007). Using the optical isomers of α-MeSpd, we found that only the *S*-α-MeSpd supported cell growth during prolonged polyamine deprivation, probably because it was the only isomer able to act as hypusine precursor (Hyvönen et al. 2007).

Here, we tested optical isomers of α-MeSpd as substrates and inhibitors of human deoxyhypusine synthase in vitro and found a correlation between the substrate function for DHS and the ability to support cell growth during prolonged polyamine depletion. We further tested three additional novel *mono-C*-methylated spermidine analogs,

β-, *γ*-, and *ω*-methylated spermidines (Scheme 1), as substrates for deoxyhypusine synthase both in vitro and at the cellular level and found that *β*-MeSpd also acts as a hypusine precursor and supports cell growth during prolonged DFMO-treatment.

Materials and methods

The human prostate carcinoma cell line, DU145, was obtained from the American Type Culture Collection, USA; [1,8-^{3}H]spermidine and [1,4-^{14}C]putrescine were purchased from PerkinElmer/NEN. The synthesis of α-methylspermidine (α-MeSpd) and its enantiomers was performed as described (Grigorenko et al. 2004, 2007). Synthesis of racemic *mono-C*-methylated spermidine analogs, *β*-MeSpd and *γ*-MeSpd, was performed as described (Khomutov et al. 2011; Hyvönen et al. 2011). DFMO was obtained from ILEX oncology Inc, and aminoguanidine (AG) from Sigma.



Scheme 1 Structures of spermidine and C-methylated spermidines, hypusine, and deoxyhypusine. The carbons in spermidine are numbered. α-MeSpd, *β*-MeSpd, *γ*-MeSpd, and *ω*-MeSpd correspond to 1-MeSpd, 2-MeSpd, 3-MeSpd, and 8-MeSpd, respectively. The butylamine portion of spermidine is used for the synthesis of deoxyhypusine/hypusine, as indicated in *bold*

Cell culture

DU145 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma) supplemented with 10% heat-inactivated fetal bovine serum (Sigma), 2 mM L-glutamine, and 50 μg/ml gentamycin (Sigma). The cells were plated at a density of 1×10^6 cells/10 cm plate and incubated overnight in a humidified atmosphere at 37°C, 10% CO_2. Medium was exchanged with fresh medium containing 1 mM AG, 5 mM DFMO, and 100 μM analogs, and the cells were subcultured every 3 or 4 days. The cells were harvested by trypsinization, counted electronically with Coulter Counter model Z1, and plated again with the supplements at density of 1×10^6 cells/10 cm plate. The rest of the sample was washed with PBS, pelleted, and stored in −70°C before analyses. The cells were lysed in a buffer containing 25 mM Tris–HCl pH 7.4, 1 mM EDTA, 0.1% Triton X-100, 1 mM dithiothreitol (DTT), and Complete EDTA-free protease inhibitor cocktail (Roche). Samples were taken for polyamine measurement and the rest of the lysate was centrifuged at 12,000 ×*g* for 30 min 4°C. The supernatant fractions were used for 2D-immunoblotting of eIF5A.

Polyamine measurements

Intracellular polyamines and polyamine analogs were measured with HPLC according to a published method (Hyvönen et al. 1992).

Western blotting and 2D-immunoblotting of eIF5A, its precursor and intermediate

Protein concentrations from the supernatants were measured in 96-well plates using Coomassie Brilliant Blue staining (Bio-Rad) with bovine serum albumin as standard. For 2D analysis, 15 μg of total protein was first separated according to pI on Immobiline DryStrip (NL pH 3.0–5.6) and then electrophoresed on 15% SDS–polyacrylamide gel. Proteins were transferred to a Immobilon-FL membrane (Millipore), and the membrane was incubated with mouse anti-human eIF5A antibody (BD, 1:30,000 dilution), washed five times with PBS-T, and treated with HRP-conjugated donkey anti-mouse IgG secondary antibody (Santa Cruz Biotechnology, 1:20,000 dilution). After washing again with PBS-T, proteins were detected with HRP substrate (ECL Plus, Perkin Elmer) and scanned with a Typhoon variable mode imager (Perkin Elmer). For 1D gel Western blotting, 10 μg of total protein was separated on 15% SDS–polyacrylamide gel and

processed as above. For a loading control, donkey anti-rabbit actin antibody (Santa Cruz Biotechnology, 1:10,000 dilution) was used with eIF5A antibody for double detection from the same membrane.

