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MINIREVIEW ARTICLE

Selective regulation of polyamine metabolism with methylated polyamine analogues

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Abstract Polyamine metabolism is intimately linked to the physiological state of the cell. Low polyamines levels promote growth cessation, while increased concentrations are often associated with rapid proliferation or cancer. Delicately balanced biosynthesis, catabolism, uptake and excretion are very important for maintaining the intracellular polyamine homeostasis, and deregulated polyamine metabolism is associated with imbalanced metabolic red/ox state. Although many cellular targets of polyamines have been described, the precise molecular mechanisms in these interactions are largely unknown. Polyamines are readily interconvertible which complicate studies on the functions of the individual polyamines. Thus, non-metabolizable polyamine analogues, like carbon-methylated analogues, are needed to circumvent that problem. This review focuses on methylated putrescine, spermidine and spermine analogues in which at least one hydrogen atom attached to polyamine carbon backbone has been replaced by a methyl group. These analogues allow the regulation of both metabolic and catabolic fates of the parent molecule. Substituting the natural polyamines with methylated analogue(s) offers means to study either the functions of an individual polyamine or the effects of altered polyamine metabolism on cell physiology. In general, gem-dimethylated analogues are considered to be non-metabolizable by polyamine catabolizing enzymes spermidine/spermine-N^1-acetyltransferase and acetylpolyamine oxidase and they support short-term cellular proliferation in many experimental models. Monomethylation renders the analogues chiral, offering some advantage over gem-dimethylated analogues in the specific regulation of polyamine metabolism. Thus, methylated polyamine analogues are practical tools to meet existing biological challenges in solving the physiological functions of polyamines.

Keywords Acetylation · Carbon-methylated · Metabolic stability · Flavin-containing oxidoreductases · Ornithine decarboxylase · Polyamine metabolism

Abbreviations

AdoMet	*S*-Adenosyl-L-methionine
AdoMetDC	*S*-Adenosyl-L-methionine decarboxylase
ALAT	Alanine aminotransferase
APAO	Acetylpolyamine oxidase
BA	Benzaldehyde
dcAdoMet	Decarboxylated *S*-adenosylmethionine

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DAO	Diamine oxidase
DFMO	α-Difluoromethyl ornithine (elfornithine®)
DHS	Deoxyhypusine synthase
Me	Methyl
ODC	Ornithine decarboxylase
Orn	Ornithine
Put	Putrescine
PL	Pyridoxal
SMO	Spermine oxidase
Spd	Spermidine
SpdSy	Spermidine synthase
Spm	Spermine
SpmSy	Spermine synthase
SSAT	Spermidine/spermine-N^1-acetyltransferase

Introduction

The polyamines spermidine (Spd) and spermine (Spm) and their diamine precursor putrescine (Put) are aliphatic non-chiral organic cations having positively charged amines at physiological pH (Pegg 2009a). They are essential for many cellular functions, in some of which they can be substituted by other polyamine species or their synthetic analogues (Brooks 2012). However, many of these interactions have very definite structural requirements that are met only by one distinct polyamine or a very closely resembling analogue (Byers et al. 1992; Park et al. 2003; Hyvönen et al. 2007a). Often polyamines modulate, but cannot substitute for, the functions of metal cations; likewise metal cations can modulate polyamine interactions but are unable to functionally replace them (Williams 1997; Hibino et al. 2010). Although many cellular targets of polyamines are known, the exact molecular mechanisms in the regulation of these processes are largely unknown. This includes the regulation of the expression of polyamine metabolic enzymes and their substrate/inhibitor specificities. Therefore, continuous structure–activity studies using rationally developed polyamine analogues are warranted. Moreover, similar studies with pathogens may reveal metabolic differences between the host and parasite or microbes, and offer novel therapeutic possibilities (Birkholtz et al. 2011). However, due to the versatile and interconvertible nature of polyamines and many of their analogues, cell and animal studies are complicated, requiring the use of combinations of inhibitors and/or silencing of metabolic enzymes by genetic means (Seiler 2003a; Alhonen et al. 2009). Carbon-methylated polyamine analogues are chemical tools prepared to study structure–activity requirements in polyamine interactions or in their metabolism. Promising results in the initial animal studies and the increased metabolic stability of some analogues have promoted their further development (Räsänen et al. 2002; Järvinen et al. 2006b). The use of methylated polyamine analogues in polyamine research has been reviewed earlier (Pegg et al. 1995; Keinänen et al. 2007; Khomutov et al. 2009). Here, we present an updated review of the further development of carbon-methylated polyamine analogues and summarize the accumulated experimental data until present. Moreover, we have extended the historical coverage for this group of polyamine analogues by including earlier omitted methylated putrescine derivatives.

Polyamine metabolism

Polyamine metabolism is relatively well characterized in mammals, and it can be roughly divided in biosynthesis, interconversion and terminal deamination branches. The current view of polyamine metabolism is shown in (Fig. 1). The rate-controlling enzymes of polyamine biosynthesis are ornithine decarboxylase (EC 4.1.1.17; ODC) and *S*-adenosyl-L-methionine decarboxylase (EC 4.1.1.50; AdoMetDC). Arginine decarboxylase present in microbes and plants has not been shown to exist in mammalians (Coleman et al. 2004). Thus, ornithine is the sole known precursor of Put in mammals. Put is converted to Spd by spermidine synthase (EC 2.5.1.16; SpdSy) and Spd is converted to Spm by spermine synthase (EC 2.5.1.22; SpmSy). The rate-controlling enzyme of Spd and Spm interconversion is spermidine/spermine-N^1-acetyltransferase (EC 2.3.1.57; SSAT) and N^1-acetylated polyamines are consequently metabolized by acetylpolyamine oxidase (EC 1.5.3.13; APAO). Spm is catabolized directly without prior acetylation to Spd by spermine oxidase (EC 1.5.3.16; SMO). The terminal catabolism of polyamines is mostly mediated by Cu^{2+}-dependent diamine oxidase (EC 1.4.3.22; DAO) and semicarbazide-sensitive amineoxidase (i.e. vascular adhesion protein; EC 1.4.3.21; SSAO), but there could be some uncharacterized oxidases present, which are capable of oxidizing polyamines, especially in bovine serum used as a supplement in cell culture media.

Polyamines are readily taken up by the cells and tissues from diet and gastro-intestinal track bacteria (Milovic 2001; Wallace and Fraser 2004). These polyamines may also consist of atypical polyamines, like cadaverine, diaminopropane, 2-OH-putrescine, which all have been detected from rat or human urine (Noto et al. 1978; Satink et al. 1989; van den Berg et al. 1986). Whether these atypical polyamines modulate polyamine metabolism and what role they have in mammalian physiology has been poorly addressed.

The complex regulation of polyamine metabolism allows cells/tissues to modulate adequate polyamine supply for different physiological conditions and insults (Pegg

