

KUOPION YLIOPISTON JULKAISUJA G. - A.I. VIRTANEN -INSTITUUTTI 37
KUOPIO UNIVERSITY PUBLICATIONS G.
A.I. VIRTANEN INSTITUTE FOR MOLECULAR SCIENCES 37

AKI JÄRVINEN

α -Methylated Polyamine Analogues

Tools to Study Polyamine Metabolism and Polyamine Oxidase

Doctoral dissertation

To be presented by permission of
the Faculty of Natural and Environmental Sciences of the University of Kuopio
for public examination in Auditorium L22, Snellmania, University of Kuopio,
on Saturday 19th November 2005, at 12 noon

Department of Biotechnology and Molecular Medicine
A.I. Virtanen Institute for Molecular Sciences
University of Kuopio

Distributor: Kuopio University Library
P.O. Box 1627
FIN-70211 KUOPIO
FINLAND
Tel. +358 17 163 430
Fax +358 17 163 410
<http://www.uku.fi/kirjasto/julkaisutoiminta/julkmyyn.html>

Series Editors: Professor Karl Åkerman
Department of Neurobiology
A. I. Virtanen Institute for Molecular Sciences

Research Director Jarmo Wahlfors
Department of Biotechnology and Molecular Medicine
A. I. Virtanen Institute for Molecular Sciences

Author's address: Department of Biotechnology and Molecular Medicine
A. I. Virtanen Institute for Molecular Sciences
P.O. Box 1627
University of Kuopio
FI-70211 KUOPIO
Tel. +358 17 163672
Fax +358 17 163025
E-mail: aki.jarvinen@uku.fi

Supervisors: Professor Juhani Jänne, M.D., Ph.D.
Department of Biotechnology and Molecular Medicine
A. I. Virtanen Institute for Molecular Sciences
University of Kuopio

Professor Leena Alhonen, Ph.D.
Department of Biotechnology and Molecular Medicine
A. I. Virtanen Institute for Molecular Sciences
University of Kuopio

Reviewers: Professor Hannu Elo, Ph.D.
Division of Pharmaceutical biology
Viikki Biocenter 2
University of Helsinki

Docent Pauli Seppänen, Ph.D.
Savonia Polytechnic
Kuopio

Opponent: Professor Harri Lönnberg, Ph.D.
Department of Chemistry
University of Turku

ISBN 951-781-396-1
ISBN 951-27-0420-X (PDF)
ISSN 1458-7335

Kopijyvä
Kuopio 2005
Finland

Järvinen, Aki Juhani. α -Methylated Polyamine Analogues. Tools to Study Polyamine Metabolism and Polyamine oxidase. Kuopio University Publications G. – A. I. Virtanen Institute for Molecular Sciences 37. 2005. 63 p.
ISBN 951-781-396-1
ISBN 951-27-0420-X (PDF)
ISSN 1458-7335

ABSTRACT

The polyamines, spermidine and spermine as well as their precursor putrescine, are found in all mammalian cells. They are essential for cell growth, the maintenance of cell physiology and they participate in the regulation of cellular metabolism. Excess amounts of polyamines are associated with malignant growth whereas insufficient amounts evoke programmed cell death i.e. apoptosis. The cellular levels of polyamines are delicately controlled via a balance between synthesis, degradation, uptake and excretion. Furthermore, polyamines are effectively interconverted in concerted manner by spermidine/spermine N^1 -acetyltransferase, the enzyme that acetylates polyamines, and polyamine oxidase, which readily oxidizes acetylated polyamines. Spermidine/spermine N^1 -acetyltransferase is a very inducible enzyme when compared to polyamine oxidase which appears rather to be constitutively expressed throughout the mammalian system. All of the above metabolic events allow for fine-tuning of the amounts of each polyamine to match their use in essential cellular processes. However, the efficient interconversion of polyamines makes any assessment of the roles of each polyamine very challenging. Therefore, we utilized metabolically stable α -methylated polyamines that are able to mimic the crucial cellular functions of their natural counterparts.