Deoxyhypusine synthesis and homospermidine synthesis assays

The C-methylated spermidines were tested as inhibitors in the deoxyhypusine synthesis assay as follows. The reaction mixture contained, in 20 µl, 0.2 M glycine NaOH buffer (pH 9.5), 5 µg of eIF-5A precursor protein, 1 mM DTT, 1.0 mM NAD, 3 µCi [1,8-^{3}H]spermidine (20–32 Ci/mmol), 25 µg of BSA, and 10 units (10 ng) of recombinant human deoxyhypusine synthase. Test compounds were dissolved in water and were added to assay mixtures at 0.3 and 1.0 mM prior to addition of the enzyme. Incubations were conducted at pH 9.5 and 37°C for 1 h. The radiolabeled deoxyhypusine formed was measured after its ion exchange chromatographic separation of acid hydrolysates of the TCA-precipitated protein fractions as described (Wolff et al. 2011). The compounds were also tested as substrates for homospermidine synthesis by deoxyhypusine synthase. The reaction mixture contained, in 50 µl, 0.2 M Glycine NaOH buffer (pH, 9.5), 50 µg of BSA, 1 mM DTT, 1 µCi [^{14}C] putrescine (100 mCi/mmol), 1 mM NAD, 10 and 25 µM of spermidine or *C*-methylated spermidines, and 1.0 µg of recombinant human deoxyhypusine synthase. After incubation of the reaction mixture at 37°C for 1–1.5 h, 250 µg of carrier BSA was added and proteins were precipitated by addition of 10% TCA. After removal of the precipitates, an aliquot of TCA supernatant was analyzed by ion exchange chromatography to determine the amount of radioactive homospermidine formed, as previously described (Park et al. 2003).

Statistical analysis

Values are means ± SD. One-way analysis of variance (ANOVA) with Tuckey's post hoc test was used for multiple comparisons with the aid of a software package, GraphPad Prism 4.03 (GraphPad Software Inc). *, **, and *** refer to P values of <0.05, <0.01, and <0.001, respectively.

Results

The uptake and metabolic stability of *C*-methylated spermidines and their effects on cellular polyamine levels of DFMO-treated DU145 cells

Our previous studies indicated that at 100 µM concentration, spermidine and ω-MeSpd were degraded to toxic by-products by serum amine oxidases present in cell culture media (Hyvönen et al. 2011). Thus, growth experiments were conducted in the presence or absence of 1 mM aminoguanidine, an inhibitor of amine oxidases.

Treatment of DU145 cells with DFMO caused efficient depletion of putrescine and spermidine (Table 1). Spermidine was reduced to 5% of the normal level on day 3, and by day 6, neither putrescine nor spermidine was detectable in these cells, and spermine was reduced to 60% of the normal level. Addition of spermidine (100 µM with 1 mM aminoguanidine) increased the cellular spermidine level but not the spermine level. The four racemic *C*-methylated spermidine (MeSpd) analogs accumulated in cells to a higher level (2,400–5,131 pmol/10^6 cells) than the natural polyamines and efficiently displaced putrescine and spermidine and markedly reduced the spermine level (Table 1). The intracellular level of γ-MeSpd was higher than other analogs (5,131 and 4,364 pmol/10^6 cells on day 3 and 9, respectively) probably because it was not converted to MeSpm derivatives (as described below). When the stereoisomers of α-MeSpd were compared, the *S*-isomer accumulated in cells to a higher level than the *R*-isomer and a smaller portion of the *S*-isomer was converted to the α-MeSpm analog (Hyvönen et al. 2007). Aminoguanidine did not make a significant difference in the uptake and accumulation of α-MeSpds and the overall polyamine patterns in these cells (data not shown).

The *C*-methylated analogs were relatively stable in cells, but partial conversion to MeSpm was observed (25% conversion for α-MeSpd, 32% for β-MeSpd, and 28% for ω-MeSpd on day 9, Table 1) except for γ-MeSpd which was not converted to its MeSpm derivative. Thus, β-MeSpd seems to be the best substrate for spermine synthase, whereas γ-MeSpd is not a substrate. No spermidine was detected in cells treated with α-MeSpd, γ-MeSpd, and ω-MeSpd, but a small but detectable amount of spermidine was found in cells incubated with DFMO and β-MeSpd. This spermidine may have been generated from the β-MeSpm analog (formed from β-MeSpd by spermine synthase) by cellular polyamine oxidases. When MDL72527, an inhibitor of polyamine oxidases, acetylpolyamine oxidase (APAO), and spermine oxidase (SMO), was added to the β-MeSpd-supplemented cells, residual spermidine was completely depleted from these cells (Table 2). However, cell growth was not reduced in the presence of MDL72527 (Table 2), indicating that β-MeSpd itself can support cell growth.

The effects of *C*-methylated spermidines on growth of polyamine-depleted DU145 cells

The ability of the analogs to support cell growth during prolonged polyamine depletion was studied in DU145

Table 1 Intracellular polyamine and analog concentrations in DU145 cells treated with 5 mM DFMO, 1 mM AG, and 100 μM Spd or analogs for 3, 6, or 9 days