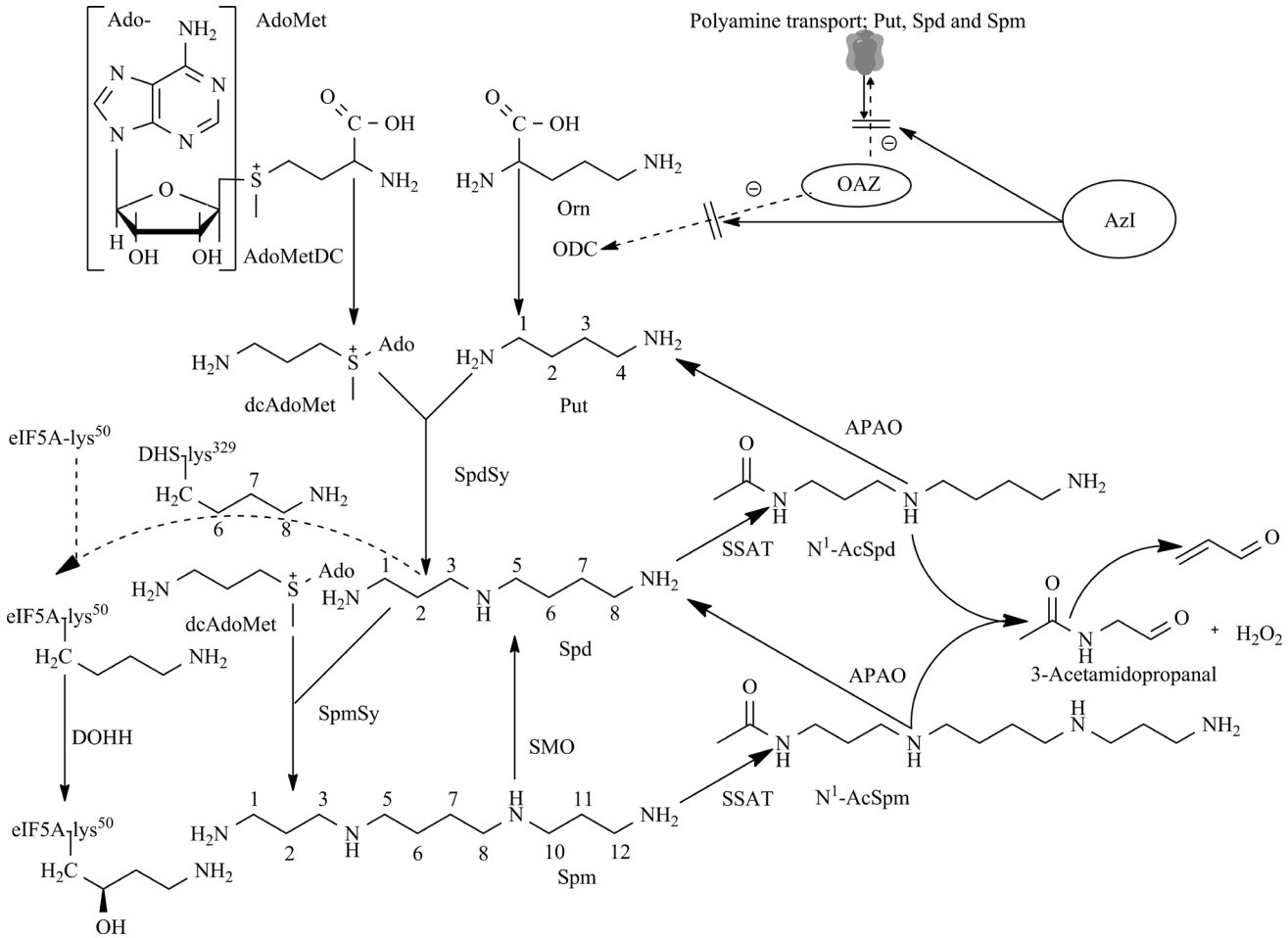


Fig. 1 Polyamine metabolism in mammals; biosynthesis and interconversion pathways and Spd mediated posttranslational modification of eukaryotic translation initiation factor 5A (eIF5A) are shown. The rate-controlling enzyme of de novo polyamine biosynthesis is ornithine decarboxylase (EC 4.1.1.17; ODC) that decarboxylates ornithine (Orn) to putrescine (Put). Put is diamine precursor for spermidine that is synthesized by spermidine synthase (EC 2.5.1.16; SpdSy) using decarboxylated-*S*-adenosyl-L-methionine (dcAdoMet) as aminopropyl donor. dcAdoMet is produced from *S*-adenosyl-L-methionine decarboxylase (EC 4.1.1.50; AdoMetDC). Spd is converted to Spm by spermine synthase (EC 2.5.1.22; SpmSy). The rate-controlling enzyme of Spd and Spm interconversion is spermidine/spermine-N^1-acetyltransferase (EC 2.3.1.57; SSAT). N^1-acetylated Spd (N^1-AcSpd) and N^1-AcSpm are consequently metabolized by peroxisomal acetylpolyamine oxidase (EC 1.5.3.13; APAO). Spm is catabolized without prior acetylation to Spd by spermine oxidase (EC 1.5.3.16; SMO) producing 3-aminopropanal and hydrogen peroxide. 3-Aminopropanal is chemically decomposing to acrolein and ammonia that are highly reactive toxic compound for the cell. Antizyme (OAZ) mediated inhibitory regulations of ODC and polyamine transport are shown as a dotted lines. OAZ itself is regulated by antizyme inhibitor (AzI) an enzymatically inactive paralogue of ODC. Spd is precursor for hypusine [2-hydroxybutyL-lysine; N^6-(4-amino 2-hydroxybutyl)-L-lysine], the unique posttranslational modification of lysine-50 residue needed for functional eIF5A protein. Active eIF5A is formed in two steps; first eIF5A precursor is hypusinated by deoxyhypusine synthase (EC 2.5.1.46; DHS), followed by hypusine hydroxylation by deoxyhypusine hydroxylase (EC 1.14.99.29; DOHH). During deoxyhypusination process lysine-329 is deoxyhypusinated at the active site of DHS and DHS is transferring the deoxyhypusine to eIF5A precursor at lys50 residue. First step of process is reversal and could be used to test substrate specificity using homospermidine assay

2009a; Casero and Pegg 2009). Accumulating evidence suggests that delicately balanced biosynthesis, catabolism, uptake and excretion of polyamines are very important for health (Casero and Pegg 2009; Brooks 2012), and disturbances in this homeostasis are associated with cancer and many other diseases (Casero and Pegg 2009; Soda 2011). It is already evident that selectively binding/targeting polyamine analogues, which have only minor interference with known regulation of polyamine metabolism, can be prepared. However, due to the versatile and interconvertible nature of polyamines, multi-targeting drugs modulating several polyamine-regulated sites are of interest in reverting deregulated polyamine metabolism to normal. The known targets and the effects of C-methylated

polyamine analogue on a given enzyme are described in detail below.

C-Methylated polyamine analogues and regulation of polyamine biosynthesis and transport

Development of C-methylated polyamine analogues for polyamine research

The α- and β-methylated ornithine derivatives, synthesized in 1978, proved to be important for the development of enzyme-activated irreversible inhibitors for ODC e.g. α-difluoromethylornithine (DFMO) (Bey et al. 1978). Knowledge of the reversible nature of enzyme catalysis and product feedback inhibition led to the synthesis of a series of product analogues including methylated putrescine derivatives as ODC inhibitors. They were tested with DAO, an enzyme terminally deaminating N^1-AcPut and Put to acetaminobutanal or 4-aminobutanal, respectively. In addition, higher polyamines Spd and Spm are degraded by DAO to toxic by-products (Hölttä et al. 1975). Gem-dimethylated spermidine derivatives synthesized in the mid-1980s (Nagarajan and Ganem 1986) were followed by 1-methylspermidine (1-MeSpd), 1-methylspermine (1-MeSpm) and 1,12-dimethylspermine (1,12-Me_2Spm) (Lakanen et al. 1992). They were soon shown to be practical research tools, especially under conditions of accelerated polyamine catabolism like in *E. coli* expressing human recombinant SSAT (Lakanen et al. 1992; Parry et al. 1995). The lack of toxicity in cell culture models and resistance to acetylation by SSAT prompted us to use 1-MeSpd also in vivo to substitute the natural polyamines (Räsänen et al. 2002).

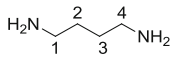
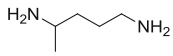
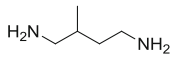
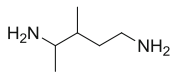
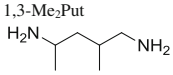
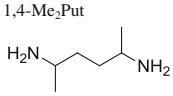
Ornithine decarboxylase, antizymes and polyamine uptake system

ODC is the rate-controlling enzyme of de novo synthesis of polyamines and thus it attracted many drug development projects in the early 1970s and is still a well-recognized target of therapeutic intervention (Fig. 1) (Seiler 2003a). The regulation of ODC is very complex and it is controlled by cellular growth factors and intracellular polyamine levels, which mainly act through induction of antizyme (OAZ) by promoting +1 ribosomal frameshift in decoding OAZ mRNA (Kahana 2009). OAZ regulates the ubiquitin-independent 26S-proteasomal degradation of ODC and the uptake and export of polyamines. Unfortunately, the polyamine transport system has not been convincingly characterized in mammalian cells, but in yeast and *E. coli,* transport proteins are known (Igarashi and Kashiwagi 2010). The regulation is even more complex, as OAZ itself interacts with antizyme inhibitor (AzI), a protein closely resembling ODC but devoid of enzymatic activity (Kahana 2009).