Metallothionein promoter driven spermidine/spermine N^1 -acetyltransferase transgenic rodents have proven invaluable for studies aimed at elucidating the mechanisms and consequences of the interconversion of the polyamines. Recombinant proteins (spermidine/spermine N^1 -acetyltransferase, polyamine oxidase and recently discovered spermine oxidase) used in this study are excellent tools to evaluate the biological stabilities of different polyamines and their analogues. Furthermore, immortalized rat fibroblast cell lines derived from syngenic and the metallothionein promoter driven spermidine/spermine N^1 -acetyltransferase transgenic rat complete the tools available for a detailed exploration of polyamine metabolism.

Both α -methylspermidine and bis- α -methylspermine are resistant to spermidine/spermine N^1 -acetyltransferase-mediated acetylation. Neither polyamine nor spermine oxidase can degrade α -methylspermidine but both oxidases use bis- α -methylspermine poorly as a substrate. *In vivo*, both compounds are able to prevent acute pancreatitis and initiate early liver regeneration in the partially hepatectomized spermidine/spermine N^1 -acetyltransferase transgenic rat. Both of these above conditions are characterized by severe depletions of the natural polyamines.

Recombinant protein studies with enantiomers of acetyl- α -methylspermidine showed that polyamine oxidase expresses stereoselectivity for the (*R*)-isomer of α -methylspermidine though the natural substrates for this enzyme are achiral. Aldehyde supplementation in the reaction buffer has been shown to enhance polyamine oxidase-mediated oxidation. Surprisingly, the stereoselectivity of the enzyme becomes also changed in the presence of different aldehydes. Benzaldehyde enhances selectively the degradation of (*R*)- and pyridoxal (*S*)-isomer of α -methylspermidine from a racemic substrate mixture in glycine-NaOH buffer at pH 9.5. The same stereoselectivity is retained in other studied buffer systems at high pH and also at pH 7.4 though at a reduced rate. There are indications that the Schiff base between aldehyde and polyamine is the target for enzymatic catalysis by polyamine oxidase. For the first time, the stereospecificity of a FAD-dependent oxidase has been shown to be guided by simple aldehyde supplementation in aqueous media. Understanding these phenomena may help clarify FAD-dependent oxidative reactions and may eventually lead to some applications in biocatalysis.

National Library of Medicine Classification: QU 61, QU 55, QU 450, WI 702, WI 805

Medical Subject Headings: polyamines/metabolism; spermidine/analogs & derivatives; spermine/analogs & derivatives; putrescine; homeostasis; metallothionein; pancreatitis/prevention & control; liver regeneration; fibroblasts; recombinant proteins; acetyltransferases; oxidoreductases; molecular conformation; aldehydes; benzaldehydes; Schiff bases; transgenes; animals, genetically modified; rats; humans

ACKNOWLEDGEMENTS

This study was carried out in the A. I. Virtanen Institute for Molecular Sciences, University of Kuopio, during 2001–2005.

I am indebted to my supervisors, Professor Juhani Jänne, M.D., Ph.D. and Professor Leena Alhonen, Ph.D. for the possibility to work under such flexible and understanding guidance. I admire the grace and professionalism of both of you.

I thank Professor Hannu Elo, Ph.D. and Docent Pauli Seppänen, Ph.D. for invaluable comments as the official referees for this thesis. I also thank Ewen MacDonald, Ph.D., for such prompt language revision of the manuscript.

I received very helpful and plentiful advice from Professor Jouko Vepsäläinen, Ph.D., and the assistance of Ale Närvänen, Ph.D. is humbly acknowledged. Alex Khomutov, Ph.D. and Kolya Grigorenko, Ph.D., the co-workers and co-authors from Moscow University are sincerely thanked.

I also want to thank Kazuei Igarashi, Ph.D., Chiba University, Japan, for inviting me into his laboratory for the next two years and for the giving me possibility to study Japanese culture.

I started working in the JJ-group in 1994 and my life has been enriched by innumerable and colorful events ever since. Many scientists and fascinating individuals have passed through the A. I. Virtanen Institute in Bioteknia I, where I have primarily worked, and the Institute of Applied Biotechnology in Bioteknia II, where I enjoyed the pleasures of teaching and supervising students for three years. If I cannot remember by name everyone who has inspired my professional journey, please, do not take offence.