Treatment	Put	Spd	Spm	MeSpd (pmol/10^6 cells)	MeSpm[a]
3 days					
AG	93 ± 25	1,065 ± 58	1,513 ± 78		
AG + DFMO	59 ± 25	55 ± 6	1,197 ± 57		
AG + DFMO + Spd	35 ± 3	2,567 ± 196	831 ± 21		
AG + DFMO + α-MeSpd	21 ± 2	47 ± 5	246 ± 26	4,223 ± 509	959 ± 119
AG + DFMO + *β*-MeSpd	20 ± 2	87 ± 5	247 ± 10	2,997 ± 108	1,231 ± 78
AG + DFMO + *γ*-MeSpd	18 ± 1	n.d.	486 ± 36	5,131 ± 159	n.d.
AG + DFMO + *ω*-MeSpd	31 ± 2	38 ± 2	241 ± 10	4,335 ± 144	720 ± 5
6 days					
AG	114 ± 23	1,109 ± 38	1,686 ± 36		
AG + DFMO	n.d.	n.d.	945 ± 20		
AG + DFMO + Spd	35 ± 4	2,681 ± 39	865 ± 19		
AG + DFMO + α-MeSpd	n.d.	n.d.	81 ± 10	3,233 ± 316	804 ± 72
AG + DFMO + *β*-MeSpd	n.d.	36 ± 5	180 ± 18	2,408 ± 95	1,212 ± 230
AG + DFMO + *γ*-MeSpd	n.d.	n.d.	159 ± 28	2,999 ± 125	n.d.
AG + DFMO + *ω*-MeSpd	n.d.	n.d.	83 ± 5	4,142 ± 292	643 ± 55
9 days					
AG	90 ± 13	932 ± 29	1,383 ± 144		
AG + DFMO	n.d.	n.d.	940 ± 96		
AG + DFMO + Spd	27 ± 1	2,459 ± 105	850 ± 3		
AG + DFMO + α-MeSpd	n.d.	n.d.	72 ± 2	3,019 ± 183	879 ± 58
AG + DFMO + *β*-MeSpd	n.d.	71 ± 4	174 ± 6	2,470 ± 173	1,174 ± 102
AG + DFMO + *γ*-MeSpd	n.d.	n.d.	94 ± 4	4,364 ± 245	n.d.
AG + DFMO + *ω*-MeSpd	n.d.	n.d.	119 ± 5	2,968 ± 211	1,131 ± 112

Results are averages ± SD, $n = 3$

n.d Not detectable

[a] All MeSpm metabolites were calculated using α-MeSpm as standard

Table 2 Cell growth and intracellular polyamine and analog concentrations in DU145 cells treated with 1 mM AG, 5 mM DFMO, and 100 μM analogs for 9 days in the presence or absence of 10 μM MDL72527

Treatment (9 days)	Growth (%)	Put	Spd (pmol/10^6 cells)	Spm	MeSpd	MeSpm[a]
AG	100 ± 1	157 ± 23	1,614 ± 18	1,932 ± 62		
AG + MDL	100 ± 2	164 ± 4	893 ± 89	2,826 ± 139		
AG + DFMO + α-MeSpd	73 ± 1	n.d.	n.d.	58 ± 15	3,934 ± 338	1,493 ± 57
AG + DFMO + α-MeSpd + MDL	76 ± 4	n.d.	n.d.	38 ± 10	4,752 ± 434	1,515 ± 83
AG + DFMO + *β*-MeSpd	49 ± 1	n.d.	183 ± 21	288 ± 37	2,718 ± 234	2,270 ± 68
AG + DFMO + *β*-MeSpd + MDL	55 ± 2	n.d.	n.d.	92 ± 4	2,117 ± 21	2,348 ± 179
AG + DFMO + *γ*-MeSpd	21 ± 2	n.d.	n.d.	565 ± 26	3,413 ± 273	n.d.
AG + DFMO + *γ*-MeSpd + MDL	22 ± 1	n.d.	n.d.	294 ± 119	3,384 ± 337	n.d.

Results are averages ± SD, $n = 3$

n.d Not detectable

[a] All MeSpm metabolites were calculated using α-MeSpm as standard

prostate carcinoma cells. Cells were depleted of natural polyamines by culturing them in the presence of DFMO, 100 μM analogs, and 1 mM aminoguanidine. 100 μM of analogs was used because the natural polyamines were most effectively displaced at this concentration (data not shown). Cells were grown for up to 18 days and replated every third day. As indicated in Fig. 1, all analogs reversed DFMO-induced growth inhibition in the first 3 days, under both conditions without (A) or with (B) aminoguanidine, but only α-MeSpd and *β*-MeSpd were effective in long-term culture. *γ*- and *ω*-MeSpds failed to support a long-term growth (Fig. 1a, b). Interestingly, aminoguanidine exerted a growth-inhibitory effect when used in combination with the analogs (Fig. 1a, b compare 12–18 day growth with or without AG for cells supplemented with α- and *β*-MeSpd).

We also compared the effects of *R*- and *S*- enantiomers of α-MeSpd in supporting long-term growth of DFMO-treated DU145 cells in the presence or absence of aminoguanidine (Fig. 1c). The *S*-α-MeSpd was supporting growth effectively (comparable to Spd) up to 12 days, whereas little growth was observed with *R*-α-MeSpd (Fig. 1c). The racemic α-MeSpd showed intermediary growth-supporting effects. Reduced growth was observed in the AG-treated cells compared with the non-AG-treated counterparts.

