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SEBAHAT UCAL

POLYAMINE ANALOGUES AS ANTICANCER AGENTS

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Polyamine Analogues as Anticancer Agents

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ABSTRACT

Polyamines are low molecular weight aliphatic polycations, found in all kingdoms of life. At physiological pH, they are positively charged, enabling them to interact with negatively charged cellular constituents, such as nucleic acids, phospholipids and some acidic protein motifs. It is recognized that polyamines play an important role in fundamental cellular processes such as replication, transcription, translation, cell proliferation and differentiation. Moreover, polyamine metabolism is often dysregulated in cancer, thus it has been, and still is, an attractive target for the development of anticancer drugs. The main focus of this thesis was to expand our knowledge in polyamine analogues as potential anticancer agents and to clarify their effects on polyamine metabolism in detail.

This thesis comprises of three original studies. In the first study, we developed a simple and straightforward synthesis of polyamine analogues such as diacetylated polyamines, N^1,N^8-diAcSpd and N^1,N^{12}-diAcSpm, which are known as early stage cancer biomarkers. In the second study, the effects of the anticancer agent, triethylenetetramine (TETA), on polyamine metabolism were investigated. Biological assays with TETA were performed to evaluate its effects on cell proliferation, polyamine metabolism and uptake in comparison to other Cu(II) chelators, D-penicillamine and tetrathiomolybdate in DU145 prostate cancer cells. The results of this study revealed that TETA is a multitargeting drug and its anticancer effect is not only attributable to its property as a selective Cu(II) chelator but also due to its significant effects on polyamine and energy metabolism.

In the third study, the catabolic pathways of *N*-alkylated polyamine analogues, *N,N'*-bis-(3-ethylaminopropyl)butane-1,4-diamine (DESpm), *N*-(3-benzyl-aminopropyl)-*N*'-(3-ethylaminopropyl)butane-1,4-diamine (BnEtSpm), *N,N'*-bis-(3-benzylaminopropyl)-butane-1,4-diamine (DBSpm) and their variably deuterated counterparts were tested *in vitro* with recombinant enzymes. Deuteration retarded the total reaction rate and changed the preferred cleavage site of both enzymes participating in polyamine catabolism i.e. spermine oxidase (SMO) and acetylpolyamine oxidase (APAO). BnEtSpm was found to be the most cytotoxic of the evaluated analogues in the tested cancer cell lines, whereas in mouse embryonic fibroblasts, DBSpm exhibited the highest cytotoxicity. Our findings showed that the analogues' antiproliferative efficacies correlated with the induction of SMO. As a result of this study, we undertook targeted polyamine analogue deuteration to demonstrate that the kinetic isotope effect could be applied to redirect analogue catabolism to SMO and APAO. Moreover, total hydrogen peroxide generation by both catabolic enzymes was decreased with the deuterated analogues as compared to the parent non-deuterated analogues. Unexpectedly, their efficacies remained almost the same regardless of deuteration of the analogue, indicating that analogue catabolism plays only a minor role in their antiproliferative action in those cell lines where basal APAO and SMO activities are low.

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Medical Subject Headings: Polyamines; Antineoplastic Agents; Cell Proliferation/drug effects; Metabolism

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TIIVISTELMÄ

Polyamiinit ovat lyhyitä suoraketjuisia polykationeita, joita esiintyy kaikissa eliökunnissa. Ne ovat fysiologisessa pH:ssa positiivisesti varautuneita, joten ne vuorovaikuttavat negatiivisesti varautuneiden solun komponenttien, kuten nukleiinihappojen, fosfolipidien ja proteiinien happamien aminohappojaksojen kanssa. Polyamiineilla on tärkeä merkitys useissa solun toiminnoissa, kuten DNA:n replikaatiossa, transkriptiossa, translaatiossa sekä solun jakautumisessa ja erilaistumisessa. Koska polyamiiniaineenvaihdunnan häiriöt ovat tyypillisiä monille syöpätyypeille, polyamiiniaineenvaihdunta on ollut ja on edelleen aktiivinen syöpälääkkeiden kehityskohde. Tämän tutkimuksen tarkoitus oli lisätä tietoamme erilaisten polyamiinijohdosten käytöstä kokeellisina syöpälääkkeinä ja selvittää niiden vaikutusmekanismeja.

Tämä väitöskirja koostuu kolmesta osajulkaisusta. Ensimmäisessä osajulkaisussa kehitimme yksinkertaisen ja suoraviivaisen menetelmän polyamiinijohdosten, kuten diasetyloitujen polyamiinien synteesiin, joista N^1,N^8-diasetyylispermidiini and N^1,N^{12}-diasetyylispermiini ovat tunnettuja syövän biomarkkereita. Toisessa osajulkaisussa vertasimme polyamiinijohdos trietyleenitetra-amiinin (TETA) vaikutuksia polyamiiniaineenvaihduntaan, solunjakautumiseen ja polyamiinien sisäänottoon kahden muun kupari(II) kelaattorin, D-penisillamiinin ja tetratiomolybdaatin kanssa DU145 eturauhassyöpäsoluissa. Tulosten perusteella TETA:n solunjakautumista estävä vaikutus ei perustunut ainoastaan sen kykyyn kelatoida kuparia(II), vaan myös sen kykyyn vaikuttaa merkittävästi polyamiini- ja energia-aineenvaihduntaan.

Kolmannessa osajulkaisussa selvitimme *N*-alkyloitujen polyamiinijohdosten, *N*,*N′*-bis-(3-etyyliaminopropyyli)butaani-1,4-diamiini (DESpm), *N*-(3-bentsyyli-aminopropyyli)-*N*'-(3-etyylaminopropyyli)butaani-1,4-diamiini (BnEtSpm), *N*,*N′*-bis-(3-bentsyyli-aminopropyyli)-butaani-1,4-diamiini (DBSpm) sekä näiden selektiivisesti deuteroitujen muotojen kataboliaa rekombinanttientsyymien avulla. Deuteraatio hidasti sekä spermiinioksidaasin (SMO) että asetyylipolyamiinioksidaasin (APAO) reaktionopeutta ja muutti johdosten primääristä pilkkoutumiskohtaa. BnEtSpm oli sytotoksisin yhdiste kaikissa testatuissa syöpäsolulinjoissa, kun taas hiiren sikiön fibroblasteissa sytotoksisin yhdiste oli DBSpm. Tulostemme perusteella polyamiinijohdosten sytotoksisuus korreloi SMO-aktiivisuuden induktion kanssa. Tutkimuksemme osoittaa, että *N*-alkyloitujen polyamiinijohdosten kohdennettu deuteraatio mahdollisti johdoksen SMO- ja APAO-välitteisen katabolian muuttamisen. Deuteraatio ei kuitenkaan merkittävästi vaikuttanut johdosten sytotoksisuuten tutkituissa solulinjoissa, mikä osoittaa, että *N*-alkyloitujen polyamiinijohdosten katabolialla ei ole suurta merkitystä niiden sytotoksisuudessa, ainakaan niissä solulinjoissa, joissa APAO- ja SMO-aktiivuudet ovat matalat.

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Yleinen suomalainen asiasanasto: polyamiinit; syöpähoidot; solunjakautuminen; lääkeaineet; aineenvaihdunta

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Kuopio, September 2017
Sebahat Ucal

List of the Original Publications

This dissertation is based on the following original publications:

I Ucal S, Khomutov A R, Häkkinen M R, Turhanen P A, Vepsäläinen J, Weisell J. Selective acetylation of primary amino groups with phenyl acetate: simple conversion of polyamines into *N,N′*-diacetyl derivatives. *Arkivoc* 7: 42-49, 2015.

II Hyvönen M T, Ucal S, Pasanen M, Peräniemi S, Weisell J, Khomutov M, Khomutov A R, Vepsäläinen J, Alhonen L, Keinänen T A. Triethylenetetramine modulates polyamine and energy metabolism and inhibits cancer cell proliferation. *Biochemical Journal* 473: 1433-1441, 2016.

III Ucal S*, Häkkinen M*, Alanne A-L, Alhonen L, Vepsäläinen J, Keinänen T A, Hyvönen M T. Controlling of *N*-alkylpolyamine analog catabolism by selective deuteration. Manuscript *submitted* *, equal contribution

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Abbreviations

ABC	ATP-binding cassette
ACN	Acetonitrile
AdoDATAD	S-adenosyl-1,12-diamino-3-thio-9-azadodecane
AdoDATO	S-adenosyl-1,8-diamino-3-thio-octane
AdoMetDC	S-adenosyl-L-methionine decarboxylase
AG	Aminoguanidine
APAO	Acetylpolyamine oxidase
APCHA	*N*-(3-aminopropyl)cyclohexylamine
Arg	L-arginine
ATCC	American Type Culture Collection
AZ	Antizyme
AZI	Antizyme inhibitor
BEHSpm	N^1,N^{14}-di(ethyl)-homospermine
BnEtSpm	*N*-(3-benzyl-aminopropyl)-*N*'-(3-ethylaminopropyl)butane-1,4-diamine
BSAO	Bovine serum amine oxidase
CGP 39937	[2,2-bipyridine]-6-6′dicarboximidamide
CGP 48664	4-amidinoindan-1-one 2′-amidinohydrazone
CHENSpm	N^1-cycloheptylmethyl-N^{11}-ethylnorspermine
CPENSpm	N^1-cyclopropyl-methyl-N^{11}-ethylnorspermine
DAO	Diamine oxidase (AOC1)
DBSpm	*N,N′*-bis-(3-benzylaminopropyl)butane-1,4-diamine
dcAdoMet	Decarboxylated AdoMet
DCFDA	2'-7-dichlorofluorescein diacetate
DENSpm	N^1,N^{11}-di(ethyl)-norspermine; BENSpm
DESpm	*N,N′*-bis(3-ethylaminopropyl)butane-1,4-diamine;N^1,N^{12}-di(ethyl)Spm; BESpm
DFMO	α-Difluoromethylornithine
DHS	Deoxyhypusine synthase

DOHH	Deoxyhypusine hydroxylase
D-pen	D-penicillamine
eIF5A	Eukaryotic translation initiation factor 5A
ESI	Electrospray ionization
FAD	Flavin adenine dinucleotide
FBS/FCS	Fetal bovine/calf serum
GABA	γ-Aminobutyric acid
GC	Gas chromatography
HPLC	High-performance liquid chromatography
IEF	Isoelectric focusing
IPENSpm	(S)-N^1-(2-methyl-1-butyl)-N^{11}-ethyl-4,8-diazaundecane
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
MAO	Monoamine oxidase
MAT	Methionine adenosyltransferase
MDL 72527	N^1,N^4-bis(2, 3-butadienyl)-1, 4-butanediamine
Met	L-methionine
MGBG	Methylglyoxal bis(guanylhydrazone)
MS	Mass spectrometry
ODC	Ornithine decarboxylase
ORF	Open reading frame
Orn	L-ornithine
PENSpm	N^1-propargyl-N^{11}-ethyl-norspermine
Put	Putrescine
PVDF	Polyvinylidene fluoride
ROS	Reactive oxygen species
SC	Semicarbazide
SLC	Solute carrier
SMO	Spermine oxidase
Spd	Spermidine
SpdSy	Spermidine synthase
Spm	Spermine

SpmSy	Spermine synthase
SpmTrien	1,12-diamino-3,6,9-triazadodecane
SSAO	Semicarbazide-sensitive amine oxidase (VAP-1/AOC3)
SSAT	Spermidine/spermine-N^1-acetyltransferase
SSAT2	Spermidine/spermine-N^1-acetyltransferase 2 (TLAT)
TETA	Triethylenetetramine;*N*,*N'*-bis(2-aminoethyl)ethane-1,2-diamine(1,8-diamino-3,6-diazaoctane; trien; trientine; Syprine®
TLAT	Thialysine N^{ε}-acetyltransferase
TLC	Thin-layer chromatography
TTM	Tetrathiomolybdate
uORF	Upstream open reading frame
VAP-1/AOC3	Semicarbazide-sensitive amine oxidase (SSAO)
3-AAP	3-acetamidopropanal
3-AP	3-aminopropanol
4-AB	4-aminobutanal
4MCHA	Trans-4-methylcyclohexylamine

1 Introduction

Ever since the initial discovery of spermine (Spm) from human semen in 1678 by Antonie van Leeuwenhoek (Leuwenhoek 1678), the polyamines have been studied extensively. The two naturally occurring polyamines, spermidine (Spd) and Spm, as well as their precursor putrescine (Put), are important molecules and essential in mammalian cell proliferation, differentiation and tissue integrity (Pegg 2009).

Polyamines are cations at physiological pH, they interact with negatively charged molecules such as DNA, RNA, phospholipids and anionic protein motifs, and thus participate in many functions in cells such as the regulation of DNA replication, transcription, translation, protein stability, functioning of various ion channels and receptors. They are synthesized *de novo* in practically all living cells or obtained from diet or produced by intestinal microbiota.

Intracellular polyamine levels are stringently regulated via polyamine biosynthesis and catabolism. They are also controlled by polyamine transport into cells, a process that is still poorly understood in mammalian cells (Nowotarski et al. 2013) though rather well characterized in *E.coli* and yeast (Igarashi and Kashiwagi 1999). High polyamine levels have been associated with various cancer types such as breast, colon, lung, prostate, and skin cancers. Additionally, in comparison to normal cells, altered levels of polyamine biosynthetic enzymes such as ornithine decarboxylase (ODC) and S-adenosyl-L-methionine decarboxylase (AdoMetDC), and the catabolic enzymes such as spermidine/spermine-N^1-acetyltransferase (SSAT), spermine oxidase (SMO) and acetylpolyamine oxidase (APAO) have been observed in cancer cells. Based on these observations and the necessity of polyamines for cell proliferation, the polyamine metabolic pathway has been viewed as an attractive target for the development of anticancer agents.

Many specific inhibitors that target enzymes of polyamine metabolic pathway have been synthesized and tested (Nowotarski et al. 2013). Among them, α-difluromethylornithine (DFMO), an irreversible enzyme inhibitor of ODC, has been the most widely studied and successful single enzyme inhibitor. Currently DFMO is in clinical use against African sleeping disease. Furthermore, it has been tested in a number of clinical trials against various cancers as a single drug and in combination with other agents or with polyamine-free diet. The most recent and important clinical trials have proved that DFMO significantly increases the survival of patients with pediatric neuroblastoma (Sholler et al. 2015).

The development of *N*-alkylated polyamine analogues as anticancer drugs goes back to the late 1980s (Casero and Woster 2001). These compounds can be classified into symmetrically and unsymmetrically substituted polyamine analogues. *N*-alkylated polyamine analogues structurally resemble the natural polyamines, and thus they are taken into the cells via the active polyamine transport system (Porter et al. 1991). Some of these analogues exhibit strong polyamine antimetabolite features, inhibit polyamine uptake and suppress their biosynthesis, and induce catabolism and efflux, resulting in almost complete depletion of all the three polyamines and eventually to cell death (Casero and Marton 2007). The names and abbreviations of polyamines in this thesis are based on the common names used in biochemistry, but IUPAC names are also shown when necessary.

Another class of polyamine analogues displaying anticancer effects is the charge-deficient isosteric polyamine analogues, such as aminooxy, aminooxa and fluorinated analogues, and analogues with an altered carbon chain length between amino groups (Weisell et al. 2014). This group of analogues consists of an important class of polyamine analogues, for example 1,12-diamino-3,6,9-triazadodecane (SpmTrien) and triethylenetetramine (TETA; 1,8-diamino-3,6-diazaoctane; trien; trientine; Syprine®). Each protonation of an amino group lowers the pK_a value of the next amino group to be protonated with the effect becoming stronger the closer are the amino groups. Thus, SpmTrien (pK_a values; 10.3, 9.5, 8.5, 6.3 and 3.3) differs from TETA (pK_a

values; 9.74, 9.07, 6.56 and 3.27); at pH 7.4, TETA carries a net charge of two (+2) whereas for SpmTrien it is three (+3), (Weisell et al. 2010a). Since it is an efficient Cu(II) chelator, TETA is used nowadays as a primary medication for the Wilson's disease (a genetic disease in which there is copper overaccumulation) (Walshe 1969, Dixon et al. 1972). More recently, TETA has attracted attention as a potential anticancer agent and it has shown efficacy against diabetic organ damage (Lu 2010). However, there have been few clinical trials conducted evaluating TETA as an anticancer drug. Clearly, a better understanding of TETA's pharmacology, its action as a polyamine analogue and its effects on the polyamine metabolism would be beneficial in order to take advantage of possible benefits of TETA in clinical cancer chemotherapy.