Past and present, senior and junior, here are some of my fellow scientists in the JJ-group: Tuomo Keinänen, B.Med., Ph.D., deserves mentioning for his ever-cheerful optimism, even in the middle of most challenging results. I also want to thank Anne Uimari Ph.D., for great advice on making good science and Marko Pietilä, Ph.D., for keeping my spirits up. Kyllikki Kaasinen, Ph.D., Tiina-Liisa Räsänen, Ph.D., Suvikki Suppola, Ph.D., Sami Heikkinen, Ph.D., Veli-Pekka Korhonen, Ph.D., every one of you is well remembered. Heartfelt thanks belong to Kirsi Niiranen, M.Sc., for introducing me to the wonders of cell culture and other joys of lab work. The delightful company of Eija Pirinen, M.Sc., Mervi Hyvönen, M.Sc., Mari Merentie, M.Sc., Maija Tusa, M.Sc., Marc Cerrada-Gimenez, M.Sc. and Teemu Kuulasmaa, M.Sc. with our latest arrivals Terhi Rouvinen M.Sc. and Susanna Boman B.Sc. are all warmly acknowledged.

The patience and skilled assistance of the technical personnel is much appreciated, individuals who seldom get enough praise. A huge "thank you" belongs to Tuula Reponen for making sure our HPLCs never run out of buffers. Anne Karppinen and Arja Korhonen deserve mentioning for carrying me through the last few hectic weeks. Maritta Salminkoski is warmly acknowledged for providing the invaluable aldehydes needed in this study. Sisko Juutinen, Anu Heikkinen, Marita Heikkinen and Eeva Hakala have been essential in keeping the wheels turning. Pekka Alakuijala, Phil.Lic. has kept our equipment running for many, many years. Special thanks go to Riitta Sinervirta for always being available and for making sure that we never run out of money.

I also want to thank the people in the Institute of Applied Biotechnology and particularly Professor Maria Halmekytö, Ph.D., Tiina Pitkänen-Arsila, Ph.D., Kati Ruotsalainen, M.Sc., Mikko Järvinen, M.Sc., Heikki Koskinen, M.Sc., Marketta Lämsä, Helena Könönen and Elina Reinikainen.

I owe deepest gratitude to Docent Riitta Keinänen, Ph.D., and Raili Rytöluoto-Kärkkäinen for helping me along the years with their inexhaustible good spirits. Extra thanks go to Jarmo Wahlfors, Ph.D., making sure that the final steps of finishing this book did not falter.

Great friends are priceless and the following are in no particular order. The Pulkkinen "brothers", Jukka, Mika and Vexi, bikers to the heart and the lively personnel of the long-gone "Monastery of Vuorikatu" including Heikki, Jontti, Junkman, Tirre and Valpuri, great memories with of all of you are colorfully painted on the canvas of my mind. Nuha, Pallo and Santeri, what games we played. Ann-Mary and Terhi, great minds think alike. Arja, Päivi, Pipa, Kepe and other spiritually young folks in Mikkeli, we have shared some great moments. Old school mates Juha, Kimmo, Marko

and Petri, I still recall the fun times with you all. Tai–ji enthusiasts – do not be square – and that applies to other martial artists, too.

Niina is sincerely thanked for patience and understanding. I cherish the peaceful and quiet moments we have shared.

The northern strongholds in Ylitornio: Mummu, Aino, Manku and Martti; and the "surrounding" areas: Arja, Aune, Kaija and Mervi with your families – I have always enjoyed your hospitalities on my all too rare journeys. I dearly embrace those moments. Sirkka, Seppo and my kid sister, Anu, blood does get thicker through the years. I love you all.

The Saastamoinen Foundation is thankfully acknowledged for the financial support for my forthcoming Japan expedition.

Kuopio, November 2005

Aki Järvinen

Mother Earth, the guardian and provider of us all, I look forward to our refreshing, inspiring and encouraging moments in the woods.