Comparison of the effects of MeSpds as substrates and inhibitors of deoxyhypusine synthase

Since all the *C*-methylated spermidines accumulated in cells, their differential effects in supporting long-term growth may be due to their differences as precursors of hypusine biosynthesis. Therefore, we tested these spermidine analogs as substrates and inhibitors of the deoxyhypusine synthase reaction in vitro. Deoxyhypusine synthase catalyzes the synthesis of deoxyhypusine in eIF5A when the eIF5A precursor protein is present as a butylamine acceptor substrate. Alternatively, in the absence of eIF5A precursor protein, it can transfer the butylamine moiety of spermidine (or its analogs) to putrescine to form *sym*-homospermidine (Park et al. 2003). α-MeSpd, *β*-MeSpd, and *γ*-MeSpd acted as a butylamine donor to the acceptor putrescine for synthesis of homospermidine, in a concentration-dependent manner with a particular increase in the case of α-MeSpd, but no homospermidine was formed from *ω*-MeSpd (Fig. 2a). The level of homospermidine formed from α-MeSpd under the reaction condition was approximately half of that formed from the natural substrate spermidine. *β*-MeSpd and *γ*-MeSpd were less effective substrates than α-MeSpd. We further tested the *C*-methylated Spd analogs as substrates for deoxyhypusine synthesis using the eIF5A precursor as an acceptor substrate and measured deoxyhypusine formed in

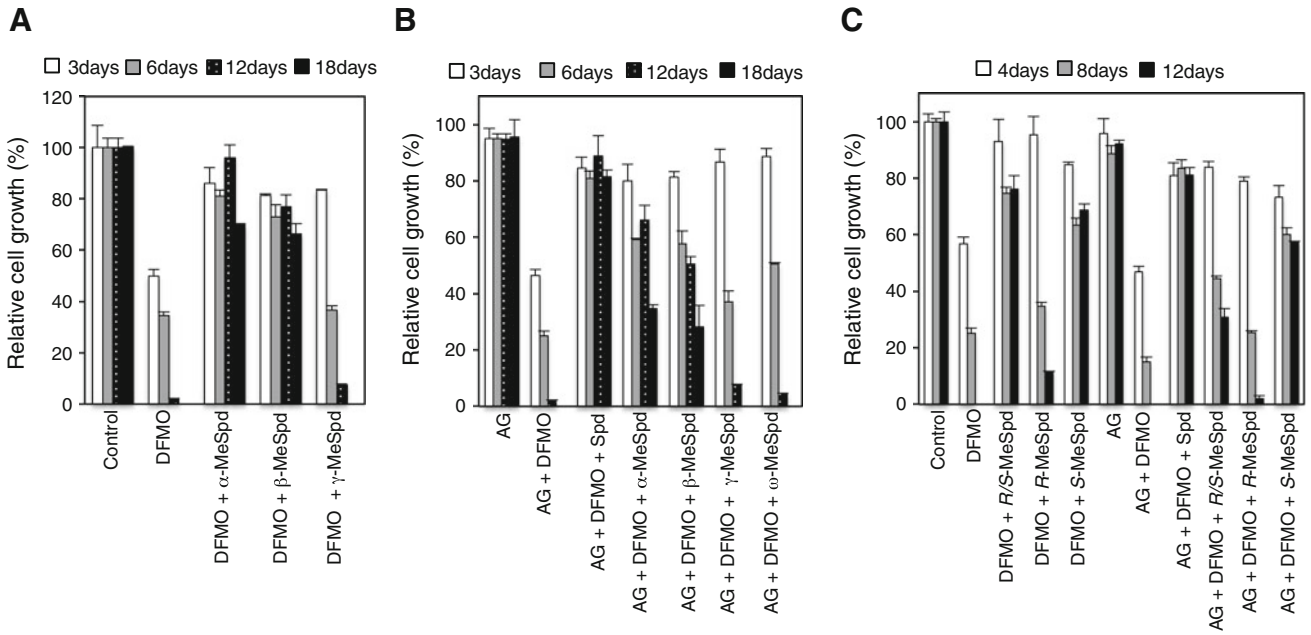


Fig. 1 Effect of the spermidine analogs on the growth of DU145 cells during prolonged polyamine depletion. Cells were treated with 5 mM DFMO and 100 μM of α-MeSpd, *β*- MeSpd, *γ*-MeSpd, and *ω*-MeSpd (each as the racemate) without (**a**) or with (**b**) 1 mM aminoguanidine (AG) for 3, 6, 12, or 18 days. **c** Cells were treated with 5 mM DFMO and 100 μM of racemic (*R/S*)-α-MeSpd, *R*-α-MeSpd, and *S*-α-MeSpd without or with aminoguanidine (AG) for 4, 8, and 12 days. Cell growth was measured by counting cell numbers after trypsinization and is expressed as % of a non-treated control. Results are averages of three independent determinations ±SD, $n = 3$

eIF5A fluorometrically after acid hydrolysis. α-MeSpd, *β*-MeSpd, and *γ*-MeSpd served as substrates for deoxyhypusine synthesis but *ω*-MeSpd did not (data not shown), consistent with the results of the homospermidine synthesis assays. The *C*-methyl spermidines acted as inhibitors of the synthesis of [^{3}H]deoxyhypusine in eIF5A in a reaction mixture containing [^{3}H]spermidine as the butylamine donor. Of the four MeSpds, α-MeSpd and *ω*-MeSpd exerted stronger inhibition than *β*-MeSpd and *γ*-MeSpd (Fig. 2b). Interestingly, *ω*-MeSpd was a good inhibitor, suggesting that it interferes with spermidine binding to the active site of DHS, while not acting as a substrate for the enzyme.