In this study, our aim was to expand our knowledge in polyamine analogues as potential anticancer drugs and to investigate their roles on polyamine metabolism in detail.

2 Review of the Literature

2.1 STRUCTURE AND PROPERTIES OF POLYAMINES

Polyamines are small polycationic organic molecules containing two or more amino ($-NH_2$) groups, which are linked to each other via hydrophobic carbon chains. They are found in all living organisms (Jänne et al. 2005). The two main mammalian naturally occurring polyamines are spermine (1,12-diamino-4,9-diazadodecane, Spm, a tetraamine), spermidine (1,8-diamino-4-azaoctane, Spd, a triamine), and their precursor diamine putrescine (1,4-diaminobutane, Put, a diamine). The chemical structures of Put, Spd, Spm and the pK_a values of primary and secondary amino groups are shown in Figure 1. Polyamines are essential for eukaryotic cell growth, survival, proliferation, differentiation and many other important functions (Casero and Marton 2007, Casero and Pegg 2009).

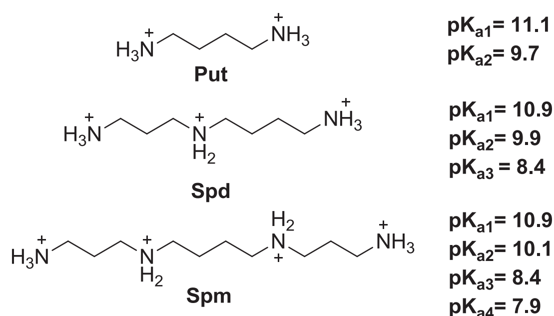

Figure 1. Chemical structures of naturally occurring polyamines Spd, Spm and their precursor, Put in their cationic forms and the pK_a values for each of their nitrogens (Bencini et al. 1999, Woster and Casero 2011).

Under normal physiological pH conditions, polyamines are protonated on each nitrogen atom (Bencini et al. 1999). Due to their pK_a values, Put is a divalent cation i.e. it has a charge of 2+, whereas Spd (3+) is trivalent and Spm (4+) is tetravalent (Figure 1). However, the charge on the polyamines is distributed along the entire length of the carbon chain, i.e. along the polyamine backbone, making the polyamines unique and differentiating them from the point charges of the cellular bivalent cations, such as inorganic cations, Mg^{2+}, Ca^{2+} and Zn^{2+} (Wallace et al. 2003). Owing to their positively charged property, they can interact not only with many negatively-charged biological macromolecules such as DNA, RNA, but also with acidic phospholipids and acidic protein motifs (Jänne et al. 2004).

2.1.1 Polyamine sources

In mammals, the polyamines originate from different sources; endogenous biosynthesis or exogenous supply from food and intestinal microorganisms. Polyamines can be synthesized from amino acids, L-ornithine, and L-methionine, and extracellular polyamines can be taken up into cells. The polyamines are detectable in normal cells but they are present at higher levels in cancer cells. The polyamine content varies in foods and beverages. Fruits and cheese are highest sources of Put, while vegetables and meat products are rich in Spd and Spm (Ali et al. 2011). Additionally, a large amount of polyamines is found in human breast milk - this is the main source of exogenous polyamines for the newborn (Plaza-Zamora et al. 2013).

2.2 POLYAMINE BIOSYNTHESIS

In mammals, the intracellular polyamine levels are tightly controlled by the combination of their biosynthesis, catabolism and active transport system at the cell membrane (Pegg 2009). The current view of mammalian polyamine biosynthesis and catabolism, transport system is shown in Figure 2.

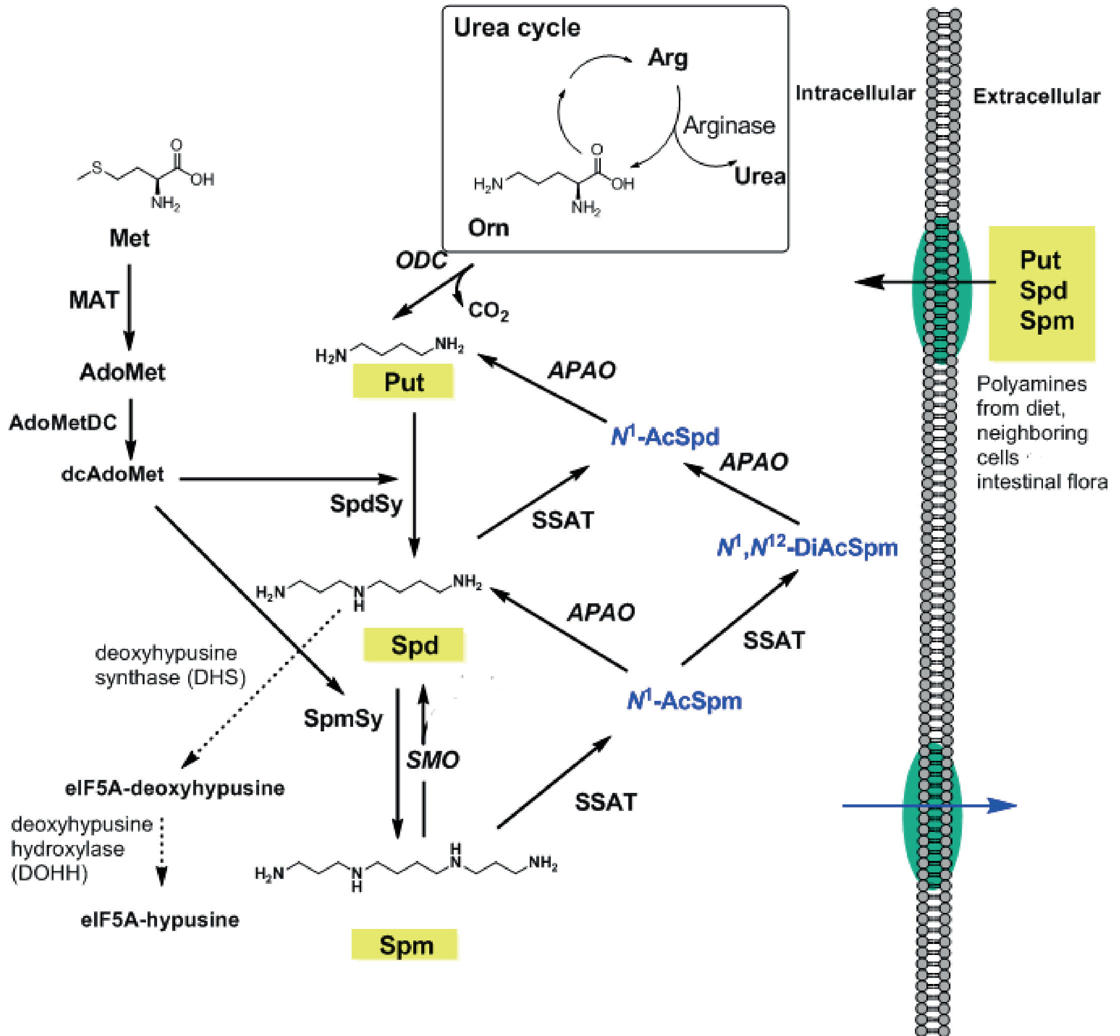

Figure 2. The main pathways of mammalian polyamine metabolism; polyamine biosynthesis, catabolism and eIF5A synthesis from Spd.

The polyamine biosynthetic enzymes are ornithine decarboxylase (ODC; EC 4.1.1.17), S-adenosyl-L-methionine decarboxylase (AdoMetDC; EC 4.1.1.50), spermidine synthase (SpdSy; EC 2.5.1.16) and spermine synthase (SpmSy; EC 2.5.1.22). Of these biosynthetic enzymes, both ODC and AdoMetDC are highly regulated at the transcriptional and post-transcriptional levels. The crystal structures of mammalian ODC (Almrud et al. 2000), AdoMetDC (Tolbert et al. 2001), SpdSy (Wu et al. 2007) and SpmSy (Wu et al. 2008) have been published; these provide very valuable information on their substrate-binding properties, catalytic mechanism and regulation.

Polyamines are synthesized from two amino acids, L-arginine (Arg) and L-methionine (Met) (Tabor and Tabor 1984). During the urea cycle, Arg is converted into L-ornithine (Orn), by the mitochondrial enzyme, arginase. Orn can then either be used in polyamine metabolism, or converted further in the urea cycle into citrulline. Put is produced by decarboxylation of Orn, a reaction catalyzed by ODC in polyamine biosynthesis. This decarboxylation reaction is considered as the first rate-limiting step in polyamine biosynthesis.

Another polyamine precursor amino acid, Met, is initially converted into S-adenosyl-L-methionine (AdoMet) by methionine adenosyltransferase (MAT), and further via the decarboxylation of AdoMet by AdoMetDC, the second rate limiting enzyme, producing decarboxylated S-adenosyl-L-methionine (dcAdoMet) to be utilized in polyamine biosynthesis. DcAdoMet is used as the aminopropyl donor by SpdSy and SpmSy to produce the higher polyamines, Spd and Spm, respectively.

The polyamine Spd is the only known natural precursor of hypusine, a crucial component of active eukaryotic translation initiation factor 5A (eIF5A) protein, which is absolutely required in cell proliferation. eIF5A is the only protein containing the unique amino acid, hypusine, which is derived from Spd by the subsequent action of deoxyhypusine synthase (DHS) and deoxyhypusine hydroxylase (DOHH) (Figure 2) (Park et al. 1997, Chen and Liu 1997). Spd is essential for the hypusination of eIF5A, but in conditions of Spd deprivation before the levels hypusinated-eIF5A are decreased, Spd and Spm can substitute for each other's functions in their macromolecular interactions. eIF5A has a long half-life, over a week in some cell lines (Bergeron et al. 1998), and therefore it takes some days *in vitro* to obtain depletion of hypusinated eIF5A when Spd is depleted. Thus, both eIF5A and polyamines affect the eukaryotic cell growth independently (Nishimura et al. 2005).

2.2.1 Ornithine Decarboxylase

The pyridoxal phosphate-dependent decarboxylase, ODC enzyme was first discovered in 1968 (Pegg and Williams-Ashman 1968, Russell and Snyder 1968). ODC has an extremely short half-life, about 10-30 min (Davis et al. 1992). ODC has an important role since it is the first rate-controlling enzyme in polyamine biosynthesis (Pegg and McCann 1982), thus it has been studied extensively (McCann and Pegg 1992). The regulation of ODC levels occurs at the transcriptional, translational and protein stability levels (Pegg 2006).

The ODC homodimer catalyzes the decarboxylation of L-Orn to Put, which is then converted into the higher polyamines. The stability of ODC enzyme is regulated by a small protein called antizyme (AZ) which is synthesized from a short open reading frame (ORF)1 and a long ORF2 by a +1 frameshifting event, as shown in Figure 3 (Hayashi and Murakami 1995). This frameshifting event is induced by polyamines, and as a result, the synthesis of AZ is increased when the polyamine levels increase. AZ binds tightly to ODC to form the ODC-AZ complex, which inhibits ODC activity and targets the complex to degradation by the 26 S proteasome without ubiquitination (Figure 3) (Pegg 2006). In addition to stimulating AZ synthesis, polyamines also inhibit AZ degradation, at least in yeasts. Polyamines regulate their synthesis by inducing the expression and blocking the degradation of AZ (Palanimurugan et al. 2004). Importantly, AZ not only promotes ODC degradation but also inhibits polyamine uptake and stimulates their efflux by some still unknown mechanism (Kahana 2009).

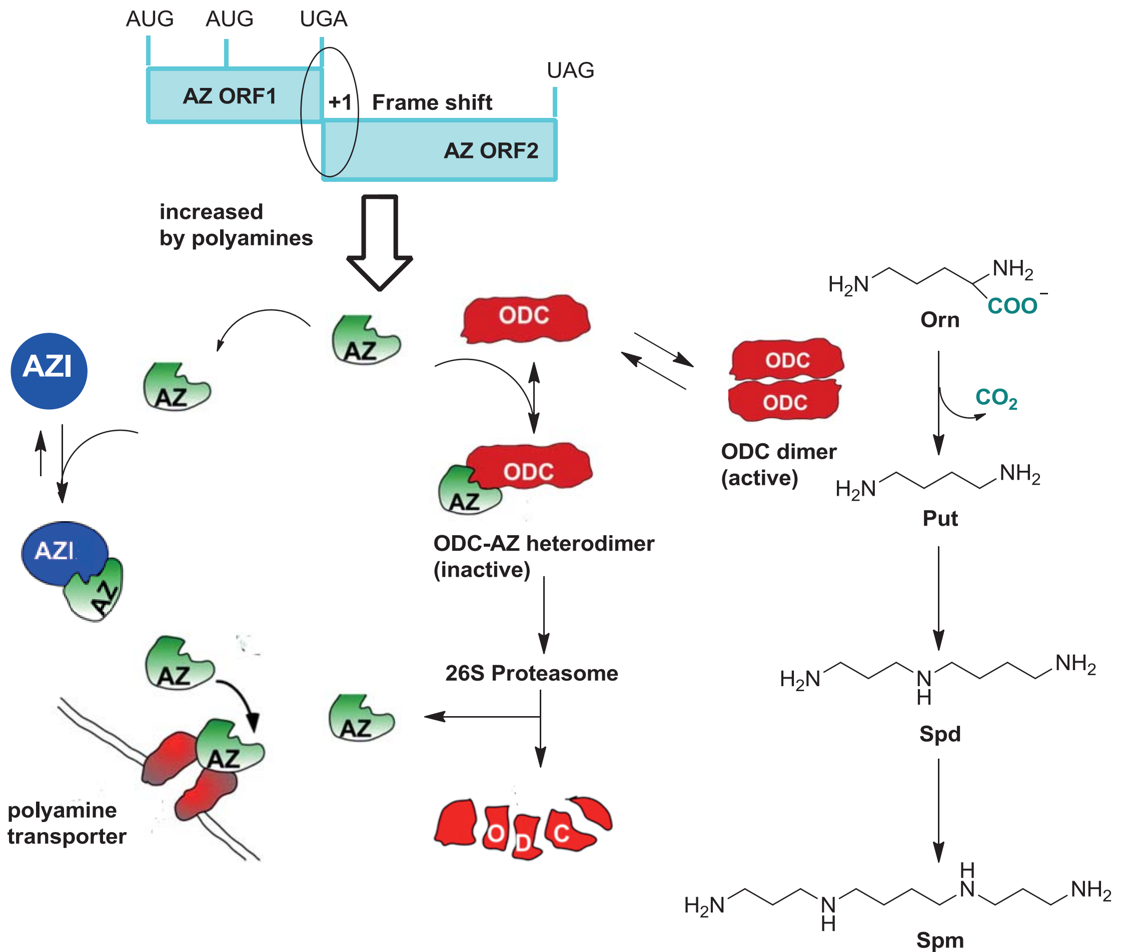

Figure 3. Role of ODC, AZ and AZI in polyamine metabolism (Pegg 2006).

There are three different genes coding for the AZs; AZ1, AZ2 and AZ3. AZ1 and AZ2 display similar tissue distributions and are expressed in most tissues and cells, although AZ2 has a much lower expression level (Murakami et al. 1992, Murakami et al. 2000). AZ3 is found predominantly in testis (Ivanov et al. 2000) and it does not promote ODC degradation (Tosaka et al. 2000).

AZs themselves are inhibited by the AZ inhibitor (AZI) which binds to AZ with higher affinity than ODC. Due to this binding, it blocks AZ activity and this leads to an elevated enzymatic activity of ODC (Fujita et al. 1982). AZIs consist of AZI1 and AZI2 which are homologous proteins to ODC. AZI1 is found in all tissues, whereas the expression of AZI2 seems to be restricted to the testis, brain and mast cells (Pitkänen et al. 2001, López-Contreras et al. 2006, Kanerva et al. 2009). While expression of AZI1 is mainly present in proliferating cells, surprisingly, the highest levels of AZI2 are found in many terminally differentiated cells like neurons and megakaryocytes. AZI2 has a role in intracellular vesicular transport (Kanerva et al. 2010), mast cell degranulation (Kanerva et al. 2009). Additionally, high AZI2 expression has been found in tissues with secretory activities and with abundant vesicle traffic i.e. in brain neurons and exocrine glands (Rasila et al. 2016).