A direct inhibitory effect of Spd or Spm or their analogues on purified ODC has not been demonstrated, but OAZ-mediated inhibition of ODC activity has been studied. In general, C-methylated polyamine analogues induce OAZ frameshifting to similar extent as the natural polyamines, and subsequently downregulate ODC activity and inhibit polyamine uptake. While direct enzyme-protein interaction studies of frameshifting are not yet possible because the molecular mechanisms of polyamine-mediated frameshifting are not known, practical in vitro models have been established in rabbit reticulocyte lysate and in cell culture using luciferase dual reporter system (Howard et al. 2001; Hyvönen et al. 2009). Moreover, since OAZ is very short-lived protein, the ability of an analogue to induce OAZ production can be tested indirectly by comparing the intracellular analogue accumulation in cells treated with and without an inhibitor of protein synthesis, such as cycloheximide (CHX) (Hyvönen et al. 2009). 1-MeSpd and 1,12-Me_2Spm can induce apoptosis and DNA fragmentation in hours in Chinese hamster ovary cells after 3 days pre-treatment with DFMO when CHX treatment is used to prevent the inhibition of polyamine transport by OAZ (Hu and Pegg 1997). These findings raise interest for selective tumor cell killing by cytotoxic polyamine analogues that do not induce OAZ production but are taken up by the polyamine transporter system and are catabolized by SMO or APAO.

Directly acting ODC inhibitors, like DFMO are efficient in cell culture models where polyamines originating from diet or gut bacteria cannot overcome the growth inhibitory effect of the drug. Among several methylated Put analogues that have been synthesized (Table 1), 2-MePut is the most efficient inhibitor of rat liver ODC having K_i of 1.0 mM (Put 3.3 mM), that is far too high to have practical applicability as compared to ODC inhibitors active at nanomolar (1-aminooxy-3-aminopropane) and micromolar (DFMO) concentrations (Ruiz et al. 1986; Seiler 2003a). Later synthesized 1,4-Me_2Put is not an inhibitor of ODC in vitro but inhibits ODC in vivo through the induction of OAZ (Moyano et al. 1990; Frydman et al. 1991), and is resistant to DAO-mediated degradation (Table 1). The effect of analogues on rat liver ODC activity (induced by injection of thioacetamide or dexamethasone) and on polyamine pools have been determined at 1 and 3 or 4 h after analogue treatment (Table 1). 1-MePut, 2-MePut and 1,4-Me_2Put have been examined also in H-35 hepatoma cells, where they all accumulate at similar levels and inhibit ODC with K_i values of Put 2.4 mM, 1-MePut 2.8 mM and 2-MePut 0.1 mM (Frydman et al. 1991). Later synthesized 2,2-Me_2Put, 2,3-Me_2Put, 2,3,3-Me_3Put and

Table 1 Structures and biological properties of methylated putrescine analogues

	K_i ODC rat liver	ODC (inhibition % of control, rat liver)	DAO[c] (pea seedling) (pig kidney) nmol/30 min	DAO[c] (pea seedling) (pig kidney) inhibition % at 30 min	SpdSy[b] relative MTA formation 1 mM/10 mM	Chick embryo survival as % at 11 days analogue + DFMO[b]
Put 	3.3 mM[a,d]	56[a]	180	407 (100)	1.0/1.0[b]	100[b]
		55[d]	120	167 (100)		
1-MePut 	2.7 mM[a]	75[a]	32	36	0.03/0.06[b]	80[b]
			7	6		
2-MePut 	1.0 mM[a]	93[a]	15	73	0.63/1.2[b]	80[b]
			13	10		
1,2-Me_2Put 	3.8 mM[d]	70[d]	Nd	Nd	Nd	Nd
	4.8 mM[a]		Nd			
1,3-Me_2Put 	2.9 mM[d]	76[d]	1	Nd	Nd	Nd
	4.3 mM[a]		1			
1,4-Me_2Put 	NI[a,d]	96[d]	0	55	0.00/0.00[b]	50[b]
			0	64		

NI, less than 10 % inhibition at 9 mM; nd, not determined; Footnote letters are referring to the following references *a* (Ruiz et al. 1986) *b* (Sarhan et al. 1987), *c* (Frydman et al. 1987) *d* (Moyano et al. 1990). DAO inhibition reference values with Put 0.5 μmol (407/167 nmol/30 min) with or without 1 μmol Put analogue in 350 μl incubation mixture

2,2,3,3-Me_4Put have K_i over 5 mM for ODC and are not efficient inhibitors of ODC in vivo. Among methylated Put analogues 1,4-Me_2Put is the most efficient in vivo in downregulating ODC activity and thus it has been synthesized as optical isomers. Interestingly, their mode of action in downregulating ODC differs as the (-)-isomer abolishes de novo ODC synthesis, while the meso-isomer strongly accelerates ODC degradation by inducing OAZ frameshifting (Moyano et al. 1990).

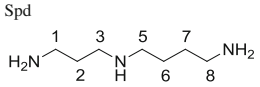
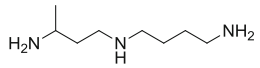
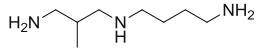
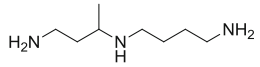
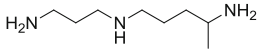
From methylated Spd analogues, 1-, 3-, and 8-MeSpd downregulate ODC in DU145 cells (Table 2), whereas 2-MeSpd is far less potent (Hyvönen et al. 2011). Earlier we showed that (*S*)-1-MeSpd and (*S*,*S*)-1,12-Me_2Spm induce OAZ frameshifting more efficiently than the other diastereomers, and that analogue ability to induce OAZ frameshifting correlates with their potency to inhibit ODC activity in DU145 cells (Hyvönen et al. 2009, 2011), suggesting that 2-MeSpd is Spd mimetic with very low OAZ induction potency.

1-, 2-, 3-, and 8-MeSpd and 1-MeSpm and 1,12-Me_2Spm analogues compete with Spd or Spm for cellular uptake (Hyvönen et al. 2009, 2011). 2-MeSpd competes as efficiently as unlabelled Spd for the transport, while 3-MeSpd has the lowest affinity (2-MeSpd > 1-MeSpd > 8-MeSpd > 3-MeSpd; Table 2) (Hyvönen et al. 2011). (*S*)-1-MeSpd and (*S*,*S*)-1,12-Me_2Spm have lowest affinity for the polyamine transport among tested isomers and diastereomers (Hyvönen et al. 2009). 1-MeSpd and 1,12-Me_2Spm have been used to study the effects of AdoMetDC inhibition on polyamine uptake in CHO cells (Byers et al. 1994b). Unfortunately, no data about polyamine transport competition or analogue regulatory effects for ODC or AdoMetDC with gem-dimethylated polyamine analogues are available.

S-Adenosyl-L-methionine decarboxylase and spermidine and spermine synthases

AdoMetDC is the second rate-controlling enzyme for the biosynthesis of the polyamines (Fig. 1) (Pegg 2009b). AdoMetDC decarboxylates *S*-adenosyl-L-methionine (AdoMet) to decarboxylated AdoMet (dcAdoMet) which then acts as aminopropyl donor for propylaminotransferases SpdSy and SpmSy. The regulation of AdoMetDC expression, like that of ODC, is very complex and has been recently reviewed in detail (Pegg 2009b). Autocatalytic cleavage of the proenzyme requires Put to produce α/β subunits which form the tetrameric active enzyme. Put also acts as an allosteric activator of the enzyme protein.