We also compared the stereoisomers of α-MeSpd as substrates and inhibitors of DHS in vitro. The *S*-isomer was a much better substrate than the *R*-isomer for DHS-mediated homospermidine synthesis (Fig. 2c), with an increase of almost four times. The *S*-isomer was also a much better inhibitor of deoxyhypusine synthesis than the *R*-isomer at 0.3 and 1 mM (Fig. 2d). The correlation between the DHS substrate activity of the stereoisomers and their activity in supporting cell growth underscores the role of hypusine synthesis in eIF5A as a critical factor in growth support by the methylated spermidines.

eIF5A modification status in DFMO-treated DU145 cells supplemented with MeSpd analogs

For all the MeSpd analogs tested, their ability to support long-term growth of polyamine-depleted DU145 cells appears to depend on their function as a precursor substrate for the hypusine modification of eIF5A. α-MeSpd and *β*-MeSpd, which are good substrates for DHS, supported long-term growth, whereas *ω*-MeSpd, which is not a substrate for DHS, did not. An exception was *γ*-MeSpd, which acted as a substrate for DHS in vitro and yet did not support long-term growth of polyamine-depleted cells. Therefore, we examined the modification status of eIF5A in the polyamine-depleted cells supplemented with *C*-methyl Spd analogs. There appeared to be an increase in the net total amount of eIF5A (modified plus unmodified) in analog-treated cells as judged from the western blot signal from 1D gels (Fig. 3a, c). It is yet unknown whether this is due to an increased translation or stability of eIF5A precursor proteins in the analog-treated cells. The relative amount of hypusine modified, unmodified, and unmodified plus acetylated eIF5A was determined by immunoblotting after 2D gel electrophoretic separation. As mentioned earlier, cells treated with 100 μM Spd or *ω*-MeSpd in the absence of aminoguanidine were not viable beyond day 2, due to their toxic oxidation products formed by serum amine oxidase. For this reason, eIF5A could not be analyzed for these samples (*ω*-MeSpd–AG, Spd–AG). Hypusinated eIF5A is normally the predominant form in control cells (Fig. 3b, d, top panels, without AG). Depletion of cellular spermidine by treatment with DFMO caused disappearance of hypusinated eIF5A with concomitant accumulation of non-hypusinated eIF5A precursors (Fig. 3b, precursor and acetylated precursor). The hypusinated eIF5A was partially

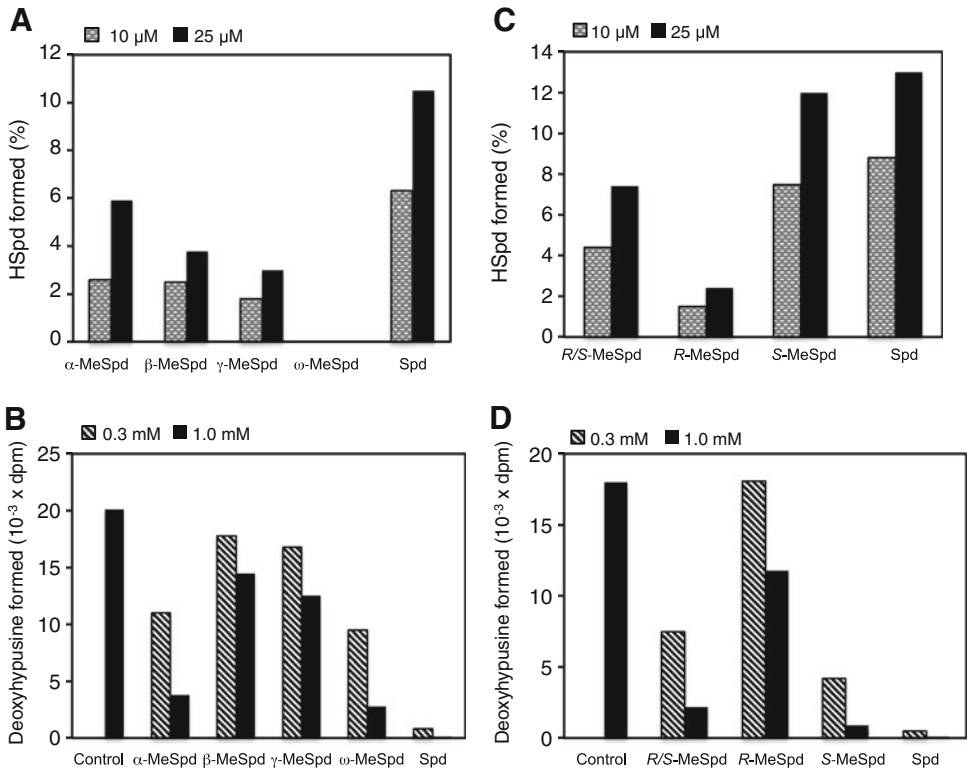


Fig. 2 Analogs as substrates and inhibitors of deoxyhypusine synthase in vitro. α-MeSpd, *β*- MeSpd, *γ*-MeSpd, and *ω*-MeSpd (each as the racemate) were tested as substrates (**a**) or inhibitors (B) of deoxyhypusine synthase as described under "Materials and methods". Racemic (*R/S*)-α-MeSpd, *R*-α-MeSpd, and *S*-α-MeSpd were also compared as substrates (**c**) and inhibitors (**d**). The experiments were repeated two or three times with similar results and representative data are shown