ABBREVIATIONS

AA	amino acid
AbeAdo	5'-{[(Z)-4-amino-2-butenyl]methylamino}-5'-deoxyadenosine
AcSPD	N^1 -acetylspermidine
AcSPM	N^1 -acetylspermine
AdoMet	(S)-adenosyl-(L)-methionine
AdoMetDC	(S)-adenosyl-(L)-methionine decarboxylase
AG	aminoguanidine
AOE-PUT	aminoxy-ethyl-putrescine
AZ	antizyme
BA	benzaldehyde
DFMO	α -difluoromethylornithine
DENSPM	diethylnorspermine
DESPM	diethylspermine
dcAdoMet	decarboxylated (S)-adenosyl-(L)-methionine
diAcSPM	N^1, N^{12} -diacetylspermine
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
DTT	dithiotreitol
eIF5A	eukaryotic initiation factor 5A
FAD	flavin adenine dinucleotide
FBS	fetal bovine serum
HPLC	high performance liquid chromatography
MeSPD	α -methylspermidine
MeSPM	α -methylspermine
Me ₂ SPM	bis- α -methylspermine
MDL 72527	N^1, N^4 -bis(2,3-butadienyl)-1,4-butanediamine
MGBG	methylglyoxal-bis(guanylhydrazone)
mRNA	messenger ribonucleic acid
MT	metallothionein I promoter
Nrf-2	Nf-E2 related transcription factor
ODC	ornithine decarboxylase
OPA	phthaldialdehyde
ORF	open reading frame
PA	polyamine
PAO	polyamine oxidase
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEST	proline (P), glutamic acid (E), serine (S) and threonine (T)
PL	pyridoxal
PL=SPM	adduct of spermine with pyridoxal
PMF-1	polyamine-modulated factor-1
PRE	polyamine response element
PUT	putrescine
P4CA	pyridine 4-carboxaldehyde
RNA	ribonucleic acid
SPD	spermidine
SPDSy	spermidine synthase
SPM	spermine
SPMSy	spermine synthase
SSAT	spermidine/spermine N^1 -acetyltransferase
SMO	spermine oxidase

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications that are referred to in the text by their corresponding Roman numerals:

- I** Järvinen A, Grigorenko N, Khomutov AR, Hyvönen MT, Uimari A, Vepsäläinen J, Sinervirta R, Keinänen TA, Vujcic S, Alhonen L, Porter CW, and Jänne J.
Metabolic stability of α -methylated polyamine derivatives and their use as substitutes for the natural polyamines.
Journal of Biological Chemistry (2005) **280**; 6595-6601
- II** Järvinen A, Cerrada-Gimenez M, Grigorenko N, Khomutov AR, Vepsäläinen J, Sinervirta R, Keinänen TA, Alhonen L, and Jänne J.
 α -Methyl polyamines: efficient synthesis and tolerance studies in vivo and in vitro. The first evidence for dormant stereospecificity of polyamine oxidase
Journal of Medicinal Chemistry (2005) **accepted for publication**
- III** Järvinen A, Keinänen TA, Grigorenko N, Khomutov AR, Uimari A, Vepsäläinen J, Närvänen A, Alhonen L, and Jänne J.
Guide-molecule driven stereospecific degradation of α -methylpolyamines by polyamine oxidase
Journal of Biological Chemistry (2005) **submitted**

Some unpublished data is also presented.