Table 2 Structures and biological properties of monomethylated Spd analogs (Hyvönen et al. 2011)

	SSAT μmol/min/mg (mouse recombinant)	SSAT productive splicing (DU145 cells)	APAO with BA (mouse recombinant)	SMO activity (DU145 cells)	Analog uptake (DU145 cells)	ODC activity (DU145 cells)	Conversion to Spm or Spm analogue
Spd 	V_{max} 4.28 ± 0.13 K_m 151 ± 15 μM	Nd	++	Nd	+++	Nd	Yes
1-MeSpd 	Ns	=DENSpm	+	↑↓	++	↓↓↓	Yes
2-MeSpd 	V_{max} 0.77 ± 0.01 K_m 132 ± 7 μM	≪DENSpm	+++	↓	+++	↑↓	Yes
3-MeSpd 	Ns C_i, K_i 52 ± 18 μM	≪<DENSpm	ns	↓↓↓	(+)	↓↓	No
8-MeSpd 	V_{max} 7.35 ± 0.10 K_m 78 ± 3 μM	=DENSpm	+	↑	+	↓↓↓	Yes

BA benzaldehyde, C_i competitive inhibitor, *Nd* not determined, *Ns* not a substrate. *Arrows* are used to describe relative suppressing activity of each MeSpd among the tested analogues, ↑↓ no effect as compared to control. SSAT mRNA productive splicing is compared to DENSpm as a reference. +++ For the best substrate of APAO or the best competitor with C^{14}-Spd in the uptake in DU145 cells. DENSpm, N^1N^{11}-diethylnorspermine

Moreover, an upstream open reading frame on the 5′-UTR of AdoMetDC mRNA coding for a six amino acid peptide (MAGDIS) regulates the production of the proenzyme in response to cellular polyamine levels.

The biological effects of methylpolyamines on AdoMetDC regulation have been relatively little studied. In Ehrlich ascites tumor cells 1 mM 1,1,4,4-Me_4Put supplement behaved similarly as control, while 1 mM treatment with 1,4-Me_2Put seemed to induce AdoMetDC after 2 days of culture (Holm et al. 1988). In the same experiment 0.5 mM 5,8-Me_2Spm suppressed efficiently both ODC and AdoMetDC. No data are available for gem-dimethylated Spd analogues, whereas 1-, 2-, 3-, and 8-MeSpd have been tested in cell culture. In DU145 cells, none of the tested analogues alone (100 μM) reduced AdoMetDC activity below the control level (Hyvönen et al. 2011). DFMO treatment is known to induce AdoMetDC activity and to cause accumulation of dcAdoMet. The intracellular levels of dcAdoMet are dependent both on analogue-mediated AdoMetDC downregulation and the substrate properties of the analogue for SpmSy. In the presence of 5 mM DFMO, 3-MeSpd caused the highest accumulation of dcAdoMet, while treatment with 2-MeSpd or Spd restored dcAdoMet to control level. 3-MeSpd is not converted to corresponding Spm analogues, while 1-, 2-, and 8-MeSpd are. 2-MeSpd is readily converted to 2-MeSpm that could explain complete disappearance of dcAdoMet as compared to 1- and 8-MeSpd (Hyvönen et al. 2011). Isomers of 1-MeSpd and 1,12-Me_2Spm suppress AdoMetDC mRNA productive translation less efficiently than Spd and Spm, respectively, (*S*)-1-MeSpd and (*R*,*R*)-1,12-Me_2Spm being the most efficient of the isomers (Hyvönen et al. 2009). Further studies with methylated putrescine analogues are of obvious interest for understanding structure–activity relationships in regulation of proenzyme mRNA translation, autocatalytic cleavage of proenzyme and allosteric activation of tetrameric enzyme.

SpdSy and SpmSy are constitutively expressed in mammalian cells and their properties have recently been reviewed (Fig. 1) (Ikeguchi et al. 2006). Several inhibitors of these enzymes have been developed (Pegg et al. 1995). Many polyamine analogues are metabolized by SpdSy and SpmSy, thus complicating studies on the biological effects of the analogues. Analyses carried out with purified bovine brain SpdSy have shown that 2-MePut is readily metabolized to 6-MeSpd, while 1-MePut is a poor substrate for the enzyme (Table 1) (Sarhan et al. 1987). 1,4-Me_2Put is not metabolized by SpdSy and, due to its resistance against DAO and most likely any cellular acetylases, it is metabolically stable. Interestingly, 1-MePut is metabolized in chick embryos to Spd analogue, initially considered to be 8-MeSpd, but later suggested to be 5-MeSpd (Sarhan et al. 1987; Hyvönen et al. 2011). In chick embryos, 2-MePut is metabolized to 6-MeSpd and consequently accumulates as MeSpm analogue in amount of 1/5 of that of 2-MePut. Of the synthesized and tested monomethylated Spd analogues,

only 3-MeSpd is not metabolized by SpmSy to respective MeSpm analogues (Table 2). Gem-dimethylated analogues 1,1-, 2,2-, 3,3- and 8,8-Me_2Spd are not metabolized to respective Me_2Spm derivatives in SV-3T3 cells although they showed minor activity at 5 mM concentration when tested as putative substrates of isolated rat prostate SpmSy (Table 3). No data about the possible conversion of 5,5-Me_2Spd to Me_2Spm derivative are available except the compound showed similar minor putative substrate activity as other gem-dimethylated Spd analogues with rat prostate SpmSy (Table 3).

C-Methylated polyamine analogues and polyamine catabolizing enzymes

N-Acetylation of methylated polyamine analogues by SSAT and other acetylases

Spd and Spm can be N^1-acetylated by SSAT to render them susceptible to excretion or back conversion to Put or Spd, respectively, by the action of acetylpolyamine oxidase (APAO; Fig. 1) (Pegg 2008). N^1-AcSpm can be also acetylated to N^1N^{12}-DiAcSpm by SSAT, and Spd can be acetylated to N^8-AcSpd by an enzyme that also acetylates Put to N^1-AcPut and is considered to be a nuclear histone acetyltransferase. Currently, there are no reports about the acetylation of any MePut analogue. Likewise, cloning and characterization of the acetylase(s) responsible for Put acetylation is still waiting to be published.

Among *gem*-dimethylated Spd analogues, 8,8-Me_2Spd is a substrate for SSAT, while 1,1-, 2,2-, 3,3- and 5,5-Me_2Spd are not (Table 3) (Nagarajan et al. 1988). 1-MeSpd and 1,12-Me_2Spm are not substrates for SSAT but act as inhibitors. Interestingly, both 2-MeSpd and 8-MeSpd are readily acetylated by the SSAT, the latter even more efficiently than its natural substrate Spd (Table 2) (Hyvönen et al. 2011). From Spm analogues, 1-MeSpm is readily acetylated by SSAT at non-methylated N^{12}-aminoterminus and further catabolized to MeSpd by APAO (Lakanen et al. 1992). We have found no evidence of N^8-acetylation of any tested analogue in cell cultures or in vivo. The MeSpd analogues differ in their ability in altering SSAT mRNA productive splicing, but their effects on SSAT activity in DU145 cells are negligible even at 100 μM concentration (Hyvönen et al. 2011). In NCI H157 lung carcinoma cells 1,12-Me_2Spm dramatically induces SSAT (20-fold) and accumulates at three times higher concentration than *N*-diethyl Spm at 10 μM. Interestingly, 1,12-Me_2Spm does not downregulate ODC in these cells, and it supports short-term cell growth in the presence of 5 mM DFMO (Yang et al. 1995). By contrast, in DU145 cells 1,12-Me_2Spm readily suppresses ODC activity and only weakly induces SSAT. Of its diastereomers, (*S*,*S*)-1,12-Me_2Spm is the most potent at inducing SSAT (two-fold as compared to control cells and cells treated with (*R*,*S*) or (*R*,*R*) diastereomers). Likewise, (*S*,*S*)-1,12-Me_2Spm is the most active in promoting SSAT mRNA productive splicing in DU145 cells (Hyvönen et al. 2009).

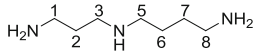
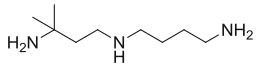
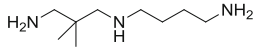
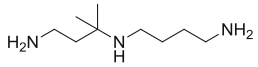
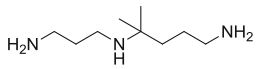
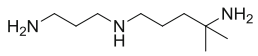
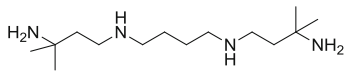
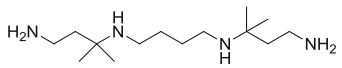
Terminal degradation of methylated polyamine analogues

The oxidative deamination of polyamines can be prevented by the combination treatment with aminoguanidine (AG, an inhibitor of DAO and SSAO) and N^1,N^4-bis(2,3-butadienyl)-1,4-butanediamine (MDL 72527), an irreversible inhibitor of APAO and SMO (Seiler et al. 1985). Serum DAO terminally deaminates Put and higher polyamines (Hölttä et al. 1975) with free primary amine(s), and therefore dimethylation renders 1,4-Me_2Put resistant towards terminal deamination (Frydman et al. 1987). So far, it has been shown that only 1-MeSpm and 8-MeSpd produce toxic metabolites under cell culture conditions without aminoguanidine supplementation. This is in agreement with earlier findings that degradation from the aminopropyl terminus produces toxic metabolite(s), e.g. acrolein. However, no data have been published about the stabilities of any methylated polyamine analogues in serum. Likewise, no data exist about DAO substrate properties with methylated Spd and Spm analogues.