restored (to approximately 30% of total eIF5A) in polyamine-depleted cells supplemented with α-MeSpd or *β*-MeSpd (Fig. 3b, right panels, without AG), but it was clearly absent in those supplemented with *γ*-MeSpd. Although hypusinated eIF5A levels were markedly reduced in cells incubated with AG compared with the non-AG-treated counterparts, hypusinated eIF5A was detected only in cells supplemented with α-MeSpd or *β*-MeSpd, not in those supplemented with *γ*-and *ω*-MeSpd (Fig. 3b, left panels, with AG). These findings indicate that of the four analogs, only α- and *β*-MeSpd were used for hypusine modification in the polyamine-depleted cells. Even though *γ*-MeSpd, as the racemate, was a substrate for DHS in vitro, it did not appear to be utilized for hypusine synthesis after its uptake into cells, because no hypusinated eIF5A was detected in cells supplemented with this analog. When we compared the eIF5A patterns in cells supplemented with stereoisomers of α-MeSpd, hypusinated eIF5A was the major form in control cells, and in DFMO-treated cells supplemented with racemic α-MeSpd, or with *S*-α-MeSpd (Fig. 3d, right panels without AG). In contrast, no hypusinated eIF5A was detected in cells supplemented with *R*-α-MeSpd. Inclusion of aminoguanidine in the culture medium drastically reduced the hypusinated eIF5A. The