2.2.2 S-Adenosyl-L-methionine decarboxylase

AdoMetDC is known as the second rate-controlling enzyme of polyamine biosynthesis, forming the dcAdoMet required for the synthesis of Spd and Spm (Pegg and McCann 1992). The regulation of mammalian AdoMetDC is controlled at both the transcriptional and translational levels (Pegg et al. 1988). The translational regulation of AdoMetDC is controlled by upstream ORF (uORF), which encodes the unique hexapeptide sequence MAGDIS (Law et al. 2001). An increased polyamine content decreases the synthesis of MAGDIS peptide by stabilizing the peptide-tRNA complex at the terminal codon. This leads to ribosome pausing and restricts its access to the downstream reading frame which encodes AdoMetDC (Raney et al. 2002). The AdoMetDC activity is decreased by Spd and Spm and increased by Put.

AdoMetDC has usually a half-life of 1-3 hours in most cell lines and tissues (Stjernborg and Persson 1993, Berntsson et al. 1999). In *Crithidia fasciculate,* the half-life of this enzyme is exceptionally short, about 3 min. (Nasizadeh and Persson 2003).

2.2.3 Spermidine synthase and spermine synthase

SpdSy and SpmSy, the best known aminopropyltransferases, are more stable than either ODC or AdoMetDC. They have similar properties and both are active as dimers consisting of two identical subunits (Wu et al. 2007, Wu et al. 2008). Put is converted to Spd by SpdSy and it is then converted into Spm by SpmSy. In these reactions, the formation of polyamines Spd and Spm depends on the availability of dcAdoMet as an aminopropyl donor (Ikeguchi et al. 2006).

2.3 POLYAMINE CATABOLISM

Intracellular polyamine concentrations are also highly regulated by catabolism via the conversion of Spd and Spm back to their precursor, Put. There are several enzymes involved in the polyamine catabolism i.e. SSAT (EC 2.3.1.57), flavin adenine dinucleotide (FAD)-dependent amine oxidases, acetylpolyamine oxidase (APAO; EC 1.5.3.13) and Spm oxidase (SMO; EC 1.5.3.16) and Cu(II) containing amine oxidases, semicarbazide-sensitive amine oxidase (SSAO/VAP-1/AOC3; EC 1.4.3.21), and diamine oxidase (DAO, AOC1, EC 1.4.3.22).

In the polyamine back-conversion pathway, Spd and Spm are initially acetylated to N^1-AcSpd and N^1-AcSpm by SSAT, and then oxidized by APAO to yield Put and Spd, respectively. SSAT enzyme acetylates also N^1-AcSpm to form N^1, N^{12}-diAcSpm which is then oxidized by APAO to produce N^1-AcSpd. Additionally, Spm is directly converted to Spd by the action of SMO (see Figure 2 on page 4) (Vujcic et al. 2003).

The extracellular formation of these compounds is more toxic than their intracellular formation due to the lack of protective enzymes, exogenous catalase and aldehyde dehydrogenases. Additionally, acrolein and H_2O_2 can be produced extracellularly from Spd or Spm in cell culture medium containing bovine serum amine oxidase (BSAO) that is able to oxidize the terminal amino group of polyamines. As fetal bovine/calf serum (FBS/FCS) contains high amounts of BSAO activity, and is the most commonly used serum in cell culture, care should be taken in differentiating polyamine/analogue cytotoxicity caused by BSAO from intracellular mechanisms of cytotoxicity. Thus, when using BSAO-degradable polyamines/analogues, experiments should be carried out in the presence of an amine oxidase inhibitor, such as aminoguanidine (AG) or semicarbazide (SC) (Sharmin et al. 2001).

2.3.1 Spermidine/spermine-N^1-acetyltransferase 1

SSAT is the key enzyme in the polyamine catabolism/back-conversion and it has a very short half-life (less than 30 min) (Matsui and Pegg 1981, Persson and Pegg 1984). Although the level of SSAT is generally very low, SSAT induction can occur rapidly in response to polyamines, polyamine analogues, toxic agents, environmental stress factors and certain drugs. SSAT is active as a dimer

consisting of two identical subunits with molecular mass of 20 kDa. The enzyme is highly regulated at transcription, mRNA processing, translation and also protein stabilization levels. SSAT catalyzes the acetylation of both Spd and Spm to form *N*-acetylated polyamine derivatives, utilizing acetyl coenzyme A (Ac-CoA) as the source of the acetyl group (Figure 4).

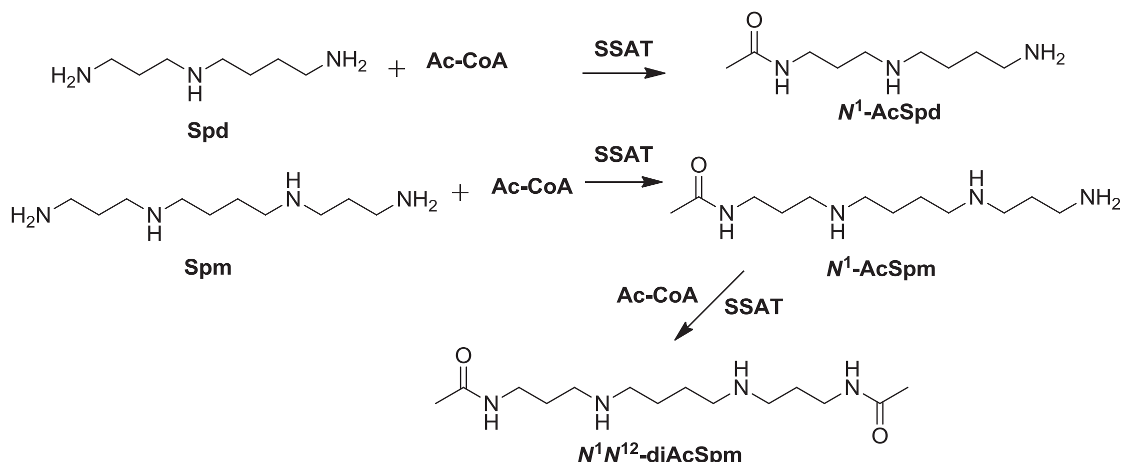

Figure 4. Reactions catalyzed by SSAT. Spd, Spm and also N^1-AcSpm are physiological substrates for SSAT enzyme. Ac-CoA is the acetyl group donor for the acetylation of Spd and Spm in which SSAT transfers the acetyl group from Ac-CoA to N^1-positions of Spd and Spm.

Additionally, SSAT is able to use N^1-AcSpm as a substrate to produce N^1,N^{12}-diAcSpm (see Figure 2 on page 4). On the other hand, those compounds with terminal aminobutyl groups such as N^1-AcSpd and Put, are not substrates for SSAT (Pegg 2008). Instead, N^1-AcSpd can be further acetylated by spermidine-N^8-acetyltransferase into N^1,N^8-diAcSpd (see chapter 2.3.5).

In addition to the acetylation of higher polyamines by SSAT, the enzyme can deactivate eIF5A via a selective acetylation of hypusine and/or deoxyhypusine residue of eIF5A (Lee et al. 2011).

2.3.2 Spermidine/spermine-N^1-acetyltransferase 2

Human Spd/Spm-N^1-acetyltransferase 2, (SSAT-2; EC 2.3.1.57) has been identified based on its structural similarity to SSAT i.e. it shares 45% identity and 61% homology with human SSAT. However, unlike SSAT, SSAT-2 is not involved in the polyamine catabolic pathway since it acetylates thialysine and thus it has been recommended to be renamed as thialysine N^{ε}-acetyltransferase (TLAT). SSAT-2 has a longer half-life than SSAT, and it is not inducible by polyamines/polyamine analogues differing from SSAT also in this respect (Coleman et al. 2004).

2.3.3 N^1-Acetylpolyamine oxidase and spermine oxidase

APAO and SMO enzymes are the two other key enzymes in polyamine catabolism. The biochemical properties of purified APAO were first characterized by Hölttä (Hölttä 1977). APAO catalyzes the oxidation of monoacetylated polyamines N^1-AcSpd and N^1-AcSpm and also diacetylated derivative N^1,N^{12}-diAcSpm as its preferred substrates (Figure 5) (Seiler 1987, Seiler 1995). High APAO activity is present in most mammalian tissues and it has a long half-life of almost 7 days. APAO has a molecular mass of 55 kDa and its activity can be detected in all vertebrate tissues (Seiler et al. 1980).

SMO is the most recently discovered mammalian enzyme in polyamine catabolism. SMO uses Spm, and less efficiently N^1-AcSpm, as its substrates (Figure 5) (Vujcic et al. 2002). The expression of SMO is stimulated by polyamine analogues at the mRNA level (Wang et al. 2001). Among the nine known splice variants of human and mouse SMO, two isoforms µSMO and αSMO (SMO5)

are catalytically active (Cervelli et al. 2004). Human SMO isoforms have molecular masses of 62 kDa and 65 kDa (Murray-Stewart et al. 2008). In general, acetylated polyamines do not usually accumulate intracellularly because there is oxidation of the acetylated forms by APAO and/or excretion of acetylated polyamines.

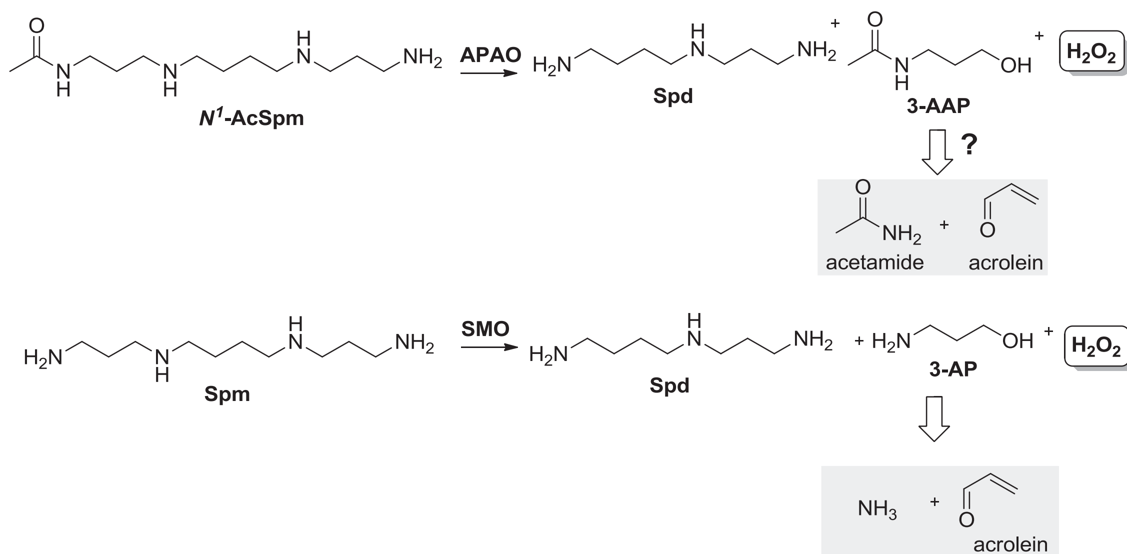

Figure 5. Oxidative degradation of Spd via N^1-AcSpm and Spd via Spm by APAO and SMO, respectively. The polyamine degradation byproducts H_2O_2, 3-AP, 3-AAP, acrolein and ammonia by SMO and APAO, are shown.

The polyamine catabolic pathway results in the generation of hydrogen peroxide (H_2O_2) as well as a number of reactive aldehydes as byproducts. The major aldehydes produced by amine oxidases are acetoamidoaldehyde 3-acetamidopropanal (3-AAP) and aminoaldehydes 3-aminopropanal (3-AP) and 4-aminobutanal (4-AB) (Figure 5, Table 1). These aldehydes are unstable; for example 3-AP is spontaneously converted into acrolein and ammonia (NH_3) (Figure 5). Several of these byproducts, e.g. acrolein, 3-AP, 4-AB and H_2O_2, are highly cytotoxic (Wood et al. 2007). They are also rapidly conjugated to proteins and lipids if not detoxified by cellular enzymes. There has been a debate about whether or not 3-AAP produced by APAO is converted to acrolein. The results from Wood et al. indicated that the aldehyde function of 3-AAP was not sufficient to express cytotoxicity, but that a free amino group would be needed to access to lysosomes where their cytotoxic actions are initiated through lysosomal membrane permeabilization and leakage of cathepsins to the cytosol. Furthermore, it has been reported that SMO byproducts are more cytotoxic than APAO's, most likely because SMO's action results in the production acrolein (Pledgie et al. 2005).

One compound, N^1,N^4-bis (2,3-butadienyl)-1, 4-butanediamine (MDL 72527), is the most specific inhibitor of both APAO and SMO (Seiler et al. 2002). Although MDL 72527 prevents the generation of H_2O_2 and aldehydes by both APAO and SMO, it is itself a lysosomotropic drug and has been shown to induce cell death and potentiate the cytotoxicity of some compounds (Dai et al. 1999, Seiler et al. 2000).

2.3.4 Other amine oxidases involved in polyamine catabolism

In addition to SSAT, APAO and SMO, also Cu(II)-containing amine oxidases, SSAO (VAP-1/AOC3) and DAO (AOC1), and FAD-dependent oxidases like monoamine oxidase (MAO; EC 1.4.3.4), can metabolize polyamines by selective cleavage of primary *N*-terminal amine

(Agostinelli et al. 2010). Furthermore, H_2O_2, reactive aldehydes and ammonia are generated as byproducts of these reactions.

DAO uses Put, and less efficiently Spd, as its substrate, whereas the SSAO enzyme prefers Spm and Spd and their acetylated derivatives (Largeron et al. 2010). Both SSAO and DAO are inhibited by SC and AG (Claud et al. 2001, Ochiai et al. 2006). There is also evidence that N^1-AcPut can be oxidized by MAO. Pargyline and selegiline are inhibitors of MAO-B enzyme, whereas clorgyline is selective for MAO-A. Importantly, the degradation of Put and N^1-AcPut by DAO and MAO, respectively, links polyamine metabolism to energy metabolism via the so- called γ-aminobutyric acid (GABA) shunt. Put and N^1-acetylputrescine are metabolized to GABA, which is then converted to succinic semialdehyde and further to succinate, which is a part of the citric acid cycle (Seiler and Al-Therib 1974). Thus, polyamines may also be used as an alternative energy source. Remarkably, one study showed that up to 70% of [^{14}C]-labelled Put was utilized in energy production in rats during 2-days' fasting (Bardocz et al. 1998).

Table 1. Polyamine sources of H_2O_2, acrolein, the reactive aldehydes, 3-AP, 3-AAP and 4-AB (Wood et al. 2007).

byproducts	polyamine sources
H_2O_2	Put, Spd, Spm
3-AP	Spd, Spm
3-AAP	N^1-AcSpd, N^1-AcSpm, N^1,N^{12}-diAcSpm
4-AB	Put, N^1-AcPut, Spd
Acrolein	Spd, Spm

2.3.5 Spermidine-N^8-acetyltransferase

Spd-N^8-acetyltransferase (Spd-N^8-acetylase) has been less extensively studied; it has not been cloned or characterised in detail. While SSAT is a cytosolic enzyme, Spd-N^8-acetylase was initially identified as a nuclear enzyme which could also acetylate histones (Libby 1978, Libby 1980, Desiderio et al. 1992). Recently, histone acetyltransferase P/CAF (E.C. 2.3.1.48) was described as an enzyme capable of producing N^8-acetylspermidine (Burgio et al. 2016). However, there is also evidence for the presence of a cytosolic Spd-N^8-acetylase, which is not inhibited by methylglyoxal bis(guanylhydrazone) (MGBG) unlike the nuclear enzyme (Wallace et al. 1988). N^8-AcSpd is not a substrate for APAO, but instead is deactylated by N^8-AcSpd deacetylase (EC 3.5.1.48) to regenerate Spd (Figure 6) (Seiler 2004). Most recently, histone deacetylase 10 (HDAC10) was identified as a robust polyamine deacetylase, Put and N^8-AcSpd deacetylase (Hai et al. 2017). The cellular functions of N^8-AcSpd are not well understood, but one study indicated that it induced neuronal differentiation of PC12 cells (Mudumba et al. 2002).