Acetylpolyamine oxidase-mediated degradation

APAO preferentially oxidizes *N*-acetylated polyamines with substrate preference of N^1-AcSpm > N^1N^{12}-DiAcSpm > N^1-AcSpd. Many *N*-alkylated polyamine analogues are also substrates for this peroxisomal enzyme (Häkkinen et al. 2010). 1-MeSpd and 1,12-Me_2Spm, are not substrates for SSAT, and therefore are resistant against APAO-mediated back conversion. However, chemically synthesized N^1-Ac-(*S*)-1-MeSpd and N^1-Ac-(*R*)-1-MeSpd are substrates for APAO in vitro, and the enzyme shows a clear chiral preference for the (*S*)-enantiomer. Furthermore, acetylation can be mimicked in vitro using aldehyde supplementation with the analogues, and under these conditions only 3-MeSpd is resistant to APAO (Järvinen et al. 2005; Hyvönen et al. 2011). Interestingly, supplementation of pyridoxal increases the degradation of (*S*)-1-MeSpd, while benzaldehyde (BA) increases the degradation of (*R*)-1-MeSpd (Järvinen et al. 2006a). Interestingly, such aldehyde-mediated stereo control is not applicable with 1,12-Me_2Spm diastereomers. In the presence of 5 mM BA, 1-MeSpm is degraded eight-fold (aminopropyl end) to five-fold (methyl end) more efficiently by APAO as compared to the reaction velocities determined without

Table 3 Structures and biological properties of gem-dimethylated polyamine analogues from *e* (Nagarajan et al. 1988), *f* (Byers et al. 1992)

Polyamine/analogue	SSAT substrate[e] at 0.1/1.0/10 mM % activity	SpmSy substrate[e] 0.05/0.5/5 mM % activity	SSAT inhibitor[e] 1/5 mM Spd at 0.1 mM	Cell growth[e,f] short-term long-term	DHS activity[f] 10/100/500 µM 3.3 µM ^{3}H-Spd
Spd 	17/66/100	45, 93, 100	0/0	+++ +++	Nd
1,1-Me_2Spd 	2/3/6	<1, 2, 7	25/78	++ –	96/61/18
2,2-Me_2Spd 	2/3/9	<1, 2, 7	49/87	+++ –	104/109/89
3,3-Me_2Spd 	2/2/2	<1, 2, 14	16/65	+++ –	101/116/109
5,5-Me_2Spd 	2/1/2	<1, 4, 10	21/64	+++ –	104/114/109
8,8-Me_2Spd 	15/64/115	<1, <1, 13	24/83	+++ –	102/94/54
1,1,12,12-Me_4Spm 	2/2/1<	<1, <1, 4	46/91	+++ Nd	Nd
3,3,10,10-Me_4Spm 	1/1/1<	<1, <1, 5	18/74	– Nd	Nd

Nd not determined, growth support–no support, +++ full restoration of growth

aldehyde supplementation (Järvinen et al. 2005). Interestingly, APAO catabolizes 1,12-Me_2Spm at three times higher velocity as compared to Spm, but in the presence of BA, the velocities are equal (Järvinen et al. 2005). Rat liver homogenates degrade methylated polyamine analogues similar to recombinant APAO and SMO, and degradation is completely prevented by supplementation of 250 µM MDL 72527 into liver homogenate. Thus, 1-methylation prevents SSAT-mediated acetylation but does not prevent APAO mediated degradation of 1,12-Me_2Spm in vitro. In the presence of 5 mM BA, MeSpd analogues are degraded by APAO in following efficacies 2-MeSpd >> Spd > 1- or 8-MeSpd >> 3-MeSpd (Table 2) (Hyvönen et al. 2011).

Spermine oxidase-mediated degradation of methylspermines

SMO catalyses the oxidative deamination of Spm to Spd without prior acetylation step by SSAT (Fig. 1). SMO was initially characterized in 2001, and it cleaves enzymatically the exo-site of secondary N^4-amine of Spm and N^1-acetylspermine, and many *N*-alkylated polyamine analogues with variable exo/endo cleavage depending on the structure of the analogue (Häkkinen et al. 2010). The exact physiological roles of SMO are not known but its deregulatory activation is harmful for the cells (Casero and Pegg 2009), most likely via the production of H_2O_2 and toxic aldehydes as reaction by-products. Interestingly, several neoplastic cells show upregulated SMO activity as compared to normal tissue, probably in response to increased requirement of Spd in rapidly growing tumour cells (Vujcic et al. 2003).

Spd acts as a product inhibitor for SMO and is not a substrate for the enzyme. SMO degrades 1-MeSpm favouring degradation at the non-methylated end (Järvinen et al. 2005). Racemic 1,12-Me_2Spm is degraded by SMO and a very strict stereospecificity for (*S*,*S*)-stereoisomer has been found, while the (*R*,*R*)-stereoisomer acts as an inhibitor for the enzyme (Hyvönen et al. 2007a). This stereospecificity allows substrate-specific determination of

SMO activity in vitro and in vivo by determining the formation (*S*)-1-MeSpd using HPLC (Hyvönen et al. 2007a).

Interactions of C-methylated polyamine analogues with DNA

The natural polyamines interact with DNA, and have a variety of effects on its conformation and stability (Brooks 2012). 1-MeSpd and 1,12-Me_2Spm appear to induce DNA B- to Z-form transition as effectively as their natural counterparts (Varnado et al. 2000). They also induce DNA condensation and nanoparticle formation like Spd and Spm, and protect against oxidative stress in vitro and in cultured cells, showing stereospecific differences (Nayvelt et al. 2010).

C-Methylated polyamine analogues in cell proliferation and differentiation

The growth-promoting action of polyamines in mammalian cells was reported already in 1960s (Ham 1964). Later it became clear that acute polyamine depletion leads to cessation of cell growth that can be reversed with polyamine or certain polyamine analogue supplementation (Pegg 1984; Lakanen et al. 1992; Byers et al. 1992). By contrast with *N*-alkylated analogues such as N^1,N^{11}-diethylnorspermine (DENSpm) which are generally cytostatic or cytotoxic, many C-methylated analogues support growth of polyamine-depleted cells, at least under short-term polyamine depletion (less than a week in cell culture) (Nagarajan et al. 1988; Lakanen et al. 1992; Hyvönen et al. 2011). However, there are also some growth-inhibitory carbon-methylated polyamine analogues. In Ehrlich ascites tumor cells grown in the presence of 1 mM 1,4-Me_2Put, 1 mM 1,1,4,4-Me_4Put or 0.5 mM 5,8-Me_2Spm, the latter had a significant inhibitory effect on cell growth (Holm et al. 1988). This inhibitory action was attributed to the depletion of total polyamine level. None of these analogues prevented DFMO-induced growth arrest.

From a set of *gem*-dimethylated Spd and Spm analogues that were studied for reversal of DFMO-induced growth arrest, all of the Spd derivatives (1,1-Me_2Spd; 2,2-Me_2Spd; 3,3-Me_2Spd; 5,5-Me_2Spd; 8,8-Me_2Spd) and 1,1,12,12-Me_4Spm, but not 3,3,10,10-Me_4Spm, were able to support the short-term growth of DFMO-treated SV-3T3 cells (Table 3) (Nagarajan et al. 1988). Early studies with 1-MeSpd, 1-MeSpm and 1,12-Me_2Spm showed that they were growth-supportive in short-term DFMO-treated cultures of L1210, SV-3T3, and HT29 cells (Lakanen et al. 1992). Collectively, these results suggest that either Spd or Spm can support the short-term cell growth without the need for metabolic interconversion.