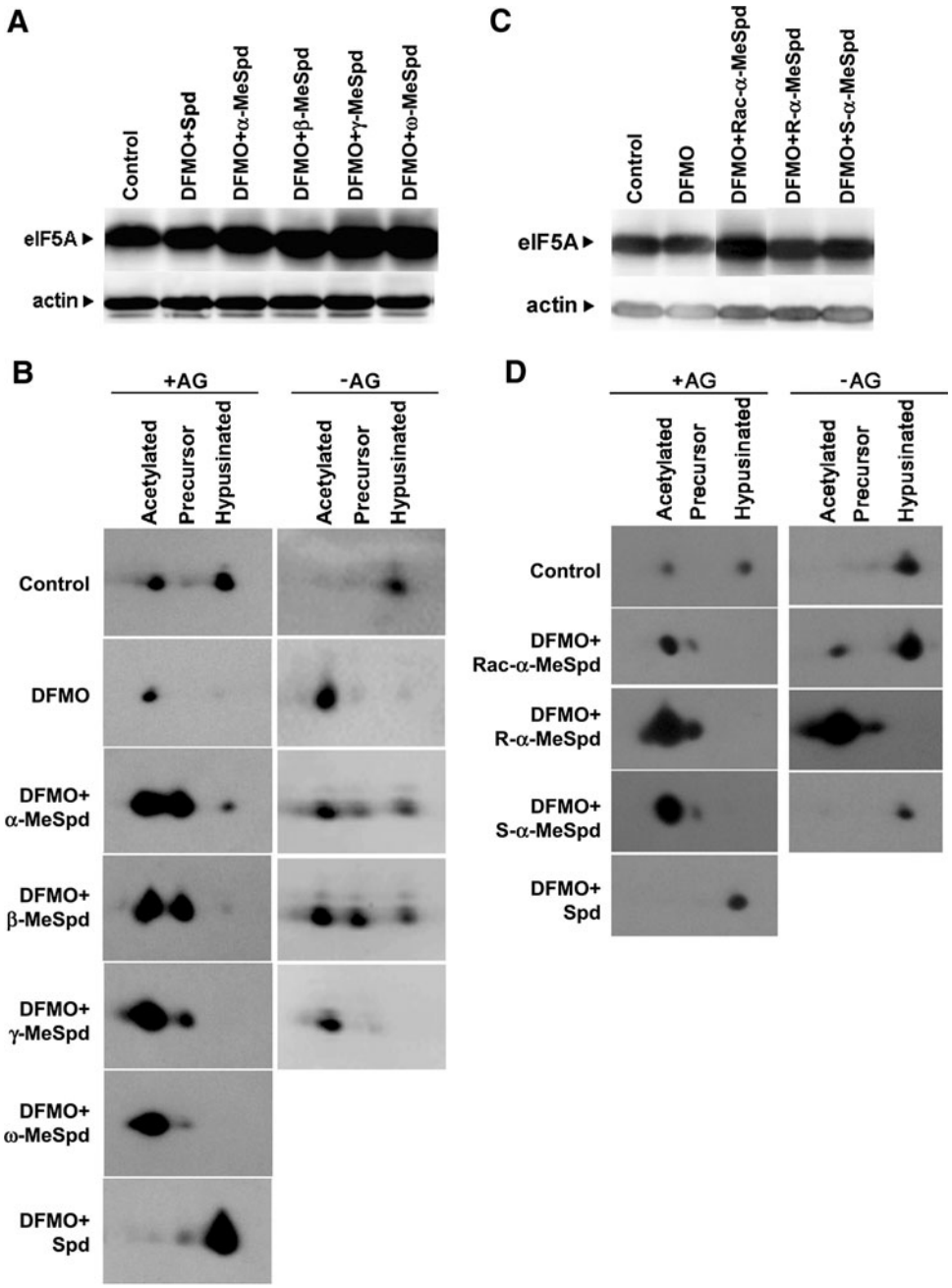


Fig. 3 Effect of the analogs on hypusine modification of eIF5A in polyamine-depleted cells. DU145 cells were treated with DFMO (5 mM) and analogs (100 μM), α-MeSpd, *β*- MeSpd, *γ*-MeSpd, and *ω*-MeSpd (each as the racemate) for 9 days (**a** and **b**) and (*R/S*)-α-MeSpd, *R*-α-MeSpd, and *S*-α-MeSpd for 8 days (**c** and **d**) with or without 1 mM aminoguanidine. Total eIF5A content was estimated by western blotting using anti-eIF5A antibody and anti-actin antibody (loading control) after SDS–PAGE (**a** and **c**) Samples in **a** are from cells not treated with AG and in **c** are all from cells treated with AG. eIF5A modification status was analyzed by western blotting after 2D gel electrophoretic separation of proteins. Areas of the gel surrounding the eIF5A protein are shown. Hypusinated eIF5A (pI, 5.37) separates from the unhypusinated precursor (pI 5.25) and the unhypusinated, acetylated eIF5A precursor (pI, 5.1) as reported previously (Park 1989)

decrease in hypusinated eIF5A in aminoguanidine-treated cells compared with that in non-treated cells is probably due to the inhibition of cellular DHS by aminoguanidine upon long-term exposure. Aminoguanidine at 1 mM indeed caused a significant inhibition of the DHS reaction in vitro (data not shown). A notable reduction in cellular growth rate in the presence of aminoguanidine may be attributable to a decrease in hypusinated eIF5A resulting from inhibition of DHS in these cells (Fig. 1). Taken together, these results demonstrate the absolute dependence of cell growth on eIF5A hypusination in the polyamine-depleted, MeSpd-supplemented DU145 cells.

Discussion

It was recently reported that a yeast mutant strain defective in polyamine biosynthesis grew at a nearly normal rate even when the bulk of cellular polyamines was depleted (to less than 0.2% of normal level of spermidine) and that the requirement for spermidine for hypusine synthesis is the primary function of polyamines in yeast cell growth (Chattopadhyay et al. 2008). On the other hand, polyamine homeostasis is crucial in the maintenance of normal growth and physiology in mammalian cells, in addition to the requirement for spermidine for hypusine synthesis (Nishimura et al. 2005). Thus, mammalian cells exhibit two phases of growth inhibition upon depletion of polyamines by treatment with DFMO (Hyvönen et al. 2007). The initial acute phase of cytostasis due to depletion of cellular putrescine and spermidine can be relieved by spermine or a number of unnatural polyamines that meet organic polycationic requirements (Nagarajan et al. 1988; Hyvönen et al. 2007). However, these analogs cannot support growth of polyamine-depleted cells in the long run, unless they serve as a substrate for hypusine modification (Byers et al. 1992, 1994; Hyvönen et al. 2007). Thus, the second phase of growth arrest, which results from depletion of hypusinated eIF5A, can only be averted by those spermidine analogs that function as a substrate for deoxyhypusine synthase to produce a biologically active form of eIF5A. The long delay in the second phase of cytostasis may be due to the fact that eIF5A is an abundant protein with a long half-life. Since deoxyhypusine synthase has a very narrow specificity for the amine substrate (the butylamine donor) as well as for the protein substrate, few spermidine analogs, including α-MeSpd and *N*-(3-aminopropyl)-1, 4-diamino-*cis*-but-2-ene (*cis*-unsaturated spermidine) have been reported to act as DHS substrates and to rescue cells from polyamine depletion-induced cytostasis (Byers et al. 1992, 1994; Hyvönen et al. 2007).