CONTENTS

1. INTRODUCTION	13
2. REVIEW OF LITERATURE	16
2.1 TOOLS TO STUDY POLYAMINE METABOLISM	16
2.1.1 <i>Cell culture</i>	16
2.1.2 <i>Experimental animals</i>	16
2.1.3 <i>Recombinant proteins</i>	16
2.2 POLYAMINES AND MAMMALIAN CELLS	17
2.2.1 <i>Polyamine synthesis</i>	18
2.2.2 <i>Polyamine interconversion</i>	20
2.2.3 <i>Terminal degradation of polyamines</i>	22
2.3 POLYAMINE ANALOGUES	24
2.3.1 <i>Analogues affecting polyamine synthesis</i>	24
2.3.2 <i>Analogues affecting polyamine catabolism</i>	25
2.3.3 <i>α-Methylated polyamine analogues</i>	26
2.3.4 <i>Polyamine transport system and polyamine analogues</i>	27
2.4 TRANSGENIC RODENTS WITH ALTERED POLYAMINE METABOLISM PRODUCED IN THE UNIVERSITY OF KUOPIO	28
2.5 OPTIMIZING ENZYME ACTION	29
2.5.1 <i>Stereospecificity of enzymes</i>	30
2.5.2 <i>Forced evolution of enzymes</i>	30
2.5.3 <i>Allosteric regulation</i>	31
2.5.4 <i>Other factors affecting enzyme reactions</i>	31
2.5.5 <i>Enzymes in organic solvents</i>	31
3. AIMS OF THE STUDY	33
4. MATERIALS AND METHODS	34
4.1 POLYAMINE ANALOGUES AND OTHER CHEMICALS	34
4.2 TRANSGENIC ANIMALS	34
4.3 IMMORTALIZED MT-SSAT TRANSGENIC RAT FIBROBLASTS	35
4.4 RECOMBINANT PROTEINS	36
4.5 LIVER EXTRACTS	36
4.6 ANALYTICAL METHODS	37
4.7 STATISTICAL METHODS	37
5. RESULTS	38
5.1 METABOLIC STABILITY OF α -METHYLATED POLYAMINE ANALOGUES	38
5.2 SYNTHESIS OF α -METHYLATED POLYAMINE ANALOGUES AND THEIR TOXICITY	38
5.3 STEREOSPECIFICALLY FLEXIBLE POLYAMINE OXIDASE	39
5.4 OTHER RESULTS	44
6. DISCUSSION	47
7. SUMMARY	52
8. REFERENCES	53

1 INTRODUCTION

Polyamines (PAs) are found in all living organisms except for a few bacteria. In mammalian cells, these small molecules are characterized by a straight carbon backbone which separates two or more amino groups. Putrescine, (PUT) found in large quantities in decomposing flesh, is the simplest of the PAs and acts as a precursor for the two longer molecules: spermidine (SPD) and spermine (SPM, Fig. 1). SPM was originally discovered in the human semen and both SPD and SPM are named accordingly. Contrary to the situation in eukaryotes, in prokaryotes a far wider variety of PAs has been found. For example, in the thermophilic bacteria, branched PAs are common (Hamana et al., 1992).

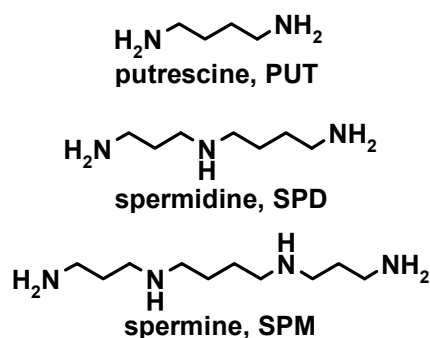


Figure 1 The three polyamines found in mammalian cells

Under normal physiological conditions, all amino groups in PUT, SPD and SPM are protonated. Due to their polycationic nature, PAs interact with negatively charged macromolecules. However, unlike the common cellular ions (Ca^{2+} , Mg^{2+} etc.), these compounds have their positive charges divided by few carbon atoms along the entire length of the molecule. This unique feature allows PAs to link to different regions of anionic macromolecules and even entirely separate molecules can be bridged by the flexible carbon chain. Both SPD and SPM fit in between the major

and minor grooves of DNA, stabilizing the nucleic acid structure. Thus, PA depletion partially unwinds DNA in nucleosomes and may reveal potential sequences for transcription regulating factors (Morgan et al., 1987). However, in cells, most of the PAs appear to form complexes with RNA (Watanabe et al., 1991). The above interactions indicate that PAs have important roles in the regulation of both transcription and translation. For example, PA depletion stabilizes both p53 mRNA and protein in rat intestinal epithelial cells, inhibiting normal proliferation (Li et al., 2001). Furthermore, PAs form complexes with proteins and phospholipids, strengthening cellular membranes. PAs also appear to regulate many membrane-bound enzymes and ion channels (Fakler et al., 1995). All these properties emphasize the importance of controlled PA levels in order to maintain the cellular functionality.