There are two distinct requirements of polyamines in cellular proliferation; "hypusine-independent" in which most of the compounds that resemble polyamines can substitute them, and the "hypusine-dependent" where only those compounds that serve as precursors of hypusine can support proliferation (Hyvönen et al. 2007a; Nishimura et al. 2005; Byers et al. 1994a; Park et al. 2003). Spd is the sole natural precursor of hypusine, the unique posttranslational modification needed for functional eIF5A protein (Fig. 1; dotted line), which is essential for eukaryotic cell growth. Active eIF5A is formed in two steps; first eIF5A precursor is hypusinated by deoxyhypusine synthase (EC 2.5.1.46; DHS), followed by hypusine hydroxylation by deoxyhypusine hydroxylase (EC 1.14.99.29; DOHH). From a set of synthetic polyamine analogues tested, only the *cis*-isomer of unsaturated Spd analogue *N*-(3-aminopropyl)-1,4-diamino-but-2-ene and 1-MeSpd were able to reverse cytostasis induced by prolonged exposure to AdoMetDC inhibitor AbeAdo (Byers et al. 1992, 1994a). The *trans*-isomer of *N*-(3-aminopropyl)-1,4-diamino-but-2-ene did function as a substrate for DHS in vitro, but failed to support long-term cell growth, apparently due to the inability of the formed deoxyhypusinated eIF5A to undergo hydroxylation.

We have shown that only the (*S*)-enantiomer of 1-MeSpd is able to support long-term growth of DFMO-treated DU145 cells, due to its ability to function as substrate for DHS in vitro and to produce hypusinated eIF5A in DU145 cells (Hyvönen et al. 2007a), thus revealing the stereospecificity of DHS reaction. The (*S*,*S*)-diastereomer of 1,12-Me_2Spm is metabolized to (*S*)-1-MeSpd by SMO, and it clearly supports proliferation longer than the other diastereomers of 1,12-Me_2Spm. Our findings are in agreement with studies conducted in *S. cerevisiae* spe2Δ auxotrophs, which completely lack spermidine (Chattopadhyay et al. 2008). From a set of polyamine analogues, only 1-MeSpd supported growth with (*S*)-enantiomer showing better growth than the (*R*)-enantiomer. From the most recently synthesized C-methylated analogues, 2-MeSpd, 3-MeSpd and 8-MeSpd, only 2-MeSpd is able to reverse DFMO-induced cytostasis of DU145 cells, although both 2-MeSpd and 3-MeSpd are substrates of DHS in vitro (Hyvönen et al. 2012). We showed earlier that polyamine transport has chiral preference for (*R*)- and (*R*,*R*)-diastereomers of 1-MeSpd and 1,12-Me2Spm, respectively (Hyvönen et al. 2009). This was also the case with racemic 3-MeSpd, where (*S*)-enantiomer was highly enriched in DU145 cells and failed to support long-term growth because it was a poor substrate for DHS (unpublished). This chiral selection may open possibilities for selective therapy against parasites with C-methylated polyamine analogues, possible affecting trypanothionein synthesis.

Polyamines are required not only for proliferation but also for differentiation of cells. Erwin et al. (1984) showed that DFMO treatment renders 3T3-L1 preadipocytes unable to differentiate to adipocytes. However, it was unclear whether differentiation block was caused by the reduced total polyamine pool or specific depletion of Put or Spd pool. Using the optical isomers of 1-MeSpd, we showed that both enantiomers of 1-MeSpd promote the differentiation of DFMO-treated 3T3-L1 cells equally well, although only the (*S*)-enantiomer is able to act as an efficient hypusine precursor (Vuohelainen et al. 2010), indicating that hypusine is not necessary for the adipogenesis of 3T3-L1 cells. The data also demonstrated that Spd cannot be substituted by Spm, since cells cultured with 1,12-Me_2Spm or with Spm plus MDL72527 did not differentiate but those grown with 1-MeSpd or Spd did. Our recent study using metabolically stable analogues 3-MeSpd and (*R*,*R*)-1,12-Me_2Spm reveals the molecular mechanisms of spermidine depletion-induced suppression of adipogenesis. We showed that Spd promotes the translation of CCAAT/enhancer binding protein β (C/EBPβ), an important early adipogenic transcription factor, by preventing the interaction of acidic nuclear phosphoprotein 32 (ANP32) with RNA-binding protein HuR and protein phosphatase 2A (PP2A) (Hyvönen et al. 2013). This study suggests that Spd can directly interact with acidic amino acid motif present in ANP32 protein(s), and that many other proteins containing similar acidic motifs could be modulated in a similar fashion by polyamines.

C-Methylated polyamine analogues in in vivo use

2-MePut as a tumor marker

One of the earliest reports on the use of methylated polyamines in vivo dates back to 1991. In the work by Moulinoux et al. (1991), healthy mice and mice with Lewis lung carcinoma xenografts were injected with 2-MePut. The compound was readily converted further to MeSpd and 6-MeSpm in vivo. MeSpd accumulated in the red blood cells of the tumor-bearing mice at levels which correlated with the tumor progression, whereas the analogue did not accumulate in the red blood cells of healthy mice. This finding indicates that 2-MePut could be used as a marker of tumor cell proliferation. Considerable accumulation of MeSpd was observed also in brain and liver of the mice both with and without tumor xenografts (Moulinoux et al. 1991). The clinical relevance of the suggestion that 2-MePut could potentially be useful as a tool in tumor diagnostics remains unproven.

Analogues without growth promoting properties: 5,8-Me_2Spd and 5,8-Me_2Spm as anticancer agents

The non-metabolizable dimethylated polyamine analogue 5,8-Me_2Spm was demonstrated to decrease cellular polyamine contents and exert an antiproliferative effect in cell culture (Holm et al. 1988). The compound inhibited polyamine synthesis through feedback regulation, but was not able to replace the polyamines in supporting growth. In a subsequent in vivo study, 5,8-Me_2Spm and the corresponding Spd derivative 5,8-Me_2Spd were used alone or in combination with DFMO to treat L1210 leukemic mice (Ask et al. 1993). Used alone, the polyamine analogues had a slight beneficial effect on the median survival time of the mice. Treatment of the leukemic mice with DFMO alone resulted in median survival of 14 days. Combined with DFMO, the Spm analogue, but not the Spd analogue, potentiated the therapeutic effect of DFMO by extending the median survival to 15.5 days and increasing the cure rate. The lack of more pronounced effect could partially relate to the finding that, contrary to the natural polyamines, the uptake of these analogues was not stimulated by DFMO in cell culture conditions (Ask et al. 1993).

Metabolism and tolerance of 1-MeSpd, 1-MeSpm and 1,12-Me_2Spm in vivo

Aiming at the use of 1-methylated analogues in animal experiments, we used transgenic rats overexpressing SSAT to investigate the metabolic stability of 1-MeSpd, 1-MeSpm and 1,12-Me_2Spm (Järvinen et al. 2005, 2006b). In these rats, any analogue susceptible to acetylation is readily metabolized in liver due to metallothionein (MT) promoter-driven expression of SSAT. We observed that all the analogues accumulated in liver: 1-MeSpd with the highest efficiency. While 1-MeSpd was not metabolized further, 1-MeSpm was converted to both 1-MeSpd and Spd (Järvinen et al. 2005, 2006b). 1,12-Me_2Spm was relatively stable, being only converted to 1-MeSpd, but to a much lesser extent than the monomethylated Spm analogue was. Obviously, the methylated Spm analogues served as substrates for APAO and SMO in vivo as they did in vitro (Järvinen et al. 2005).