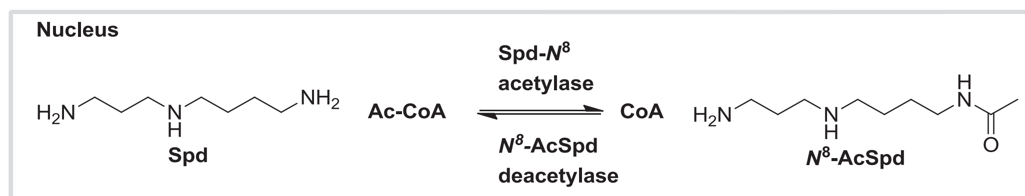

Figure 6. Acetylation and deacetylation of Spd by Spd-N^8-acetylase and N^8-AcSpd deacetylase, respectively.

2.4 POLYAMINE TRANSPORT

Polyamine transport (uptake and efflux) plays an important role in the control of intracellular polyamine levels (Figure 2 on page 4). In general, polyamine uptake is increased when the amount of intracellular polyamines decreases, whereas high levels of polyamines increase polyamine efflux out of the cell (Seiler et al. 1996). Various physiological stimuli, such as cellular proliferation and differentiation, can induce polyamine synthesis and uptake and cause substantial, several-fold increases in intracellular polyamine levels.

Some bacterial and yeast polyamine transporters have been cloned and characterized, but the mammalian polyamine transport system is not yet well characterized. However, it is known that polyamine uptake is energy-dependent and saturable (Seiler et al. 1996). Some members of the ATP-binding cassette (ABC) and solute carrier (SLC) transporter families have been postulated to function as transport polyamines (Abdulhussein and Wallace 2014). Additionally, it has been reported that glypican-1 is a vehicle for polyamine uptake. In this model, polyamines first bind to the heparan sulfate chains on glypican-1 and then they are taken up by endocytosis (Belting et al. 2003).

The major intracellular polyamines are Spd and Spm whereas Put and acetylated polyamines are the main polyamines effluxed from the cell (Wallace and Mackarel 1998, Fraser et al. 2002). Since the products of polyamine catabolism are acetylated polyamines, this indicates that polyamine catabolism and excretion are linked to each other.

2.5 THE ROLE OF POLYAMINES IN CANCER

Changes in polyamine levels and polyamine metabolic enzymes are often associated with various diseases including cancer, inflammation, stroke, renal failure and diabetes. Most notably, high polyamine levels have been found in various cancers such as breast (Kingsnorth et al. 1984, Davidson et al. 1993, Wallace et al. 2000), colon (Milovic and Turchanowa 2003), prostate (Schipper et al. 2003), skin (Gilmour 2007) and lung (Min et al. 2014) cancers. Urinary polyamine derivatives and their metabolites have been intensively studied as diagnostic markers of cancer (Russell et al. 1971, Russell 1971). Diacetylated Spd and Spm can be detected at high levels in the urine of cancer patients, and thus are considered as potential biomarkers of early stage cancer (Lipton et al. 1975, Park and Igarashi 2013).

The first polyamine biosynthetic enzyme, ODC plays an important role in cancer development. High ODC activity is observed in many cancer types as compared to normal tissues (Russell and Snyder 1968, Auvinen et al. 1992). Cell transformation by various well-known oncogenes such as *myc, ras, v-src* and *neu* is associated with constitutively elevated ODC activity. Overexpression of ODC is alone sufficient to transform NIH3T3 cells (Moshier et al. 1993), which form highly vascularized and invasive tumors in nude mice (Auvinen et al. 1997).

Increased SMO activity has been associated with various cancers such as prostate (Goodwin et al. 2008), colon (Goodwin et al. 2011) and *Helicobacteria pylori*-induced gastric carcinogenesis (Butcher et al. 2017). On the other hand, SMO activity was found to be lower in breast tumor tissues compared to normal tissue (Cervelli et al. 2010).

Based on all this information gathered over many years, the polyamine metabolic pathway has been considered to be an attractive target for anticancer drug development.

2.5.1 Single enzyme inhibitors as anticancer agents

The first synthetic single enzyme inhibitors of the polyamine metabolic enzymes were targeted against ODC, the initial rate-limiting enzyme, and against AdoMetDC, the second rate-limiting enzyme (Stanek et al. 1992, Seiler 2003). However, single enzyme inhibitors have been developed for almost all of the enzymes involved in polyamine metabolism. These compounds have proved

to be extremely useful research tools helping to elucidate the cellular functions of polyamines. In addition, the inhibitors of the polyamine biosynthetic enzymes have been extensively used in order to clarify the roles of eIF5A and polyamines in cell proliferation. Furthermore, some of these inhibitors have proceeded to clinical trials. The chemical structures of some of the more important single enzyme inhibitors in polyamine metabolism are shown in Figure 7, and their effects on polyamine metabolism are shown in Table 2. Many of these compounds have some disadvantages, e.g. they do not reduce polyamine levels to a low enough level or they have unacceptable adverse effects. To date, MDL 72527 has not been examined in clinical trials.

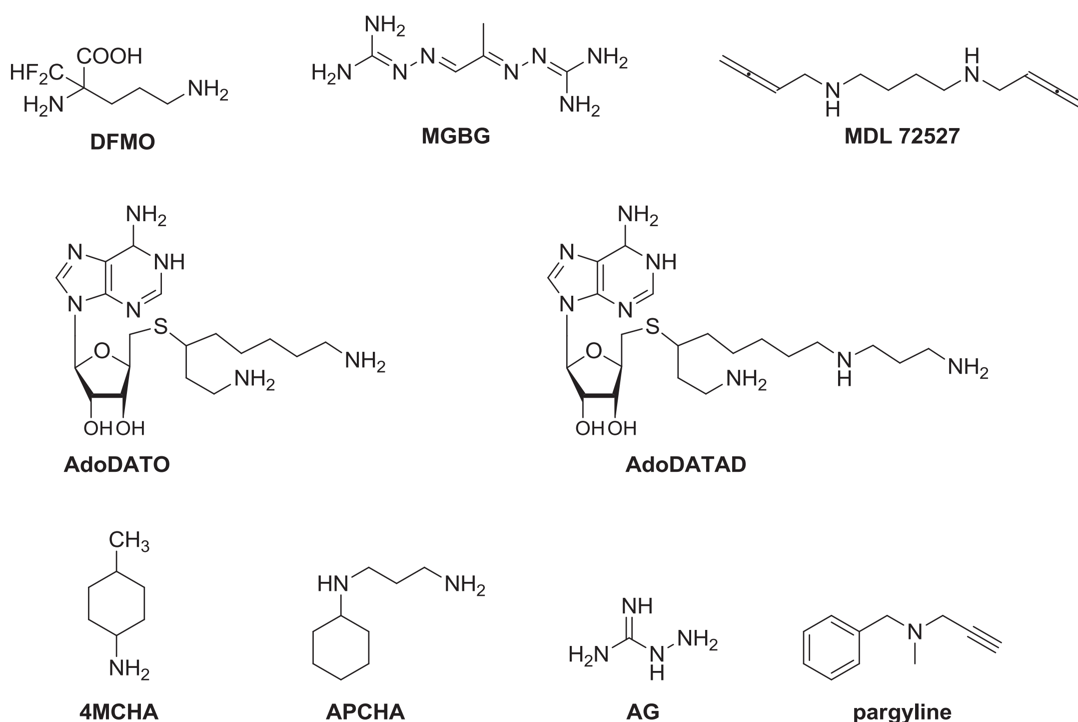

Figure 7. Chemical structures of enzyme inhibitors targeting either polyamine biosynthesis or catabolism: DFMO (ODC), MGBG (AdoMetDC), 4MCHA and AdoDATO (SpdSyn), APCHA and AdoDATAD (SpmSyn), MDL 72527 (APAO and SMO), AG (DAO and SSAO), Pargyline (MAO).

Table 2. Polyamine metabolic enzyme inhibitors and their effects on polyamine metabolism.

Inhibitor	name	enzymes affected	effect on intracellular polyamine pools	reference
DFMO	α-difluoromethylornithine	ODC	Put↓ Spd↓ Spm↔/↓/↑	(Metcalf et al. 1978)
CGP 48664	4-amidinoindan-1-one 2′-amidinohydrazone	AdoMetDC	Put↑ Spd↑ Spm↔	(Regenass et al. 1994)
CGP 39937	[2,2-bipyridine]-6,6′-dicarboximidamide	AdoMetDC	-	(Stanek et al. 1993)
MGBG	Methylglyoxal-bis(guanylhydrazone)	AdoMetDC	Put↑ Spd↑ Spm↓	(Porter et al. 1980)
AdoDATO	S-adenosyl-1,8-diamino-3-thio-octane	SpdSy	Put↑ Spd↓ Spm↑	(Holm et al. 1989)
AdoDATAD	S-adenosyl-1,12-diamino-3-thio-9-azadodecane	SpmSy	Put↑ Spd↑ Spm↓	(Pegg et al. 1989)
4MCHA	trans-4-methylcyclohexylamine	SpdSy	Put↑ Spd↓ Spm↑	(Beppu et al. 1995)
APCHA	*N*-(3-aminopropyl)cyclohexylamine	SpmSy	Put↑ Spd↑ Spm↓	(Beppu et al. 1995)
MDL 72527	N^1, N^4-bis (2, 3-butadienyl)-1, 4-butanediamine	APAO, SMO	Put↓ Spd↓ Spm↑ Acetylated polyamines↑	(Bolkenius et al. 1985, Duranton et al. 2002)
GC7	N^1-guanyl-1,7-diaminoheptane	DHS	deoxyhypusinated eIF5A↓ hypusinated eIF5A↓	(Lee et al. 2002)

2.5.1.1 The ODC inhibitor DFMO

DFMO was first synthesized as an irreversible enzyme inhibitor of ODC in 1978 (Metcalf et al. 1978). Of all the polyamine-based enzyme inhibitors, DFMO has been the most successful enzyme inhibitor; it acts as “suicide substrate” for ODC (Figure 7, Table 2) (Bey et al. 1978). DFMO is recognized by ODC as a substrate that binds to the active site of ODC. However, DFMO is cytostatic in mammalian cells, leading a reduction in cell proliferation in the absence of cell death (He et al. 2017).

The effects of DFMO treatment on cell proliferation and polyamine content *in vitro* have been extensively investigated. One interesting study examined the roles of active hypusinated eIF5A and polyamines in cell proliferation. Mouse mammary carcinoma FM3A cells were treated with DFMO or the combination of DFMO with the SpmSy inhibitor, APCHA. Both treatments inhibited cell growth already from the first treatment day. DFMO alone markedly decreased the levels of Put and Spd, and the formation of active eIF5A started to cease when the amount of Spd was below a significant level after 12 h incubation with DFMO. The combination of DFMO and APCHA (see Figure 7 on page 12) significantly depleted the levels of Put and Spm and markedly reduced the level of Spd, but did not have any effect on the active eIF5A concentrations until the third day when the Spd level was depleted to 7 nmol/mg of protein. It was concluded that a decrease in either active eIF5A or polyamines inhibited the cell growth, evidence that eIF5A and polyamines have independent roles in cell growth (Nishimura et al. 2005). The combined treatment of DFMO with AdoMetDC inhibitor CGP 48664 (4-amidinoindan-1-one 2'-

amidinohydrazone) revealed that depletion of polyamine levels resulted in rapid cell growth inhibition in human neuroblastoma cell lines (Wallick et al. 2005).

Previous studies showed that *in vitro* DFMO treatment has usually a cytostatic effect, which leads to a cessation of cell proliferation in the absence of cell death, rather than cytotoxic effects, and that it prevents cell growth through depletion of Put and Spd (Seidenfeld 1985, Porter and Bergeron 1983). However, the intracellular Spm level in DFMO-treated cells is not usually decreased but may even be increased. In addition, DFMO increases polyamine uptake *in vitro* and *in vivo*. DFMO has been reported to inhibit cell growth in animal models of various cancer types including bladder, colon, liver, skin and pancreas (Esmat et al. 2002).

Currently DFMO is in clinical use against the advanced form of African sleeping sickness (trypanosomiasis) and used as a cream for facial hirsutism in women. DFMO has been the most extensively studied single enzyme inhibitor as an anticancer agent, both alone and in combination with other drugs. The combination chemoprevention therapies are often more effective than a single agent on its own.

At least three phase II chemoprevention studies of DFMO have been completed in patients with colon cancer. DFMO has been shown to be safe and highly effective in the primary chemoprevention of human colon cancer, both as a single agent (Love et al. 1998, Meyskens et al. 1998) and in combination with sulindac, a nonsteroidal anti-inflammatory drug (NSAID) (Meyskens et al. 2008). Remarkably, in one phase IIb/III trial, high-risk patients for colon cancer treated for 3 years with DFMO and sulindac had 92% and 70% reductions in the incidence of advanced adenocarcinomas and adenomas, respectively. The high effectiveness of this combination, as compared to DFMO alone, might be related to the ability of sulindac to induce polyamine catabolism (Babbar et al. 2003). There is one completed phase III clinical study with DFMO (500 mg/m^2/day) in skin cancer (Bailey et al. 2010). The authors concluded that DFMO as a single agent or in combination with potential anticancer agents to prevent skin cancer should be investigated more intensively.

The most recent clinical trials have shown that DFMO significantly increases the survival and prevents relapses of neuroblastoma in children (Sholler et al. 2015). The side effects have been claimed to be minimal and the outcomes have been very positive. As a result, DFMO is currently undergoing several clinical trials for the treatment or prevention of high-risk neuroblastoma in children, both as a single agent and in combination with other agents.

2.5.1.2 AdoMetDC inhibitors

Various single enzyme inhibitors such as methylglyoxal-bis(guanylhydrazone) (MGBG) (Porter et al. 1980) and MGBG analogs, (2,2-bipyridine)-6,6′-dicarboximidamide (CGP 39937) and 4-amidinoindan-1-one 2′-amidinohydrazone (CGP 48664) (Regenass et al. 1994) have been investigated as mammalian AdoMetDC inhibitors (Table 2) (Thomas et al. 1996).

MGBG, a structural analog of Spd, inhibits proliferation and decreases the intracellular Spd and Spm levels while increasing Put levels. Despite its potency as an AdoMetDC inhibitor, its effect as an anticancer agent was limited by its mitochondrial toxicity, interference with polyamine transport and the induction of SSAT (Williams-Ashman and Schenone 1972, Nass 1984).

CGP 48664, a competitive inhibitor of AdoMetDC, is a more effective anticancer agent than CGP 39937. It exerts less mitochondrial toxicity and has been evaluated in several phase I and II clinical studies for various cancer types. It has shown some efficacy in non-Hodgkin's lymphoma (Eskens et al. 2000, Zhou et al. 2000, Paridaens et al. 2000, Pless et al. 2004). CGP 48664 depletes both Spd and Spm levels and thus inhibits cell growth (Table 2) (Regenass et al. 1994).

2.5.1.3 SpdSy and SpmSy inhibitors

In addition to ODC and AdoMetDC enzyme inhibitors, a number of SpdSy and SpmSy inhibitors have been developed (Pegg et al. 1995). S-Adenosyl-1,8-diamino-3-thio-octane (AdoDATO), trans-4-methylcyclohexylamine (4 MCHA) (Dufe et al. 2007), are inhibitors of SpdSy, and S-

adenosyl-1,12-diamino-3-thio-9-azadodecane (AdoDATAD) (Woster et al. 1989), *N*-(3-aminopropyl)cyclohexylamine (APCHA), are SpmSy inhibitors, (see Figure 7 on page 12, Table 2 on page 13) (Woster 2006). AdoDATO and AdoDATAD have shown only minor growth inhibition in cancer cell lines (Holm et al. 1989, Pegg et al. 1989).