In prokaryotes, PAs appear to stabilize nucleic acids under extreme conditions (Terui et al., 2005) and are also used as carbon and nitrogen sources. In eukaryotes, detoxification processes and regulation of fundamental cellular processes appear to be the main roles for PAs. Recently numerous terminally *N*-alkylated PA analogues have been studied in PA metabolism associated effects but these PA mimetics have limited stabilities. Therefore, the development of metabolically stable PA derivatives would be extremely useful in elucidating the exact roles of each individual PA. Several apparently stable and differently methylated PA analogues were introduced in 1980s (Nagarajan and Ganem, 1986) but due to their limited supply these compounds have so far been studied only *in vitro*. The methylation apparently protects these PA derivatives against acetylation as well as degradation by mono- and diamino oxidases, making them metabolically far more stable than

their natural counterparts (Nagarajan et al., 1988). Spermidine/spermine N^1 -acetyltransferase (SSAT, EC 2.3.1.57) is considered as the key enzyme in interconversion of PAs and transgenic rodents over-expressing SSAT are characterized by enhanced PA catabolism (Alhonen et al., 2000; Pietilä et al., 1997; Suppola et al., 1999). We previously demonstrated that α -methylated SPD (MeSPD, Fig. 2) can mimic the cellular functions of SPD and can prevent acute pancreatitis in the mouse metallothionein I (MT) promoter driven SSAT transgenic rats (Räsänen et al., 2002).

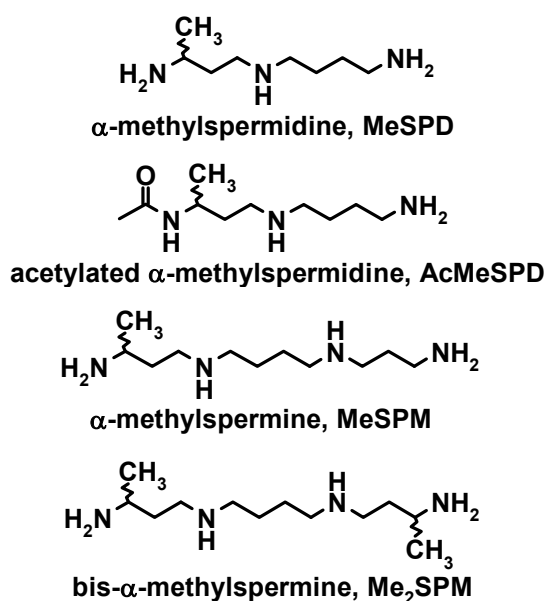


Figure 2 Four studied α -methylated polyamine analogues.

In vitro studies with recombinant human SSAT showed that a single methyl group is indeed enough to prevent acetylation of MeSPD. Neither recombinant human polyamine nor human spermine oxidase (PAO, EC 1.5.3.11 and SMO, respectively) could utilize MeSPD as a substrate. However, the SPM derivative with a single α -methyl group (MeSPM, Fig. 2) was readily degraded by hSMO but bis- α -methylated SPM (Me₂SPM, Fig. 2) was more resistant to hSMO-mediated oxidation. On the other hand, hPAO used Me₂SPM more readily than MeSPM, though

both were poor substrates for this enzyme. SPD has been considered as the essential PA to sustain cellular functionality. Hepatic PAs are severely depleted in the MT-SSAT transgenic rat after hepatectomy. However, Me₂SPM treatment prior to the operation prevented the delay in liver regeneration in these animals. In fact, Me₂SPM appeared to be a more effective drug than MeSPD on a molar basis. Extensive *in vivo* studies showed that the MT-SSAT transgenic mice tolerated all of the α -methylated PA analogues.

Many enzymes preferentially use one isomer as their substrate(s). Natural substrates for PAO are achiral but since the studied α -methylated PA analogues were chiral we suspected that PAO might express stereospecificity. The enantiomers of acetylated MeSPD (AcMeSPD, Fig. 2) were indeed degraded differently by hPAO with (*R*)-AcMeSPD being a far better substrate than the (*S*)-isomer.