The biological tolerance towards these three analogues was studied in SSAT transgenic mice and in their wild-type littermates (Järvinen et al. 2006b). The transgenic mice expressed the same MT–SSAT transgene as described above for the transgenic rats. The methylated analogues accumulated in a dose-dependent fashion in liver, pancreas and kidney of the mice of both genotypes. Hepatic Spd was markedly depleted in the analogue-treated mice, apparently as a result of SSAT induction. This was most notable in

wild-type and transgenic mice treated with 1-MeSpd, whereas 1,12-Me_2Spm did not seem to induce hepatic SSAT. In pancreas, 1-MeSpm apparently degraded to Spd derivatives in the wild-type mice, while in transgenic mice Spd was further catabolized to Put. The three analogues were well tolerated in the mice, and as judged by unaltered the plasma α-amylase (EC 3.2.1.1) and alanine aminotransferase (EC 2.6.1.2; ALAT) activities, no functional or structural damage occurred in pancreas and liver, respectively. The general tolerance was in the range of the tolerance of the natural polyamines in mice (Järvinen et al. 2006b). Similar to mice, tissue-specific differences in the stability of the methylated polyamines were also seen in MT–SSAT transgenic rats (Järvinen et al. 2006b). In addition to liver metabolism, 1-MeSpm was readily degraded in rat kidney. However, degradation of the Spm analogues was not detected in rat pancreas (Järvinen et al. 2006b). The increased metabolic stability and the acceptable tolerance of 1-MeSpd and 1,12-Me_2Spm clearly offer means to use these compounds to replenish and maintain depleted polyamine pools in a whole body setting, as described below in more detail.

Restoration of liver regeneration by 1-methylated polyamine analogues

The most traditional model to investigate the role of polyamines in rapid growth is rat liver regeneration after partial hepatectomy. Our studies with the MT–SSAT transgenic rats revealed the requirement of certain critical levels of Spd and/or Spm for proper initiation of liver regeneration (Alhonen et al. 2002). Induction of SSAT by partial hepatectomy depleted substantially hepatic Spd and Spm concentrations and delayed considerably the initiation of liver regeneration in the transgenic rats. To assess the growth-promoting properties of 1-methylated polyamines in vivo, we administered these compounds to the rats prior to partial hepatectomy (Räsänen et al. 2002; Järvinen et al. 2005). 1-MeSpd enhanced the postoperative SSAT activity further from the level seen in untreated rats. However, the analogue accumulated effectively in the transgenic liver and completely restored early liver regeneration as judged by liver weight gain and the number of proliferating cells (Räsänen et al. 2002). In the same experimental setting, 1,12-Me_2Spm readily accumulated in the transgenic liver and stimulated hepatic DNA synthesis close to the level seen with the effective dose of 1-MeSpd. Although the dimethylated Spm analogue was converted to 1-MeSpd to limited extent, the level of the latter alone was not high enough to be attributed to the stimulation of DNA synthesis (Järvinen et al. 2005). Thus, both 1-MeSpd and 1,12-Me_2Spm can be considered functional analogues that fulfil the requirement of the natural polyamines in the process of liver regeneration. In addition to racemic 1,12-Me_2Spm, the (*R*,*R*)- and (*S*,*S*)-diastereomers of the compound restored the delayed liver regeneration in the transgenic rats (Grigorenko et al. 2005). The diastereomers accumulated in the liver at similar quantities but their metabolism differed in the sense that only (*S*,*S*)-1,12-Me_2Spm was converted to MeSpd. Apparently, the stereo configuration of 1,12-Me_2Spm stereoisomers was not critical for the physiological functionality (Grigorenko et al. 2005).

Effect of 1-methylated polyamine analogues on the development of acute pancreatitis

Induction of polyamine catabolism in the MT–SSAT transgenic rats was followed by rapid and extensive depletion of spermidine and spermine resulting in acute necrotizing pancreatitis (Alhonen et al. 2000). This initial finding suggested that polyamines are essential for pancreatic integrity. In the presence of high SSAT activity, natural polyamines are, however, not the way to replenish the depleted polyamine pools. Therefore, we pretreated the rats with the metabolically stable 1-MeSpd. Along with effective accumulation in pancreas, the analogue prevented the development of pancreatitis completely in the transgenic rats with induced SSAT despite the depletion of the natural polyamines (Räsänen et al. 2002). When 1-MeSpd or 1,12-Me_2Spm was administered after the induction of pancreatitis, less tissue damage was seen in the analogue-treated animals when compared with the untreated ones (Hyvönen et al. 2006a). The beneficial effect of 1,12-Me_2Spm is attributable to the parent compound itself and to its oxidation product 1-MeSpd found in the pancreatic tissue. Both (*R*,*R*)- and (*S*,*S*)- diastereomers of 1,12-Me_2Spm alleviated the development of pancreatitis as judged by the reduction of plasma α-amylase activity in the rats after the induction of the disease but similar to liver metabolism, only (*S*,*S*)-1,12-Me_2Spm was converted to MeSpd in the pancreas (Grigorenko et al. 2005). As our transgenic model is a lethal model of acute pancreatitis, the most dramatic effect exerted by the Spd and Spm analogues was long-term prevention of pancreatitis-associated mortality irrespective of partial early pancreatic damage (Hyvönen et al. 2006a).

Acute pancreatitis in general is associated with premature trypsinogen activation, inflammation, oxidative stress and, in severe cases, multi organ damage leading to death. With the aid of 1-methylated polyamine analogues, we were able to demonstrate that the mechanism of polyamine catabolism-induced acute pancreatitis also included trypsinogen activation (Hyvönen et al. 2006a; Merentie et al. 2007). Activation of pancreatic lysosomal hydrolase cathepsin B, a known activator of trypsinogen, was seen as early as 2 h after induction of pancreatitis in MT–SSAT

transgenic rats. Pretreatment of the rats with 1,12-Me_2Spm mostly prevented cathepsin B activation as well as the activation of pancreatic trypsin (Hyvönen et al. 2007b). Early signs of disease development were changes in tissue ultrastructure, including dilatation of rough endoplasmic reticulum and appearance of autophagosomes (Hyvönen et al. 2007b). The levels of typical inflammatory mediators, such as TNF-α and IL-6, were also found to be elevated in polyamine catabolism-induced acute pancreatitis, and the increases were prevented by pretreatment with 1,12-Me_2Spm (Merentie et al. 2007). Oxidative stress, on the other hand, appeared to be less clearly involved in the disease development, although indicative changes in lipid peroxidation and superoxide dismutase activity—both affected by analogue pretreatment—were observed (Merentie et al. 2007). The most clear-cut therapeutic effect of 1-MeSpd was found to be linked to its potency to prevent the development of intravascular coagulopathy that causes lethal systemic complications in rats with induced pancreatitis (Hyvönen et al. 2010b). Apart from pancreatic destruction, the rats exhibited symptoms of liver and kidney damage which could be alleviated by treatment with 1-MeSpd. In addition, coagulopathy that was induced in the transgenic rats by another mechanism, namely lipopolysaccharide, could likewise be ameliorated with 1,12-Me_2Spm pretreatment, strengthening the view that polyamines play a critical role in hemostasis (Hyvönen et al. 2010b).

Since our rats with transgene-induced SSAT activation represent an extreme model of polyamine catabolism-mediated pancreatitis, it was of interest to explore other pancreatitis models to see if activation of polyamine catabolism was associated with their mechanism of induction. Indeed, polyamine depletion was seen in arginine- and cerulein-induced pancreatitis in wild-type rats, but the favorable effect of 1-MeSpd pretreatment could only be seen in the former model (Hyvönen et al. 2006a). Treatment of rats with taurodeoxycholate induced severe necrotizing pancreatitis with partial depletion of Spd and Spm as a result of SSAT activation (Jin et al. 2008). 1,12-Me_2Spm had a transient beneficial effect, but was not able to prevent tissue damage when pancreatitis progressed (Jin et al. 2008, 2011). In this model, activated polyamine catabolism was clearly not a preceding event to trypsin activation and emerged together with development of necrosis. Although the latter was alleviated with 1,12-Me_2Spm, the former was not (Jin et al. 2011). The analogue was unable to ameliorate multi organ injury or prevent mortality caused by taurodeoxycholate but rather seemed to cause renal toxicity at the dose used to treat the rats (Jin et al. 2011). Yet another model, namely acute pancreatitis caused by L-ornithine, manifested SSAT activation and Spd depletion but only after onset of acute pancreatitis (Biczo et al. 2010). Hence, it was not surprising that pretreatment or treatment with 1-MeSpd had no effect on the outcome, despite the obvious accumulation of the analogue in pancreas (Biczo et al. 2010). Taking these findings together, activation of polyamine depletion is associated with development of acute pancreatitis but its impact in the initiation and progression of the disease depends on the trigger of the pathogenic process. It is noteworthy that human tissue samples from pancreatitis patients had depleted polyamine levels (Hyvönen et al. 2006a) but the relation of this finding to the development of human pancreatitis remains to be seen.