The effects of 4MCHA or APCHA on polyamine metabolism were investigated in rat tissues. Oral administration of these agents in drinking water for a period of 10 days or 4 months significantly decreased Spd/Spm ratios (as much as a 95% decrease) with a compensatory increase in the Spm/Spd ratio in rat tissues. Thus, the total higher polyamine pool remained unchanged. Therefore, no changes in the growth of treated rats were observed. A high activity of AdoMetDC was observed with unchanged activity of ODC (Shirahata et al. 1993).

The effects of these compounds on polyamine biosynthesis and cell growth have also been investigated in rat hepatoma HTC cells. It was found that treatment with 4MCHA or APCHA decreased markedly Spd and Spm levels with a compensatory increase in other polyamines, resulting in the same net amount of polyamines. On the other hand, when the cells were treated with 250 μM of 4MCHA or APCHA for 8 days, Spd and Spm levels were reduced below 2% and 1% of those in control cells, respectively, while the total polyamine level did not change extensively because there was a huge increase in the amount of Put (Beppu et al. 1995).

Another interesting study showed that the combination of DFMO and APCHA strongly inhibited the growth of FM3A cells and P388 leukemia cells, compared to a single DFMO treatment. The suppression in cell growth with DFMO was potentiated by APCHA, leading to a decline in Spm levels, and a reduction in the total amount of Spd and Spm (He et al. 1995).

2.5.2 Polyamine analogues as anticancer agents

A wide variety of polyamine analogues have been synthesized and evaluated. The most widely studied polyamine-based anticancer agents have been the terminally di-*N*-alkylated polyamine analogues. Another important group of polyamine analogues are charge-deficient analogues which have been synthesized to investigate the importance of the molecule's charge distribution.

In these compounds, small structural changes have resulted in surprisingly significant changes in biological activity. It is already known that polyamine analogues represent a potentially effective class of compounds for preventing cancer cell growth because of their ability to interfere with polyamine metabolism.

2.5.2.1 Symmetrically and unsymmetrically *N*-alkylated polyamine analogues

Due to the limitations of the single enzyme inhibitors, an alternative approach - the development of *N*-alkylated polyamine analogues as potential anticancer agents - has been investigated; their history extends back to the late 1980s (Casero and Woster 2001). A huge number of polyamine analogues, structurally similar to the natural polyamines, have been synthesized in order to achieve depletion of all intracellular polyamine levels and to elicit high cytotoxicity (Figure 8) (Casero and Marton 2007). These polyamine analogues are actively taken up via the polyamine transport system into cells in the same way as natural polyamines (Porter et al. 1991). The molecular charge and presence of terminal amines were found to be important for the transport of these analogues (Porter et al. 1985). *N*-Alkylated polyamine analogues have been classified into two groups named symmetrically and unsymmetrically substituted polyamine analogues (Figure 8). Although the synthesis of symmetrical terminally alkylated polyamine analogues is relatively straightforward, the synthesis of unsymmetrical terminally alkylated polyamines analogues is more complicated due to the requirement that there should be selective protection and/or deprotection of internal and external nitrogens.

The first generation of *N*-alkylated polyamine analogues were symmetrically terminally substituted Spd or Spm analogues. One Spd analogue, N^1,N^8-di(ethyl)Spd (BESpd; DESpd), was shown to inhibit ODC activity and found to be cytotoxic especially in human large cell undifferentiated lung cancer cells. However, the corresponding Spm analogue, N^1,N^{12}-di(ethyl)Spm (BESpm; DESpm), has been found more effective in some cells. The *N*,*N*'-

di(ethyl)Spm analogues have been able to decrease ODC activity, deplete intracellular polyamine levels and induce cell death (Porter et al. 1987). The cytotoxic effects of many polyamine analogues have been speculated to be partly mediated via the induction of SSAT and/or SMO and partly via depletion of the natural polyamines and competition for their important macromolecular interactions (Pledgie-Tracy et al. 2010).

Furthermore, some of the most widely studied and successful analogues such as N^1,N^{11}-di(ethyl)-norspermine (BENSpm; DENSpm) and N^1,N^{14}-di(ethyl)-homospermine (BEHSpm), decreased the levels of all three intracellular polyamines and exerted cytotoxic effects in several cell lines (Bernacki et al. 1992, Chang et al. 1992). Especially, DENSpm possessed multiple mechanisms to interfere with the polyamine pathway; for example, up-regulation of SSAT and SMO, down-regulation of ODC and polyamine uptake. Thus, DENSpm decreased intracellular polyamine levels and induced apoptosis and cell death in several cancer cell types. DENSpm went on to phase I and II clinical trials in the treatment of various types of cancer. In phase I clinical trials in patients with advanced non-small cell lung cancer, the compound was proven to be safe at a daily dose of 185 mg/m^2 for 5 days with minimal toxicity (Hahm et al. 2002), but, unfortunately, in phase II clinical trials in patients with metastatic breast cancer, it showed little evidence of clinical activity (Wolff et al. 2003).

There are also conformationally restricted *N*-alkylated polyamine analogues, such as (N^1,N^{12})di(ethyl)-6,7-dehydrospermine (PG11047), which has a structure based on DENSpm, modified by the introduction of a double bond into the central 4-carbon methylene chain. PG11047 has shown good anticancer efficacy in various cell and animal models, and underwent a phase I clinical trial between 2005 - 2009 in subjects with advanced refractory solid tumors (Dredge et al. 2009).

The second generation polyamine analogues, unsymmetrically substituted polyamine analogues initially described and developed in 1993 (Saab et al. 1993) such as N^1-propargyl-N^{11}-ethyl-norspermine (PENSpm), N^1-cyclopropyl-methyl-N^{11}-ethylnorspermine (CPENSpm), N^1-cycloheptylmethyl-N^{11}-ethylnorspermine (CHENSpm), and (S)-N^1-(2-methyl-1-butyl)-N^{11}-ethyl-4,8-diazaundecane (IPENSpm) (Figure 8). This class of compounds has more structural diversity than the previously described molecules, containing aromatic groups, unsaturations, stereochemistry and heteroatoms.

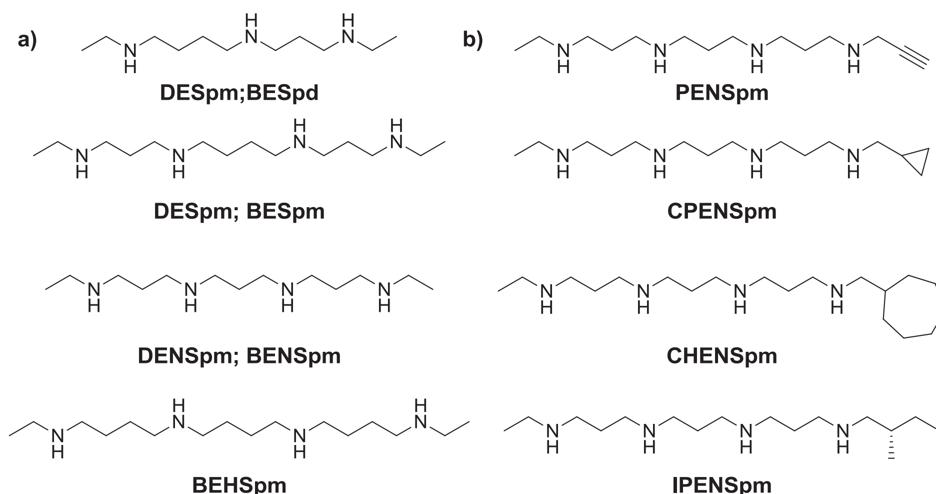

Figure 8. Examples of a) symmetrically substituted and b) unsymmetrically substituted polyamine analogues.

Both CPENSpm and PENSpm compounds were found to exert greater cytotoxicity and SSAT induction than BESpm (Saab et al. 1993). CHENSpm and IPENSpm showed a more impressive antitumor activity *in vitro* but with only lower SSAT induction and less polyamine depletion (Wu et al. 1996).

2.5.2.2 Charge-deficient polyamine analogues, TETA and SpmTrien

Another important class of polyamine analogues are charge-deficient isosteric polyamine analogues. A few different methods have been utilized to decrease the basicity of the amino groups in the polyamine backbone and therefore to deplete the net charge of the polyamine analogues. The concept has been that it would be an advantage to alter the basicity of polyamine analogues causing the steric disturbance but nonetheless having an analogue that would still resemble the natural polyamines. The first method involved the introduction of one or two electronegative fluorines in the polyamine backbone. An alternative way was to replace the carbon next to nitrogen with the more electronegative oxygen, e.g. NH_2CH_2- for NH_2O-; the third known way involves a reduction of the length of carbon chain to two between amino groups. In the latter case, $(\text{-N-C-C-N-})_n$ (n>2) chain nitrogens are difficult to protonate, since maintaining positive charges next to each other separated by only two bonds requires a rather low pH (Weisell et al. 2010a). Another alternative method involves the use of conformationally restrained cyclic amines instead of a non-branched carbon chain. The charge-deficient polyamine analogues have been valuable tools for biochemists trying to clarify the importance of the charge in polyamine binding and recognition.

The synthetic polyamine analogues, triethylenetetramine (1,8-diamino-3,6-diazaoctane; TETA; trien; trientine; Syprine®) and 1,12-diamino-3,6,9-triazadodecane (SpmTrien) are charge-deficient analogues of Spd and Spm, respectively. TETA was firstly synthesized in Berlin, Germany in 1861 and its dihydrochloride salt was produced in 1896 (Lu 2010). The pK_a values of SpmTrien (10.3, 9.5, 8.5, 6.3 and 3.3) and TETA (9.74, 9.07, 6.56 and 3.27) are markedly lower than those of Spm (10.9, 10.1, 8.4 and 7.9) and Spd (10.9, 9.9 and 8.4) (Weisell et al. 2010a), thus TETA has only two positive charges (+2) at physiological pH (Bencini et al. 1999), whereas SpmTrien has three (+3) positive charges at pH 7.4 (Weisell et al. 2010a). Both TETA and SpmTrien are efficient Cu(II) chelators while the natural polyamines Spm and Spd do not possess this property at physiological pH (Khomutov et al. 2007, Weisell et al. 2014). TETA is used as an alternative drug for Wilson's disease in patients who are intolerant to primary medication with D-pen (Walshe 1969, Dixon et al. 1972) but it has also been investigated as an anticancer agent (Lu 2010).

TETA dissolves in aqueous solutions and is administered as either a dihydrochloride or a dissuccinate salt. TETA is metabolized in cells into its monoacetylated N^1-AcTETA and diacetylated N^1,N^8-diAcTETA forms (Lu et al. 2007a). The *in vivo* acetylation of TETA can be catalyzed by either SSAT, which mediates the acetylation of Spd and Spm, or its homologue enzyme, SSAT-2, to generate two known metabolites, N^1-AcTETA and N^1,N^8-diAcTETA. TETA has a K_m value of 2.5 mM and V_{max} of 3.96 μmol/min/mg for human recombinant SSAT2, and a K_m value of 83 μM and a V_{max} value of 0.90 μmol/min/mg for human recombinant SSAT, as determined by using a paper (P81) disc method (Cerrada-Gimenez et al. 2011). Furthermore, the enzyme kinetics with mouse recombinant SSAT1 were determined for TETA with values of K_m of 169 μM and V_{max} of 1.37 μmol/min/mg. Based on studies with SSAT overexpressing and deficient mice as well as siRNA experiments in liver cells, it was observed that despite the similarities in the structures of TETA and SpmTrien, TETA is mainly acetylated by SSAT2, whereas SpmTrien has an aminopropyl group which is preferred by SSAT (Hyvönen et al. 2013).

The synthesis and biological characterization of novel charge-deficient polyamine analogs have been reported (Figure 9). These include SpmTrien, N^1-AcSpmTrien, N^{12}-AcSpmTrien, and N^1,N^{12}-diethylSpmTrien (N^1,N^{12}-Et_2-SpmTrien) (Khomutov et al. 2007).

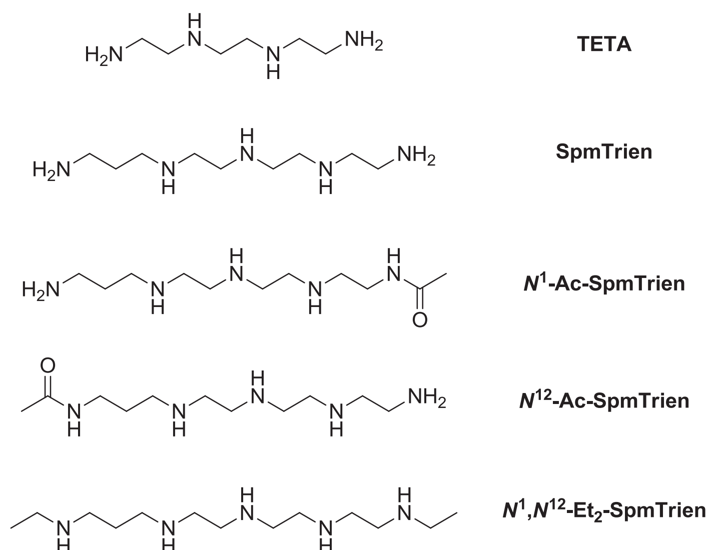

Figure 9. Chemical structures of TETA and SpmTrien and SpmTrien derivatives.

2.5.2.3 The characteristics of TETA as anticancer agent

TETA is a well-tolerated drug, possibly due to its low bioavailability and rapid acetylation and excretion (Lu et al. 2007b). A number of preclinical *in vivo* and *in vitro* studies of TETA have been carried out. TETA has been used in clinical trials for the treatment of diabetic organ damage (Lu 2010). However, so far, only two clinical trials of TETA in cancer have been reported. In the first study, TETA was orally administered for 12 weeks in a single dose of 250 mg per day before a meal or a total daily dose of 750 mg, in 3 divided doses, to 12 patients with hepatocellular carcinoma (HCC), which is the most common type of liver cancer. According to this study, TETA could sufficiently reduce the Cu(II) level in the liver tissue, which could be effective in the treatment of HCC since an increased level of Cu has been associated with HCC development (Fukuda et al. 2004). In the second study, TETA was given in combination with carboplatin to patients with various advanced malignancies who had failed to respond to primary therapy with platinum-based agents. The results showed that the combination was well tolerated and had antitumor activity, especially in patients in whom copper and/or ceruloplasmin levels were reduced (Fu et al. 2014).

In preclinical studies, TETA has been shown to effectively inhibit the cell growth in various cancer types including neuroblastoma (Lombardo et al. 2003), HCC (Yoshii et al. 2001, Moriguchi et al. 2002), hela (Yin et al. 2004), breast (MCF 7) (Lixia et al. 2008, Wang et al. 2017), and fibrosarcoma (Kadowaki et al. 2009) cancer cells; its mechanisms of action include anti-angiogenesis (Hitoshiyoshiji et al. 2003, Hitoshiyoshiji and Shigekikuriyama 2005), telomerase inhibition (Yin et al. 2003, Liu et al. 2008), and induction of apoptosis (Kadowaki et al. 2009). In animal studies on Long-Evans cinnamon rats, short-term administration of TETA dramatically inhibited the development of hereditary hepatitis (Sone et al. 1996). In addition, TETA in combination with ascorbic acid, prevented cancer growth in nude mice implanted with MCF7 breast cancer cells (Wang et al. 2017).

Copper is believed to play an important role in cancer. For example, there is data indicating that high copper concentrations are involved in the development and proliferation of cancer (Harris 2004, Brewer 2001). Like TETA, TTM and D-pen, other selective Cu(II) chelators, are currently being investigated as anticancer agents (Helsel and Franz 2015).

2.5.3 Polyamine and polyamine analogue analysis

The accurate measurement of polyamines and their analogues is required in order to investigate their intra- and extracellular content and their metabolism within the cells. Polyamine analogues can be distinguished from the natural polyamines by chromatographic methods such as with the simple and rapid method of thin layer chromatography (TLC), but also gas chromatography (GC), ion-exchange chromatography and high-performance liquid chromatography (HPLC) have been exploited (Teti et al. 2002). HPLC methods using post-column *o*-phthalaldehyde (OPA) or pre-column dansyl chloride derivatization have been mostly used due to their high sensitivity and reproducibility and also ease of automation. The disadvantages of the dansylation method include the long derivatization times, labor-intensivity and the instability of the derivatives (Acheampong et al. 2011). Diacetylated polyamines are detectable with the dansyl approach, but not with the OPA method as the latter technique detects only molecules with primary amino groups.