Aldehyde supplementation increases the reaction rates of PAO (Hölttä, 1977). However, the ability to predetermine the stereoselectivity of hPAO-mediated oxidation of the chiral substrate with appropriate aldehyde supplementation was totally unexpected. Benzaldehyde (BA) supplementation resulted mostly in degradation of (*R*)-MeSPD by hPAO whereas (*S*)-MeSPD was exclusively used by hPAO when pyridoxal (PL) was included into the reaction mixture. In the presence of the above aldehydes, the same stereoselective degradations of MeSPM isomers and Me₂SPM diastereomers were also observed. The current study suggests that the Schiff base formed between the aldehyde and the PA may be the target for recognition by PAO.

The stereospecific nature of enzymatic oxidation with supplementary guide molecules is a unique finding. The present results may be used to develop PAO specific inhibitors. Also the reaction mechanisms of FAD-dependent oxidases might be understood better in the light of this

study. Furthermore, as the demand for tools to produce enantiomerically pure compounds as part of drug synthesis increases, PAO might represent a model enzyme for further exploitation.

2 REVIEW OF LITERATURE

2.1 TOOLS TO STUDY POLYAMINE METABOLISM

The PAs have been extensively studied since the beginning of the last century but the first report about SPM dates back to the 17th century. However, the exact roles of PAs in different cellular functions have proved hard to clarify. This is most likely due to the abundance of these molecules and due to their polycationic nature, their strong association with multiple cellular organelles (Igarashi and Kashiwagi, 2000). It is not possible to accurately measure the amounts of free PAs in cells with the current methods and their compartmentalization within intact cells remains a mystery. The regulation of PA homeostasis has proven to be complicated and highly variable from cell type to cell type let alone from species to species. Some approaches used in the PA related studies are presented with a critique.

2.1.1 Cell culture

The number of published research papers on PAs easily passes five digits and out of all these papers a significant proportion deals with cell culture studies. Innumerable cell lines have been derived from both human and animal tissues; moreover, several embryonic cell lines have been established from both intact and genetically manipulated animals. During the past years, these techniques have given rise to specifically engineered cells where a selected gene can either be over-expressed or knocked out.

In general, studies with a single cell type can give very clear answers to well defined questions. However, cultured cells are devoid of all tissue-tissue interactions and with time tend to adapt to their environment, losing some essential features required *in vivo* or the cells may acquire new properties. It is essential to measure intracellular

and occasionally even extracellular levels of each drug as they may compete for the same cellular transport system(s). Furthermore, the compounds studied *in vitro* are quite often administered at high doses and at concentrations not necessarily relevant to conditions prevailing *in vivo*. Accordingly, *in vitro* results have rather limited value if one wishes to extrapolate the results to the situation in intact animals and humans.

2.1.2 Experimental animals

Different rodent models have proven very valuable. However, one always has to bear in mind the species-specific differences and the fact that no single animal model can perfectly reflect the metabolism of humans.

The past two decades have witnessed a large number of genetically modified animal studies (Jänne et al., 2004) but they have their own limitations. When a transgene is introduced into the host genome it can integrate in the middle of an active gene possibly knocking it out. It is extremely common that the transgene expression results in some compensatory effects in the organism but such metabolic adjustments may not always be sufficient and an abnormal phenotype may well be observed. The heredity of transgene(s) is also sometimes problematic and does not necessarily follow Mendelian law in live offspring.

2.1.3 Recombinant proteins

Recombinant protein production in bacteria, yeasts and different cell cultures has advanced significantly during the last few years. The desired protein(s) can be purified on a large scale with a few steps by using commercially available kits (Terpe, 2003). Such pure enzymes are excellent tools to study enzyme kinetics under precisely controlled conditions. However, one should be

aware that the enzymes in their natural cellular environment will be exposed to multiple effectors that are absent in test tubes.

Lately, the traditional practice of tissue extract studies has more or less been discarded in favor of more modern approaches. However, such

extracts reflect the cellular metabolism better than any cell line in an artificial buffer system.