Other animal studies with 1-MeSpd

The protective and growth supportive functions of 1-MeSpd in vivo were observed in two additional experimental systems. Carbon tetrachloride (CCl_4) caused moderate hepatic damage in wild-type rats and severe hepatic and pancreatic damage in MT–SSAT transgenic rats in response to activation of polyamine catabolism in these tissues (Hyvönen et al. 2010a). Pretreatment of the rats with 1-MeSpd reduced liver damage in the wild-type animals and prevented both hepatic and pancreatic injury in the transgenic rats. The analogue clearly promoted tissue regeneration, manifested as an increased number of regenerating hepatocytes in the rats (Hyvönen et al. 2010a).

Polyamines have been also shown to play a critical role in the growth of skin and hair follicles. In mouse, the resting, telogen-phase hair follicles with low polyamine levels could be pushed to enter the growth phase with a cutaneous application of 1-MeSpd (Fashe et al. 2010). The natural epidermal spermidine pool was partially replaced by the analogue and, with overall elevated polyamine pools, the application site in skin expressed features of anagen, the rapid growth phase of hair cycle (Fashe et al. 2010). The findings emphasize the role of adequate polyamine pools in normal hair cycle regulation and suggest that stable polyamine analogues might be used to treat hair loss.

Novel polyamine targets to study with the aid of C-methylated analogues

Methylated polyamine analogues, especially those that are metabolically stable, allow the structure–activity studies of various polyamine metabolism-related physiological and pathophysiological processes. It has been shown that viral infections disturb polyamine metabolism and some viruses adopt a part of polyamine metabolism to support their replication (Hyvönen et al. 2006b; Kaiser 2012). Furthermore, some microbes promote their secondary pathological effects and escape host defence system by disturbing

polyamine metabolism (Chaturvedi et al. 2012). Likewise, microbes can acquire resistance towards antibiotics by protective polyamine biofilm production (Shah and Swiatlo 2008). Polyamine analogues may offer practical therapeutic possibilities against viral/microbial diseases or in alleviating their secondary pathological processes (Korovina et al. 2012). The differences in the host and pathogen polyamine metabolism are especially of interest, because compounds interfering only with the pathogen polyamine metabolism are supposedly less toxic to the host (Mandal et al. 2011).

Pancreatic beta cell damage causes diabetes and consequently peripheral organ damage after several years of deregulated glucose metabolism. Interestingly, the inflammatory response is alleviated and beta cells are protected by inhibiting the formation of hypusinated eIF5A in streptozotocin-induced diabetes model (Kaiser 2012; Maier et al. 2010). Selective binding of (*S*)-1-MeSpd over (*R*)-1-MeSpd by DHS might be exploitable in several experimental settings (Hyvönen et al. 2007a).

Practically, most insults that induce inflammatory response affect the regulation of polyamine metabolism. In cancer, deregulated polyamine metabolism may affect the generation of extracellular matrix (protein glycosylation, heparan sulphate production) to support tumor progression and metastasis, and to promote the escape from host immune defence (Kaiser 2012). In addition, increased circulating polyamine and copper levels are also associated with cancer. Recently, Spd has been also linked to longevity via activation of autophagy in several experimental models (Eisenberg et al. 2009).

The regulation of cell energy metabolism and possible metabolic reprogramming by the analogue treatment is especially of interest. Furthermore, the maintenance of cell organelles (especially mitochondria), cytoskeleton architecture, protein glycosylation (including regulation of autophagy) and alterations in acetyl- and phosphoproteome equilibrium are subjects of further studies.

Polyamine analogue development and testing

Investigation of the metabolism and interactions of polyamines with the aid of structural mimetics and synthesis inhibitors have been active for almost half a century. These studies have provided invaluable information for polyamine research, but many old analogues/inhibitors have unfortunately been forgotten or are no more available. Thus, currently available data are mostly limited to metabolic enzymes and regulatory pathways known at the time when these drugs were initially synthesized and tested. However, further testing of these analogues with the currently available methods and currently known metabolic pathways may reveal important information. These analogues include antimetabolites, such as *N*-alkylated polyamine analogues, charge isosteric polyamine analogues, analogues having variable-length carbon chains and variable amine content to alter the spatial arrangement of charged amines, and unsaturated polyamine analogues (Seiler 2003a, b; Casero and Marton 2007).

Among C-methylated polyamine analogues, some methylated Put derivatives (e.g. 1,4-Me_2Put, including its enantiomers) should be studied further. Moreover, novel spermine analogues with 2, 3 or 5 (and/or 8, 10, 11) methyl-modified sites are needed to study their effects on polyamine metabolism. The effect of larger groups, e.g. ethyl instead of methyl to increase lipophilicity and steric hindrance, or hydrophilic (-OH) as substituents, should also be studied systematically in the future. Methyl substitution of hydrogen is well-known modification in drug development projects, affecting greatly the ADME properties of given drug (Barreiro et al. 2011). So far, there are not many examples of substituting hydrogen with methyl in aliphatic carbon chain in drugs without cyclic or heterocyclic structures. The same principles seem to be valid for C-methylated polyamine analogues, thus strongly encouraging their further development (Barreiro et al. 2011). It is obvious that we need also parallel computer modelling experiments to fit obtained structure–activity data with known target structures.

Conclusions

Increasing knowledge of the cellular functions of polyamines and the importance of their metabolism for cellular physiology maintain continuous need for novel polyamine structural mimetics having specific metabolic characteristics. Structure-specific methylation offers various possibilities to alter the behaviour of the parent polyamine analogue in cellular metabolism and its biological target sites (Bachrach 2005).

In general, gem-dimethylation renders the analogue metabolically more stable as compared to monomethylated polyamine analogue. None of the tested gem-dimethylated Spd analogues are metabolized to corresponding Me_2Spm derivative in SV-3T3 cells, while all monomethylated analogues except 3-MeSpd are metabolized to MeSpm derivative. Mono- and gem-dimethylation at 1- or 3-position of Spd prevents SSAT-mediated N^1-acetylation of the analogue; whereas gem-dimethylation and monomethylation at 8 position of Spd accelerates acetylation velocity as compared to Spd. 1-Methylation or 1,1-gem-dimethylation of carbon next to primary amine group prevent oxidative terminal deamination. APAO and SMO, which cleave polyamines at N^4-exo site (C_3–N_4 bond) are highly influenced by the presence and the chirality of methyl-substituent(s) at 1 (and 12) position. The (*S*,*S*)-diastereomer of 1,12-Me_2Spm is a substrate of SMO and

thus can be used to assay its activity, whereas the (*R*,*R*)-diastereomer is metabolically stable competitive inhibitor. Among the reviewed analogues, 2-MeSpd does not significantly downregulate ODC through induction of OAZ, and therefore it can be used as a tool to study the molecular mechanism of OAZ frameshifting process. 1-, 2-, and 3-MeSpd are substrates for DHS, however, 3-MeSpd do not produce functional eIF5A in cell cultures. 1,1- and 8,8-Me_2Spd (8-MeSpd) act as inhibitors of DHS. In addition, DHS shows stereospecificity, strongly preferring the (*S*)-1-MeSpd and (*R*)-3-MeSpd. These chiral differences can be exploited to differentiate between hypusine-dependent and independent cellular processes. Thus, in polyamine metabolizing or catabolizing enzymes, the site of enzyme action (including binding amine[s]) i.e. adjacent carbons are most prone to Me substitution.

In conclusion, polyamine metabolism and interactions can be selectively modulated by analogues where hydrogen(s) of the carbon backbone have been substituted with methyl group(s). Replacement of the intracellular natural polyamines with their metabolically stable analogues offers a sophisticated way to investigate the functions, molecular mechanisms and novel targets of the individual polyamines, and facilitates their structure–activity studies. 1-MeSpd, 1-MeSpm and 1,12-Me_2Spm and their stereoisomers have already proved to be in vivo tolerable and feasible surrogates of natural polyamines in animal experiments. In the future, this class of compounds may be of therapeutic value in the treatment of parasitic diseases, cancer or other polyamine metabolism-related disorders.

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