Several other techniques such as spectrophotometric (Vandenabeele et al. 1998), electrochemical (Ma et al. 2004), fluorescence (Paproski et al. 2002), and chemiluminescence (Liu et al. 2003) methods have been described for the determination of polyamines. Mass spectrometry (MS) has also been applied such as in the published liquid chromatography-tandem mass spectrometry method (LC-MS/MS) (Ducros et al. 2009).

Another recent interesting study reported an analysis method for assaying free, monoacetylated and diacetylated polyamines from human urine by LC-MS/MS using deuterium-labelled polyamines as the internal standand for each analyte. It was claimed that this method could be utilized to study the polyamine levels in the urine samples of cancer patients (Häkkinen et al. 2013). It has also been used for analysing polyamines and *N*-alkylated polyamine analogues and their degradation products from cell and medium samples (Häkkinen et al. 2010).

2.6 STABLE ISOTOPE-LABELED COMPOUNDS

Stable isotope-labeled molecules have been commonly exploited tools in both biomedical and chemical research. They are most widely used as internal standards in MS quantification to enable the exact quantification of drugs and metabolites by pharmacologists and toxicologists (Chokkathukalam et al. 2014). Stable isotopes are also useful in the analysis of absorption, distribution, metabolism and excretion (ADME) studies of drugs, toxic agents and structure of their metabolism products (Mutlib 2008).

2.6.1 Deuterium-labeled derivatives

Researchers first began to include heavy hydrogen into drugs to improve the properties of drugs at the beginning of the 1960s. A deuterated drug refers to a small medicinal molecule in which hydrogen (H) atom(s) in the drug molecule has/have been replaced by deuterium (D), a heavy isotope of H (Mullard 2016). Due to the kinetic isotope effect, deuterated drugs might have significantly lower metabolic rates, and thus a longer half-life allowing less frequent dosing (Sanderson 2009, Gant 2013). Therefore, deuterium labeling plays an important role in the examination of the secondary and tertiary structures, it has also effects on the kinetics of the drug molecules i.e. their metabolism, other aspects of pharmacokinetic as well as toxicity. Deuterated drugs have also been utilized in investigations of metabolism of drugs and toxic substances in humans (Kushner et al. 1999). In general, these compounds have been used to prevent the formation of toxic catabolites by redirecting drug catabolism and/or to slow down its catabolism *in vivo*.

Generally, a C-D bond is more difficult to break down than its C-H counterpart. The explanation for this phenomenon is based on the so-called zero point energy in which the heavier deuterium atom has less vibrational energy than hydrogen. In practice, the C-D bond is

kinetically more difficult cleave than the corresponding C-H bond which decreases the metabolic rate. Thus, the replacement of hydrogen with deuterium in compounds can introduce important alterations in their metabolism and therefore have beneficial effects in their biological effects. The decreased metabolic rate makes less frequent dosings possible. This is exemplified in in the case the first FDA approved deuterated drug called deutetrabenazine, a deuterated analogue of tetrabenazine (Fernandez et al. 2017, Paton 2017). Deutetrabenazine is dosed twice daily compared to tetrabenazine which needs to be given three times each day. In phase III clinical trials, the heavy drug deutetrabenazine exerts less severe side effects in comparison with the parent drug (Mullard 2016). As a result of these findings, it has been speculated that significant clinical improvements might be achievable with certain deuterated derivatives (Timmins 2014).

3 *Aims of the Study*

Investigations into the roles of polyamines and the regulation of their metabolic enzymes, synthesis of new analogues and clinical trials and experiments have been ongoing for decades. However, in this field, there are several open questions. In this thesis, I have attempted to resolve a couple of important questions related to the selective preparation of diacetylated polyamines (publication I), polyamine metabolism-related anticancer effects of known Cu(II) chelating drug TETA (publication II), and anticancer effects of *N*-alkylated polyamine analogues and their variably deuterated derivatives (manuscript III).

(I) In the case of the preparation of natural polyamines, one obvious need is to develop a fast one-pot method to synthesize selectively terminally acetylated symmetric polyamines like N^1,N^8-diAcSpd and N^1,N^{12}-diAcSpm. The problem encountered in this case was how can one develop a reagent which reacts selectively only with primary amino groups in the starting polyamine. There are several methods available for this purpose in the literature, but they either require the use of protection groups, long reaction time (days) or their selectivity is poor.

(II) Since copper plays an important role in cancer development and progression, Cu(II) chelators, including TETA, have been postulated as potential anticancer drugs. However, although TETA has been in clinical use for decades for the treatment of Wilson's disease, a complete understanding of its pharmacology and its effects on the polyamine metabolism is lacking. In this study, the effects of TETA as an anticancer agent on polyamine pathways were studied in comparison with two other Cu(II) chelators, D-pen and TTM.

(III) Like copper, polyamines are essential for cellular proliferation and thus their metabolism represents a potential anticancer target. Since they are anticancer agents that target polyamine metabolism, terminally *N*-alkylated polyamine analogues have attracted the attention of researchers due to their abilities to interfere with multiple steps in polyamine metabolism. Many of these analogues have been earlier shown to be degraded by APAO and/or SMO, giving rise to the question of whether the analogues themselves, or their metabolic products/by-products, or both, are the cause of their cytotoxicity. In an attempt to resolve this question, selective deuteration of DESpm, BnEtSpm and DBSpm was used to modify the catabolism of these analogues. Their cytotoxicity and effects on polyamine metabolism were investigated in cell culture.

4 Materials and Methods

Many different chemical, molecular, cellular and biological methods were used in this study. The main methods used in this study are described in the following chapters. Details of the methods used are described in the original papers I-III.

4.1 NMR SPECTROSCOPY (I)

^{1}H and ^{13}C NMR spectra were recorded using a Bruker AVANCE DRX spectrometer operating at 500.1 and 125.8 MHz, respectively. ^{1}H and ^{13}C NMR chemical shifts (δ) are reported in parts per million (ppm) and referenced to solvent signals at 7.26 (residual solvent proton signal) and 77.16 ppm in $CDCl_3$, respectively, or sodium 3-(trimethylsilyl)-1-propane sulfonate (TSP) for D_2O. Normal $^{3}J_{HH}$ coupling constants are indicated with the letter "*J*" and given in Hz.

4.2 SYNTHESIS OF DIACETYLATED POLYAMINE COMPOUNDS (I)

All of the chemicals were purchased from commercial sources and used without further purification. All reactions were performed under an argon atmosphere.

Amine (1.33 mmol) containing one primary amino group and phenyl acetate (1.2 eq, 1.60 mmol) were stirred for 0.5 or 1 h in 5 mL of acetonitrile (ACN). ACN was evaporated *in vacuo* and the conversion was monitored by ^{1}H NMR spectroscopy. The synthesis of N^{1},N^{8}-diacetylated Spd hydrochloride and N^{1},N^{12}-diacetylated Spm hydrochloride was achieved by using 0.5 h reaction time and 2.2 equivalents of phenyl acetate.

Positive electrospray ionization (ESI) mass spectra were obtained with an Applied Biosystems/MDS Sciex QSTAR XL spectrometer.

4.3 CELL CULTURE EXPERIMENTS (II, III)

The human prostate cancer (DU145, 22RV1, PC3), breast cancer (MCF7), and placental choriocarcinoma (JEG3) cell lines were obtained from American Type Culture Collection (ATCC). DFMO was obtained from ILEX Oncology Inc, USA. [^{14}C]-labelled Spm tetrahydrochloride (specific activity 112 mCi/mmol) and Spd trihydrochloride (specific activity 113 mCi/mmol) used in these studies were obtained from GE Healthcare. [^{14}C]-Put dihydrochloride (specific activity 100 mCi/mmol) was purchased from American Radiolabelled Chemicals.

The cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) with GlutaMAX containing 4.5 g/l glucose (Gibco) and supplemented with 10% heat-inactivated fetal bovine serum (FBS, Sigma-Aldrich) and 50 µg/mL gentamycin (Sigma-Aldrich). The cells were incubated in a humidified atmosphere (+37 °C, 5% CO_2). The cells were collected with 0.25% trypsin/1 mM EDTA/ phosphate-buffered saline (PBS)-solution and counted in a Coulter Counter model Z1 (Coulter Electronics). Cell pellets were solubilized in homogenization buffer and incubated for 20 min on ice. Samples for Cu(II) analysis and polyamine measurement were taken, the rest of the lysate was centrifuged for 20 min at 13000 rpm (16060 ***g***) at +4°C and the obtained supernatant was used for enzyme activity assays, Western blotting and also isoelectric focusing (IEF)-immunoblotting.

4.3.1 Western blotting and IEF-immunoblotting of eIF5A (II)

Samples containing 50 µg of protein per lane were separated on 15% SDS-polyacrylamide gels and then transferred to polyvinylidene fluoride (PVDF) microporous membrane. AZ1 (OAZ1) protein was detected using rabbit anti-mouse OAZ1 antibody and HRP-conjugated goat anti-rabbit secondary antibody. The signal was detected with ECL Plus reagent and the membrane was scanned with Typhoon 9400 imager. IEF-immunoblotting of eIF5A was done as previously described on slab-IEF-gels pH 4-7 (Hyvönen et al. 2007).

4.3.2 Uptake experiments (II, III)

The cells were plated onto 6-well plates (0.25 x 10^6 cells/well) and incubated with the tested compounds **(II)**. The wells were then washed with warm PBS and incubated for 10 min with a pre-warmed (+37°C) labelling mixture containing 10 μM [^{14}C]-labelled Spd, Spm or Put (specific activity 25 mCi/mmol). The wells were washed twice with ice-cold PBS and the cells were solubilized with 0.5 M NaOH and the lysates were used for radioactivity determination. The counts were normalized to theDNA amount in the sample.

Competition experiments **(III)** of *N*-alkylated polyamine analogues with [^{14}C]-labelled Spm (10 µM, 25 mCi/mmol) were performed as described previously (Hyvönen et al. 2009).

4.3.3 Conversion of [^{14}C]-Put to [^{14}C]-CO_2 (II)

DU145 cells on 10-cm plates were plated and treated with TETA or SC for 24 h. The plates were washed with warm PBS and incubated with [^{14}C]-Put in growth medium diluted 1:10 with PBS (containing ~2.5 mM glucose) for 6 h. A Whatman 3MM filter paper square containing Solvable (PerkinElmer) was kept on the plate ceiling during incubation. Then, 1/10 medium volumes of 2M citric acid were added. After a further 15 min incubation, the papers were removed and counted for radioactivity. The cells were lysed in 0.5 M NaOH and a portion of the lysate was used to count radioactivity and the DNA amount.

4.3.4 Detection of intracellular ROS (II)

Cells were plated onto black, clear bottom 96-well plates and treated for 3 days with the tested compounds. Then, 2'-7'-dichlorofluorescein diacetate (DCFDA) was added to obtain a final concentration of 10 µM, and the incubation was allowed to continue for 1 h. Fluorescence at excitation at 495 nm and emission at 529 nm was read. Wells containing medium, compounds and DCFDA but no cells were used as controls for medium/compound autofluorescence. Fluorescence intensity was normalized to the protein amount in the well.

4.4 ANIMAL EXPERIMENTS (II)

SSAT-deficient mice (Niiranen et al. 2006) on CH57Bl/6J background were injected intraperitoneally (i.p.) with aminoguanidine (AG, 25mg/kg) (an inhibitor of Cu(II)-containing amine oxidases), or AG+TETA (300mg/kg). After 4 h, the mice were injected i.p. with 0.1 µCi of [^{14}C]-Put and euthanized 1 h later. Perfusion with ice-cold PBS was performed, tissues were collected, snap-frozen in liquid nitrogen and stored at -80°C until analyzed. The tissues were homogenized in ice-cold lysis buffer using TissueLyzer at 30 Hz for 2.5 min (Millipore), a portion of sample for polyamine measurement was taken and the rest of the homogenate was centrifuged for 20 min at 13000 rpm (16060 *g*) at +4°C. The obtained supernatant was used for enzyme activity assays.

To measure tissue accumulation of [^{14}C]-Put, 50 mg of tissue was dissolved in 300 µl of Solvable (PerkinElmer) at 65°C. The radioactivity of samples was analyzed by scintillation counting using liquid scintillation cocktail (Optiphase 'SuperMix'). The counts were normalized to protein amount in the sample. All animal procedures followed the European convention for the

protection of vertebrate animals used for experimental and other specific purposes (ETS No. 123, 170).

4.5 ENZYME ACTIVITY ASSAYS (II, III)

4.5.1 SSAT and amine oxidase assays

SSAT assay was done essentially as described (Libby et al. 1989). The measurement is based on the use of radioactive Ac-CoA, from which radioactive acetylgroup is transferred to Spd by SSAT enzyme. Acetylated Spd, which is cationic, is bound to cation-binding P81 filter paper, and radioactivity is counted after washing away the non-bound label. SMO, APAO, DAO, and SSAO activities were measured as described ealier with some modifications (Wang et al. 2001). The reaction is based on horseradish peroxidase (HRP)-coupled assay to detect the formation of H_2O_2. Additionally, SMO activity was assayed by measuring the conversion of (*S*,*S*)-Me_2Spm to (*S*)-MeSpd with HPLC. Details of sample volumes, preparation, and specific activities of the compounds are described in the original papers.

4.5.2 ODC and AdoMetDC activity assays

ODC activity was assayed as described earlier (Janne and Williams-Ashman 1971). The ODC assay is based on the use of [^{14}C]-*L*-ornithine (specific activity 100 µCi/ml), from which radioactive CO_2 is released by the action of the ODC enzyme. Released radioactive CO_2 is trapped by base-containing folded papers. The AdoMetDC activity assay is technically identical to that of ODC, but with a different reaction mixture and with [^{14}C]-decarboxylated *S*-adenosyl-*L*-methionine used as the substrate.

4.6 DNA AND PROTEIN CONCENTRATIONS (II, III)

DNA concentrations were measured with a PicoGreen reagent kit (Invitrogen) according to the manufacturer's instructions, using dilutions of calf thymus DNA (Sigma-Aldrich) as standards. Protein concentrations were measured by using Bio-Rad protein kit with dilutions of bovine serum albumin (BSA) (Bio-Rad Laboratories) as standards.

4.7 POLYAMINE, POLYAMINE ANALOGUE AND COPPER ANALYSIS (II, III)

The polyamine levels were measured by the HPLC-based method as described earlier (Hyvönen et al. 1992). The sample for polyamine determination was precipitated in sulfosalicylic acid (SSA) (5% (w/v) final concentration) solution containing 10 µM diaminoheptane (DAH) as the internal standard. The samples were vortexed and then centrifuged for 30 min at 13000 rpm (16060 *g*) at +4°C. The supernatants were filtered through a 0.22 µm filter and run with HPLC with post-column OPA derivatization as described previously (Hyvönen et al. 1992). For analysis of *N*-alkylated analogues (III), the samples were pre-derivatized with dansyl chloride before HPLC analysis (Kabra et al. 1986). The results were normalized to cell, protein or DNA amount in the sample. Protein and/or DNA concentrations were measured from polyamine sample pellets dissolved in 0.1 M NaOH.

Cu levels were measured using ZEEnit 700 GFAAS (Analytik Jena AG) from 100 µl cell lysates and tissue homogenates, which were first decomposed with 500 µl of Suprapur HNO_3 using microwave-assisted acid-hydrolysis and diluted to 3 ml with 0.01% Triton X-100 (v/v).

4.8 STATISTICAL ANALYSIS (II, III)

Data are expressed as the mean ± S.D. One-way analysis of variance (ANOVA) with Dunnet's *post hoc* test being used for multiple groups comparisons (unless otherwise indicated) with the aid of GraphPad Prism 5.03 software (GraphPad software Inc., USA). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

5 Results and Discussion

In this chapter, the main results are shown and summarized. The detailed results and discussion can be found in the original papers I-III.

5.1 SYNTHESIS OF DIACETYLATED POLYAMINES (I)

5.1.1 Diacetylated polyamines

Acetylation of polyamines by SSAT is known as one of the key catabolic steps that regulates polyamine levels (Figure 2 on page 4). Acetylated polyamines are not only needed as standards for spectroscopic and biochemical analyses but they are also advantageous in clarifying how they affect polyamine metabolism.