The *C*-methylated derivatives of spermidine used in this study apparently fulfill the polycationic functions of the natural polyamines. Despite the methyl substitution, these compounds retain the charge distribution of natural polyamines. Their effects on growth were apparently not mediated via their conversion to spermidine, since no spermidine was detected in cells treated with α-, γ-, or ω-MeSpd. A trace amount of spermidine was found only in cells treated with β-MeSpd, but it was diminished by co-treatment with MDL72527, an inhibitor of cellular polyamine oxidases, without abolishing the growth-supporting effects. Our results demonstrate that only the two analogs, α- and β-MeSpd, which serve as substrates for hypusine modification in cells (Fig. 3b), can substitute for the natural polyamine, spermidine, in supporting long-term growth, whereas γ- and ω-MeSpd, which do not act as a precursor of hypusine in cells (Fig. 3b), cannot. The correlation is also evident in the cases of the *R*- and *S*-isomers of α-MeSpd. Only *S*-α-MeSpd, which is an effective substrate for DHS in vitro and in cells, can support long-term growth, but not the *R*-isomer. Neither α- nor β-MeSpd was as effective as spermidine as a DHS substrate or in supporting long-term cell growth. However, it is noteworthy that growth was maintained at a relatively high level (Fig. 1a, b), in the α- and β-MeSpd-supplemented cells, when hypusinated eIF5A was far below its normal level (Fig. 3b). The sustenance of such growth in spite of the marked reduction in hypusinated eIF5A is probably attributable to the abundance of eIF5A and its long half-life. Our data with *C*-methylated spermidine analogs reinforces the absolute requirement for hypusine modification of eIF5A in mammalian cell proliferation. The differential growth effect of MeSpds may reflect the specificity of the deoxyhypusine synthase reaction in cells.

It is interesting to consider specific interaction of these methylated spermidines with the active site of deoxyhypusine synthase. *S*-α-MeSpd, β-MeSpd, and γ-MeSpd were substrates for DHS, whereas *R*-α-MeSpd and ω-MeSpd were not. The active site of DHS has been probed with many derivatives of spermidine, diaminoheptane, or diaminooctane with different substitutions at various carbons or nitrogen (Jakus et al. 1993). Furthermore, the crystal structure of DHS complexed with its cofactor NAD and an inhibitor, N^1-monoguanyl-diaminoheptane (GC7), bound at the spermidine binding site (Umland et al. 2004) revealed that spermidine fits into a narrow tunnel (of about 15 Å), which does not tolerate bulky substitutions on its carbon backbone. The two terminal amino groups (basic) of spermidine spaced at the proper distance are critical for enzyme binding. The secondary amino group is not required for binding, but its proper positioning is critical for the DHS reaction, such that only spermidine bound in a proper orientation (butylamine side at the bottom of the tunnel) would be cleaved by DHS. The fact that all the *C*-methylated analogs inhibited the deoxyhypusine synthase

reaction to a certain extent suggests that they could fit into the narrow tunnel in the proper or the opposite orientation. However, only those that bind in the right orientation for the *N*-*C*5 bond cleavage are expected to work as a substrate for DHS. This may explain why ω-MeSpd is an inhibitor but not a substrate. The remarkable specificity of DHS revealed using these *C*-methyl analogs and stereoisomers reflects a tight topology of its active site pocket. It is conceivable that one stereoisomer of β-and γ-MeSpd is preferred as a substrate as is the case for α-MeSpd. Molecular modeling of the active site of DHS using these stereoisomers will provide new insights into the fine specificity of DHS.

The ability of the methylated spermidines to support a long-term growth correlated with their ability to act as substrates for DHS in vitro with the exception of γ-MeSpd that acted as a substrate for DHS in vitro, but failed to support long-term growth. When we examined the modification status of eIF5A in cells supplemented with γ-MeSpd, hypusinated eIF5A was not detectable even in cells not treated with aminoguanidine. It is puzzling that γ-MeSpd transported into cells was not used for hypusine modification, while racemic γ-MeSpd functioned as substrate for DHS in vitro. It is possible that there is difference in stereospecificity for cellular uptake of γ-MeSpd and for its DHS reaction. Indeed, a difference in cellular uptake was found for the stereoisomers of α-MeSpd (Hyvönen et al. 2007, 2009). In this case, a stereoisomer of γ-MeSpd transported into cells may not work as a substrate for DHS. Interestingly, γ-MeSpd differs from other MeSpds in that there was no methylspermine derivative formed from intracellularly accumulated γ-MeSpd, whereas all other MeSpds were partially converted to the corresponding methylated spermine derivatives. Whether there are differences in stereo specificity in the transport of γ-MeSpd and in the DHS or spermine synthase reactions awaits further investigation with individual stereoisomers of this compound.

In addition to their use in the deoxyhypusine synthase reaction, the *C*-methylated spermidine analogs are useful tools in the investigation of other cellular processes involving polyamines. α-MeSpd, β-MeSpd, and ω-MeSpd are substrates for spermine synthase, whereas γ-MeSpd apparently is not (Table 1). Furthermore, these analogs displayed different substrate activities toward spermidine/spermine acetyltransferase 1 (SSAT1) and the polyamine oxidase (APAO) (Hyvönen et al. 2011). Of the methylated spermidines, γ-MeSpd is the most stable metabolically, not being a substrate for APAO, SSAT1, and spermine synthase (Hyvönen et al. 2011). Thus, a methyl group at the third position of spermidine may either restrict the reactions involving the cleavage of *C*3-*N* or *N*-*C*5 bonds of the spermidine backbone (see Scheme 1), or interfere with the proper binding to the active site of these enzymes. These spermidine analogs provide new insights into the spermidine binding site of deoxyhypusine synthase and warrant development of new stereoisomers with distinct properties as tools for further investigation of polyamine transport, polyamine biosynthesis/metabolism, and hypusine biosynthesis.

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