The selective acetylation of primary amino groups in the presence of secondary amino groups or other nucleophilic groups in a polyamine compound is still challenging. However, ethyl trifluoroacetate has been successfully used in the selective protection of primary amino groups of Spm (Blagbrough and Geall 1998). The acetylating agent, phenyl acetate, is known to react efficiently with aliphatic diamines (Bruice and Willis 1965) and has been exploited for mono-acylation of polyamines in the presence of water (Pappas et al. 2009). In addition, *N*-methoxydiacetamide has been used for the selective acetylation of primary amines in the presence of alcohols or secondary amines (Kikugawa et al. 1990).

Diacetylated Spd and Spm (N^1,N^8-diAcSpd and N^1,N^{12}-diAcSpm) have been recognized as promising metabolites for the detection of various cancers (Hiramatsu et al. 1997, Samejima et al. 2010, Nakayama et al. 2012). The chemoselective acetylation is the most efficient way to synthesize these compounds. Chemoselective acetylation of Spd has been achieved by using *N,N*-diacetylaminoquinazolinone (Atkinson et al. 1996) which is not commercially available. Thus, there is a need for a simple and selective acetylation method to prepare e.g. N^1,N^8-diAcSpd and N^1,N^{12}-diAcSpm in a one-pot procedure (Figure 10). In the next chapter, the novel synthetic method of these compunds will be described in detail.

5.1.2 The novel synthesis method of N^1,N^8-diAcSpd and N^1,N^{12}-diAcSpm

Based on above literature, a theoretical knowledge of good leaving groups and our preliminary NMR tests, we decided to study the use of phenyl acetate as a selective acetylation regent for primary amino groups. Moreover, it was speculated that this reagent could potentially make it possible also to add other carbonyl moieties (RCO-) to primary polyamines, since the PhO^- (phenoxy) group is a much better leaving group than RO^- (alkoxy).

A good conversion of both primary amino groups of both Spd and Spm was achieved within 0.5 h using a 2.2-fold molar excess of phenyl acetate as a selective acetylating agent (Figure 10). After the acetylation step, the solvent ACN was evaporated *in vacuo* and the synthesized polyamines were dissolved in EtOH and converted into their hydrochloride salts by passing HCl gas into the reaction mixture. In the initial trials to precipitate the polyamines, we detected an undesired cleavage of the acetyl groups. This cleavage could be prevented by adding HCl gas into the reaction mixture over 0.5 h with efficient cooling to 0 ºC. Both of the products were isolated by simple filtration, resulting in the target compounds with >95% purity in reasonable yields (50% for N^1,N^8-diAcSpd and 41% N^1,N^{12}-diAcSpm) without any further steps. NMR spectroscopy was used to analyse the purity and chemical structures of compounds.

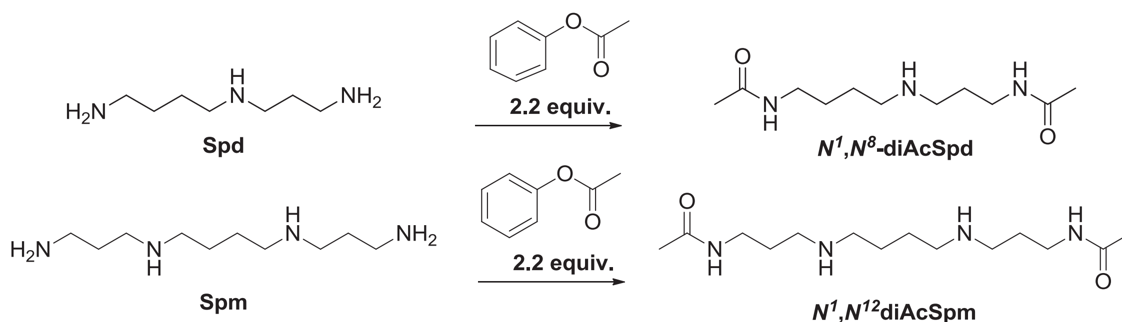

Figure 10. Synthesis of N^1,N^8-diAcSpd and N^1,N^{12}-diAcSpm using phenyl acetate.

5.1.3 The use of the diacetylated polyamines

Mono- and diacetylated polyamines are natural products of SSAT-catalyzed reactions, and they are recognized as potential cancer biomarkers. In particular, both N^1,N^8-diAcSpd and N^1,N^{12}-diAcSpm are present at high levels in the urine of human cancer patients (Hiramatsu et al. 1997). Additionally, N^1,N^{12}-diAcSpm is a more sensitive cancer biomarker than N^1,N^8-diAcSpd (Umemori et al. 2010). Since these polyamine compounds are available now to be used as standards, this provides the opportunity to analyze the levels of free and acetylated polyamines from urine samples and to investigate the differences in the excretion of these compounds between human cancer patients and healthy individuals. In addition, they may be also used to investigate how these compounds affect polyamine metabolism, cell proliferation and differentiation.

5.2 ANTICANCER EFFECTS OF TETA VIA POLYAMINE AND ENERGY METABOLISM (II)

TETA is known to exert antiproliferative effects in multiple cell lines, but earlier its mechanism of action was thought to be solely attributable to Cu(II) chelation, despite the fact that it is an analogue of the polyamine, Spd. In this study, the effects of TETA on polyamine metabolism were investigated in detail. Table 4 summarizes the main findings.

Table 4. The effects of TETA on polyamine metabolism.

- polyamine pools: Put ↓, Spd ↓, Spm ↓
- polyamine uptake *in vitro* and *in vivo* ↓
- polyamine uptake *in vitro* in the presence of DFMO ↓
- ODC activity *in vitro* and *in vivo* ↓
- AZ1 ↑
- hypusinated eIF5A ↓
- SMO activity *in vitro* and *in vivo* ↓
- SSAO/DAO ↓
- [^{14}C]-Put conversion to [^{14}C]-CO_2 (via TCA cycle) ↓

5.2.1 Inhibition of cell proliferation, polyamine uptake and hypusination of eIF5A

In our previous study, we showed that treatment with low doses of TETA (50 µM) in DU145 cells for a short time exerted only minor effects on both cell proliferation and polyamine metabolism (Weisell et al. 2010b). Therefore, in this present study, higher concentrations of TETA were used. First, we studied the effects of TETA (1 mM) in DU145 prostate cancer cells on cell proliferation and polyamine metabolism in comparison with other Cu(II) chelators, D-pen (1 mM) and tetrathiomolybdate (TTM, 50 µM). Short term (1-2 days) treatment of DU145 cells with TETA, D-pen and TTM did not evoke any cytotoxicity, but after a longer time, i.e. 7 days' treatment, especially TETA and TTM clearly decreased the growth of the cells. Furthermore, both of these compounds also decreased intracellular copper levels efficiently. D-pen had almost no effect on cell proliferation even after 7 days' treatment, because most probably it did not deplete intracellular copper levels as efficiently as could be achieved by treatment with TETA and TTM. On the other hand, we demonstrated that TETA exerted significant effects on intracellular polyamine levels; TETA depleted intracellular Put and Spd levels, and even decreased Spm levels after 4-7 days of treatment. Unlike TETA, D-pen and TTM in some undetermined manner increased the intracellular polyamine levels. Since cancer cells have high polyamine levels and increased uptake to maintain rapid cell growth (Soda 2011), depletion of polyamine levels and inhibition of their uptake is considered as a desirable feature for an anticancer drug.

Furthermore, we studied the mechanisms by which TETA decreased polyamine pools. TETA is a polyamine analogue which likely has an effect on polyamine transport. The pre-treatment of DU145 cells with 1 mM TETA before the uptake assay produced a high reduction of [^{14}C]-Spd, [^{14}C]-Spm and [^{14}C]-Put uptake. However, D-pen, even at a high (2.5 mM) concentration in DU145 cells, did not have any effect on the [^{14}C]-Spd uptake, which might be because it is a less potent copper chelator than TETA or TTM. On the other hand, TTM dose-dependently inhibited [^{14}C]-Spd uptake in DU145 cells likely due to its copper chelating properties, as polyamine uptake is copper-dependent.

Similarly as the situation in DU145 cells, TETA decreased the [^{14}C]-Spd uptake in MCF-7 breast adenocarcinoma cells and JEG-3 placental choriocarcinoma cells. D-pen and TTM exerted no effect on the uptake of [^{14}C]-Spd in MCF-7 cells while both compounds slightly reduced the [^{14}C]-Spd uptake in JEG-3 cells.

DFMO treatment increases polyamine uptake in cancer and normal cells. Therefore, preventing the DFMO-induced increase in polyamine uptake is a desirable feature in combination chemotherapy. Research groups are working to develop polyamine uptake inhibitors; these will be used in combination with DFMO or on their own (Muth et al. 2014). In this study, one of the most important findings was that TETA completely prevented the DFMO-induced increase in [^{14}C]-Spd uptake. It is well known that even as a single agent, DFMO has been shown to be efficient in the treatment of some cancer types. Both TETA and DFMO are well tolerated and are already being used in the clinic for various applications.

Additionally, we found a 2-fold increase in the intracellular copper level after one day of DFMO treatment. Based on our findings, Cu(II) is required for polyamine uptake.

Hypusinated eIF5A is required for eukaryotic proliferation, and Spd is the only known natural precursor of hypusine (Park et al. 2010). The fact that TETA decreased Spd levels indicated that it might inhibit the cell growth through the inhibition of the hypusination of eIF5A. Our results revealed that treatment of DU145 cells for 4-7 days' with TETA depleted the level of hypusinated eIF5A and caused the accumulation of the precursors forms, whereas this did not occur after treatment with D-pen or TTM.

5.2.2 Induction of AZ1 and downregulation of ODC

Polyamine transport (uptake and export) are regulated by a small class of inhibitory proteins called AZs that also bind to ODC and form an ODC-AZ complex which is then targeted for degradation (Mangold 2005). Three AZs are currently known with AZ1 (OAZ1 in **II**) being the

most abundant. Polyamines also stabilize AZ proteins by inhibiting their degradation in the proteasome (Palanimurugan et al. 2004).

In order to investigate whether TETA induced AZ1, a Western blotting method was used to assay cells cultured in the presence or absence of TETA and the proteasomal inhibitor, MG132. MG132 was used in order to block AZ1 degradation. We found that TETA induced the synthesis of the AZ1 protein, and dramatically decreased ODC activity. D-pen did not inhibit ODC activity whereas TTM after 7 days' treatment decreased ODC activity. TTM had no effect on intracellular polyamine levels; this might be because of the low concentration of TTM (50 µM) which was used; this exerted only a minor inhibitory effect on polyamine uptake.

5.2.3 Inhibition of polyamine oxidation

We next studied the effect of TETA on polyamine catabolism. It is known that polyamines can be metabolized by SMO, DAO and SSAO while generating H_2O_2 and reactive aldehydes as byproducts (Seiler 2004). Typically, cancer cells have intense H_2O_2 production and high levels of oxidative stress; low concentrations of H_2O_2 have been shown to stimulate their proliferation (Burdon 1995). In human colorectal cancer, DAO activity was found to be significantly lower compared to normal tissues and polyamine levels were observed to be significantly higher in comparison to normal surrounding tissues (Linsalata et al. 1993). Moreover, in another study, a higher DAO activity and elevated polyamine levels were found in human ovarion, cervical and endometrial carcinomas compared to their normal surrounding tissues (Chanda and Ganguly 1995). Additionally, SSAO inhibitors have been found to suppress tumor growth in mice (Li et al. 2013).

It was shown previously that the metabolites of TETA, called N^1-AcTETA and N^1,N^8-diAcTETA, inhibited SMO *in vitro* (Weisell et al. 2010b). In the present study, we examined TETA and its metabolites, N^1-AcTETA and N^1,N^8-diAcTETA, in comparison with D-pen and TTM as inhibitors of pig kidney DAO and bovine plasma SSAO, and found that TETA inhibited both enzymes i.e. DAO and SSAO, in a dose-dependent manner. As it is known that DAO and SSAO are copper-dependent enzymes, it was speculated that they could be inhibited by other Cu(II) chelators like D-pen and TTM. Indeed, D-pen and TTM dose-dependently inhibited both SSAO and DAO. Furthermore, N^1-AcTETA was found to be as an efficient inhibitor of DAO as TETA itself, but much less inhibitory towards SSAO, whereas TETA inhibited SSAO more efficiently than DAO. N^1,N^8-diAcTETA had only a minor effect on both DAO and SSAO enzyme activities.

We also studied the effect of TETA on polyamine oxidation in DU145 cells. TETA decreased SMO and DAO/SSAO activities in DU145 cells. Furthermore, TETA decreased the intracellular ROS. Decreased polyamine oxidation was associated with a general reduction in intracellular ROS, which was measured by DCFDA staining.

5.2.4 Effects of TETA on energy metabolism

Polyamines play a role in energy metabolism, being used as an energy source via the tricarboxylic acid (TCA) cycle (Seiler and Al-Therib 1974) as already described in chapter 2.3.4. Since the concentrations of highly consumed nutrients, such as glucose, are generally lower in cancer cells compared to normal cells, cancer cells may use this alternative pathway to meet their increased energy requirements (Andersson et al. 1980). One piece of supporting information from another study was that DAO activity was greater in highly tumorigenic cell lines than in their poorly tumorigenic counterparts (Thomasset et al. 1982). Here we found that TETA not only inhibited DAO/SSAO activity in DU145 cells, but it also efficiently decreased the production of [^{14}C]-CO_2 from [^{14}C]-Put during a 6 h incubation in low-nutrient medium.

5.2.5 Effects of TETA *in vivo*

Another important finding was that TETA inhibited [^{14}C]-Put uptake also *in vivo*, in kidney, lung and liver already 4 h after a single injection (300 mg/kg. i.p.). The uptake of TETA in brain was

slightly, but not statistically significantly, decreased, possibly because of its poor penetration through the blood-brain barrier. Furthermore, TETA decreased renal ODC and SMO activities.

5.3 THE DEUTERATED *N*-ALKYLATED POLYAMINE ANALOGUES AND THEIR PARENT ANALOGUES (III)

Many terminally *N*-alkylated polyamine analogues are metabolized by SMO and APAO enzymes (Bergeron et al. 1995, Bergeron et al. 1997). Although both SMO and APAO might have a significant effect on the biological efficacy of polyamine-based drugs, there have been very few metabolic studies conducted with both purified enzymes and *N*-alkylated polyamine analogues (Lawson et al. 2002, Wang et al. 2003, Wang et al. 2005). Previously, the compounds called DESpm, BnEtSpm, DBSpm and their degradation have been studied *in vitro* with recombinant SMO and APAO, as well as in DU145 prostate cancer cells (Häkkinen et al. 2010). However, it has been postulated that H-D exchange might be used to redirect the oxidative catabolism. Thus, a number of selectively deuterated polyamine analogues were synthesized to prove the alteration of catabolic pathways (Figure 11).

In this study, the substrate properties of the *N*-alkylated Spm analogues compared to their deuterated analogues were studied using human recombinant SMO and APAO. Furthermore, the biological assays were conducted to evaluate cellular growth and catabolism in cancer cells as well as in non-cancerous mouse fetal fibroblasts (MEFs). The results of this study provide significant data of the substrate properties of these analogues, especially their catabolism by these two crucial enzymes; this information will be beneficial for the future drug design.

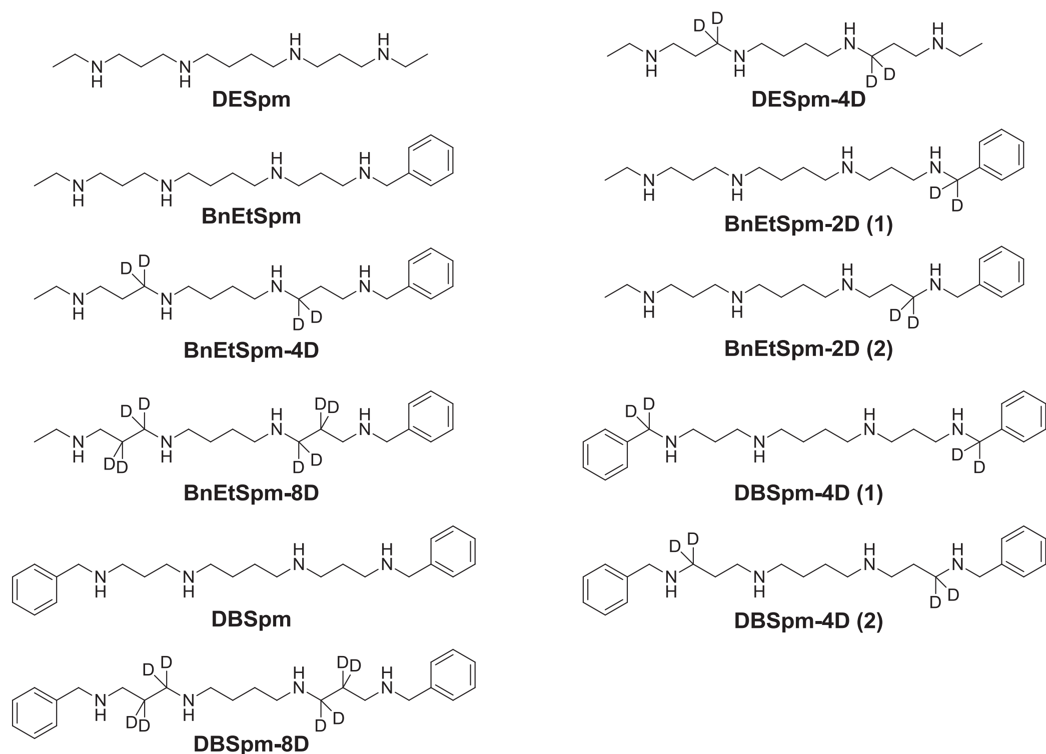

Figure 11. Chemical structures of *N*-alkylated Spm analogues and their variably deuterated polyamine derivatives.

5.3.1 *N*-Alkylated Spm analogues and their metabolites as substrates and inhibitors for amine oxidase enzymes

First, we screened the tested analogues using enzymatic coupled homovanillic acid-HRP assay to measure the degradation of the analogues and their metabolites by SMO, APAO, SSAO, DAO, MAO-A and MAO-B. The results were compared based on H_2O_2 release from the enzymes' natural substrates (Spm, *N*[1]-AcSpd, Spm, Put and p-tyramine, respectively) set as 100%.

DESpm was found to be the best analogue substrate for SMO, producing 22% of H_2O_2 while DESpm-4D generated only 4% of H_2O_2. Both BnEtSpm and DBSpm were equally good substrates for SMO, producing 12% of H_2O_2. The deuterated analogues BnEtSpm-8D and DBSpm-4D(1) decreased H_2O_2 generation to 6% and 7%, respectively. The metabolites of the analogue, EtSpm were found to be as good a substrate for SMO as Spm, and some reactivity towards BnSpm (15%) and BnSpd (4%) could be detected.

According to our results with APAO, DBSpm produced almost 100% of H_2O_2 and deuteration at the preferred cleavage sites decreased H_2O_2 production to 82% (DBSpm-8D) and 70% (DBSpm-4D(1)). The other tested analogue, BnEtSpm, generated 40% of H_2O_2 and unexpectedly only the di-deuteration at the exo-*N*[1] site of the analogue, BnEtSpm-2D(1) decreased significantly H_2O_2 generation to 18%. DESpm was found to be the weakest substrate for APAO, producing only 17% of H_2O_2 which diminished to less than 2% with DESpm-4D. The metabolites of this analogue, BnSpd (68%), BnSpm (64%) and EtSpm (6%) were substrates for APAO. Thus, deuteration at the preferred cleavage site decreased H_2O_2 generation. The analogues were better substrates for APAO in comparison to SMO.

Additionally, for SSAO, only DESpm showed some substrate-related properties, producing 5% of H_2O_2 in comparison to Spm. Mono-*N*-alkylated EtSpm and BnSpm were substrates of SSAO. Only EtDAP was metabolized by DAO, generating 30% H_2O_2 in comparison to Put.

The analogues and their metabolites were very poor substrates for human recombinant MAO-B, all producing <1% of H_2O_2 as compared to p-tyramine. Under the same experimental conditions, they were also poor substrates for human recombinant MAO-A, generating only <3% of H_2O_2, with the exception of BnDAP, which produced 23% H_2O_2.

Based on our findings, we showed that the main enzymes responsible for the degradation of the analogues were APAO and SMO while SSAO, DAO, MAO-A and MAO-B degraded only some of their metabolites which had free amino termini.

We next studied these analogues and their metabolites which had shown poor substrate properties, for their effects as inhibitors of SSAO, DAO, APAO and SMO. We used concentrations of 50 μM substrate (Spm, Put, *N*[1]-AcSpd and Spm, respectively) for each enzyme and 50 and 500 μM of inhibitor. Based on our findings, SSAO was inhibited especially by BnSpd and also in decreasing rank order by EtSpd and DBSpm. DAO was inhibited dramatically by EtSpd and BnSpd. EtSpd was found to be the most efficient inhibitor of APAO whereas in the case of SMO, there was approximately 50% inhibition detected for 500 μM EtSpd, BnSpd and Spd.

5.3.2 *N*-Alkylated Spm analogues as substrates of human recombinant SMO and APAO

We studied the reaction products and kinetics with human recombinant SMO and APAO in more detail. DESpm to EtSpd is degraded by SMO and APAO (Häkkinen et al. 2010). The DESpm to EtSpd conversion by SMO was reduced from 20.0 ± 4.2 to 6.6 ± 0.3 μmol/min/μmol by deuteration. The kinetic values for DESpm to EtSpd degradation were measured; the following values were obtained K_m 23.6 μM, V_{max} 43.1 for DESpm compared to K_m 21.2 μM, V_{max} 6.7 for DESpm-4D. Furthermore, the degradation of DESpm to EtSpd by APAO was decreased from 42.1 to 12.9 μmol/min/μmol with deuterated DESpm, while the degradation of DESpm to EtSpm was increased from 0.5 to 3.8 μmol/min/μmol.

The degradation of BnEtSpm to EtSpd by SMO was reduced from 6.8 μmol/min/μmol down to 0.9 μmol/min/μmol with BnEtSpm-4D and to 1.0 μmol/min/μmol with BnEtSpm-8D, but slightly increased to 7.4 μmol/min/μmol with BnEtSpm-2D(1). Additionally, the degradation of

BnEtSpm-8D to BnSpd was reduced from 3.0 to 0.6 μmol/min/μmol. In the case of the degradation of BnEtSpm to EtSpd by APAO, this was decreased from 46.1 to 32.4 μmol/min/μmol with BnEtSpm-4D and -8D. There were no differences with the degradation of deuterated BnEtSpm-2D(2) and DBSpm-4D(2) analogues compared to their non-deutarated counterparts.

With DBSpm-8D, the production of BnSpd by APAO was decreased from 48.7 to 30 μmol/min/μmol and BnSpm production was increased from 3.7 to 11.8 μmol/min/μmol. Unlike DBSpm-8D, DBSpm-4D(1) increased BnSpd production to from 48.7 to 70.1 μmol/min/μmol, while decreasing the production of BnSpm from 3.7 to 0.5 μmol/min/μmol.

In summary, we observed that 3-6 fold reductions in the reaction velocity but only slight changes in affinity with the tested deuterated analogues in comparison to their non-deuterated analogues. In addition, the preferred cleavage site by SMO and APAO could be effectively changed by selective deuteration.

5.3.3 Uptake competition of analogues with Spm

All the tested analogues competed with [^{14}C]-labelled Spm for polyamine transport. Additionally, of all the tested analogues, the best competitor with Spm for the uptake was found to be the unsymmetrically substituted BnEtSpm; there was no difference in the affinity between its deuterated BnEtSpm-4D and BnEtSpm-8D isoforms. One interesting finding was that heavily deuterated DBSpm-8D had lower affinity for the transport than the parent analogue DBSpm. Therefore, it might be possible to change affinity for the polyamine transport system by deuteration of the carbon chain.

5.3.4 The effects of deuteration on polyamine metabolism

Treatment with 25 μM of DESpm and DESpm-4D decreased the activities of the two polyamine biosynthetic enzymes, ODC and AdoMetDC, induced SSAT and reduced the intracellular polyamine levels in DU145 prostate cancer cells within 1-3 days more significantly than the other tested analogues. Interestingly, no clear differences in the growth inhibition were observed between deuterated and non-deuterated analogues of DESpm. In addition, BnEtSpm was the best inducer of SMO activity. BnEtSpm and its deuterated analogues, BnEtSpm-4D and BnEtSpm-8D, were the most cytotoxic agents in DU145 cells exposed to 25 μM concentration for 3 days.

Additionally, we studied these analogues in isolated wild-type mouse primary fetal fibroblasts (MEF) as non-cancerous reference cells. These cells were more resistant to the analogue-mediated growth inhibition, thus 50 μM concentrations were used. Interestingly in MEF cells, DBSpm was found to be the most cytotoxic analogue. Similar to the situation in DU145 cells, the antiproliferative activity of the analogues did not correlate with either SSAT activities or polyamine levels, but did correlate with the induction of SMO activity. In MEFs, deuteration significantly affected the toxicity of the analogues; when tested at their IC_{50} values - BnEtSpm-2D(1) was most toxic of the BnEtSpm isotopomers and DBSpm-8D was less toxic than other DBSpm isotopomers, probably due to the high basal APAO activity.

Furthermore, we tested the non-deuterated parent analogues also in PC3, 22RV1 (prostate) and MCF7 (breast) cancer cell lines. We found that at 10 μM, BnEtSpm was the most cytotoxic in these cell lines and the cytotoxicity of the analogue correlated similarly with its ability to induce SMO activity rather than with its effects on SSAT activity or with total Spd+Spm level.

5.3.5 The mechanisms of growth inhibition of analogues

We also tested whether analogue-mediated growth inhibition could be prevented by pretreatment with inhibitors MDL 72527 or SC. Fetal bovine serum contains amine oxidases such as DAO and SSAO, which can act on the free primary amino groups of Put, Spd and Spm generating H_2O_2 and the highly toxic compound, acrolein, as by-products. It is known that the compound SC is an inhibitor of these enzymes. We found that SC had an increasing effect on the growth inhibition evoked by BnEtSpm, whereas it exerted no clear effects on the efficacies of either DESpm or DBSpm.

The treatment of DU145 cells with 250 µM of MDL 72527 for 2 days reduced the cytotoxic effect of BnEtSpm while it dramatically increased the cytotoxicity of DBSpm. Even alone, 250 µM MDL 72527 displayed a growth inhibitory effect, this is possibly due to its known lysosomotropic properties. At lower concentrations, such as at 10 µM, it had almost no effect on BnEtSpm or DBSpm efficacy, but it did partly reverse the growth inhibition caused by DESpm.

None of the analogue metabolites were found to be toxic at 10µM. However, at higher concentrations, i.e. 100µM, both EtSpm and BnSpm exhibited dramatic antiproliferative effects in DU145 cells. SC completely prevented the growth inhibition caused by EtSpm (a substrate of SSAO and SMO), and partly that of BnSpm (a substrate of SSAO, APAO and SMO), whereas 10 µM MDL 72527 prevented the growth inhibitory effect of BnSpd (a substrate of APAO and SMO) and BnDAP (a substrate of SSAO and MAO-A). The interpretation of the results with MDL 72527 are complicated, because MDL 72527 is a polyamine analogue and competes for uptake with natural polyamines and their analogues. In addition, in our *in vitro* analyses MDL 72527 inhibited also other oxidases, DAO and SSAO. Furthermore, some benzylated analogues have been shown to be taken up via additional mechanisms than polyamine transporter. Therefore further studies using cell lines deficient of various polyamine oxidases are warranted.

6 Conclusions and Future Perspectives

This thesis has expanded our initial knowledge of different aspects of the properties of polyamines. The main findings of papers 1-3 were as follows;

Paper 1: Although there are numerous methods available for the acetylation of primary amino groups, the novel approach that we developed achieved simple and selective acetylation based on phenyl acetate. In our method, there is no need to use any catalysts or specific supplementary reagents, such as indium triiodide (Ranu and Dutta 2003), or *N*-heterocyclic carbenes (Movassaghi and Schmidt 2005), and the compound can be obtained without any further purification step. The method developed here is simple, inexpensive and straightforward in comparison with previously published acetylation methods. Moreover, it seems that the developed phenyl acetate method is not only rapid but also highly selective for primary alkyl amino groups in preference to other nucleophilic groups like secondary alkyl amines, primary hydroxyl groups and primary aromatic amino (anilines) groups. Another considerable advantage of this present method is that no further time-consuming purification steps are required. One of the most important achievements of this work was the straightforward synthesis of the diacetylated polyamines N^1,N^8-diAcSpd and N^1,N^{12}-diAcSpm with good yields by our selective acetylation method with its simple purification procedure. Since diacetylated polyamines are recognised as early cancer biomarkers, these compounds can be used as analytical standards and also in biological assays as well as in cellular and animal models when investigating their biological functions.

Paper 2: The effects of TETA, a charge-deficient polyamine analogue of Spd, on the polyamine pathway were studied in comparison with two other Cu(II) chelators and anticancer agents, TTM and D-pen. Based on our findings, the treatment of DU145 prostate cancer cells with TETA inhibited polyamine biosynthesis and uptake, which led to a decrease in the intracellular levels of Put, Spd and hypusinated eIF5A. Moreover, TETA also inhibited polyamine oxidation by SMO and DAO/SSAO, and limited the use of Put as an energy source. It is important to inhibit the catabolic enzymes which generate the toxic byproducts, for example, H_2O_2, 3-AP and 3-AAP by SMO and APAO. Furthermore, rapid *N*-acetylation of TETA by SSAT2 and SSAT consumed cellular Ac-CoA pools, which potentially affected a variety of cellular pathways. One of the most important outcomes emerging from this study was that TETA decreased Put uptake and also ODC and SMO activities *in vivo*. Moreover, the study suggested that DFMO, the most widely studied single enzyme inhibitor of ODC, in combination with TETA could help in overcoming the problem of compensatory polyamine uptake from external sources and improve the blockade of biosynthesis. As single agents, both DFMO and TETA have been evaluated in different clinical applications, but their combination therapy should be first evaluated in experimental animal to determine whether this would achieve synergism. In summary, this study revealed that TETA is a promising multi-functional drug which inhibited cell proliferation both by chelating Cu(II) and by regulating polyamine and energy metabolism. Furthermore, based on our findings, further studies with TETA in combination with DFMO *in vivo* are warranted.

Paper 3: Degradation of terminally *N*-alkylated Spm analogues DESpm, BnEtSpm, DBSpm and their variably deuterated counterparts and their effects on polyamine metabolism and proliferation were studied. According to our findings, in comparison to their parent analogues, deuterium substitution in polyamine analogues at the preferred cleavage site, decreased more efficiently *in vitro* H_2O_2 production with two recombinant enzymes, SMO and APAO. Deuteration also switched the cleavage site so that the presence of deuterium at 1 position reduced debenzylation and deethylation, whereas deuteration at the 3,4 positions promoted

debenzylation and deethylation. One conclusion emerging from this study is that analogue degradation by APAO/SMO is not the major determinant of the compound's cytotoxicity. However, in those cells with high basal APAO/SMO activity, it is possible to control the oxidative catabolism of *N*-alkylated polyamines by selective deuteration. One of the most important findings of this work was that the antiproliferative efficacy of the analogues did not correlate with their ability to decrease ODC, AdoMetDC, induce SSAT or deplete intracellular polyamine levels but it did correlate with their ability of induce SMO. Furthermore, unsymmetrically substituted BnEtSpm was the most cytotoxic analogue in DU145, PC3, 22RV1 and MCF7 cancer cell lines; in contrast, in syngenic fibroblasts, the most toxic analogue was DBSpm. These deuterated analogues are interesting compounds which should be examined further in *in vivo* studies. These findings may be exploited in future drug development and discovery.

7 References

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Polyamines are low molecular weight aliphatic polycations, found in all kingdoms of life. Moreover, polyamine metabolism is often dysregulated in cancer, thus it has been, and still is, an attractive target for the development of anticancer drugs. This thesis focuses on to expand our knowledge in polyamine analogues as potential anticancer drugs and to investigate their roles on polyamine metabolism in detail.

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