2.2 POLYAMINES AND MAMMALIAN CELLS

The PA homeostasis is maintained via an intricate metabolic system in mammals. The

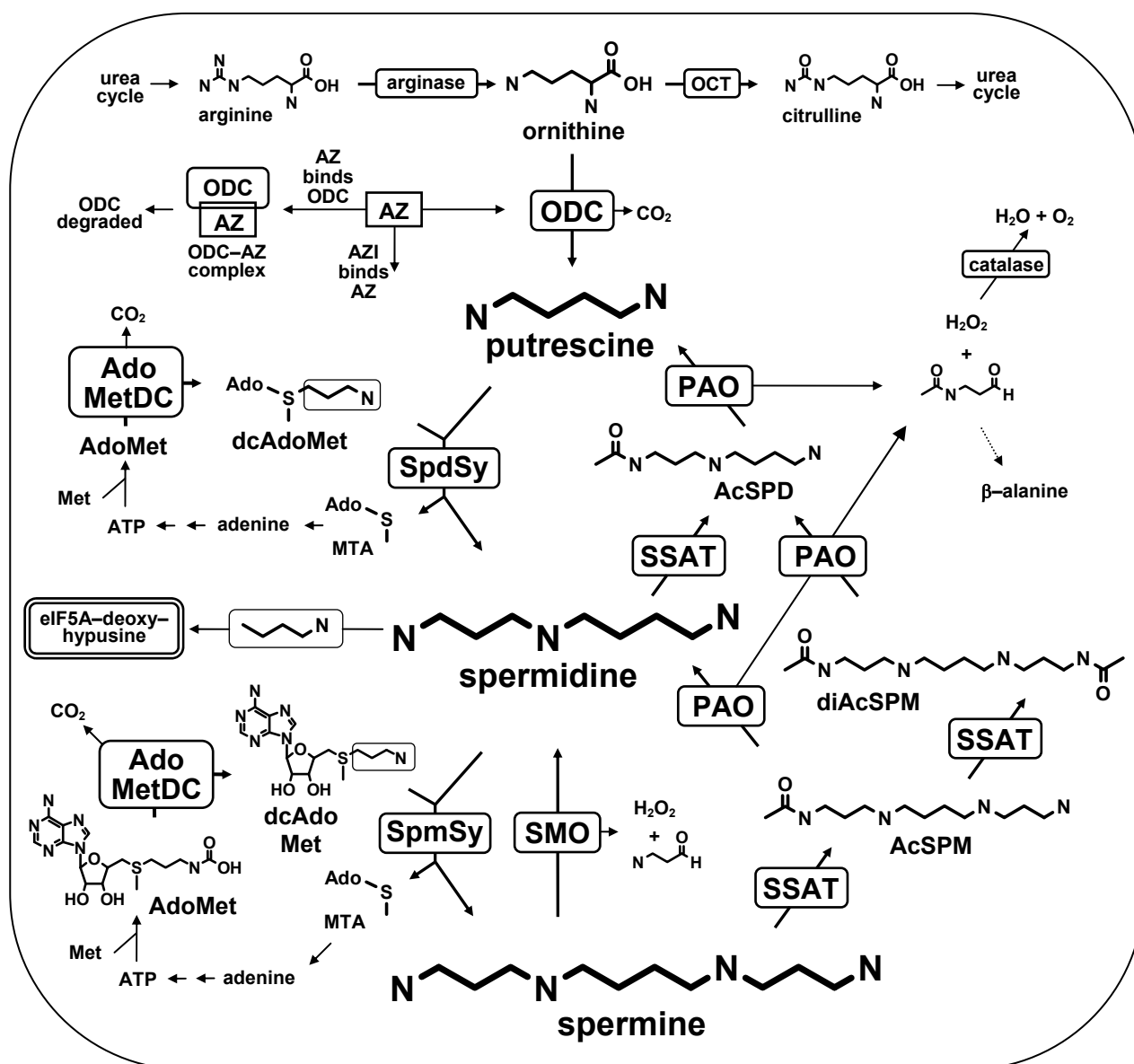


Figure 3 Polyamine biosynthesis and interconversion in mammalian cells

AcSPD, N^1 -acetylspermidine; AcSPM, N^1 -acetylspermine; AdoMet, (S)-adenosyl-(L)-methionine; AdoMetDC, (S)-adenosyl-(L)-methionine decarboxylase; ATP, adenosinetriphosphate; AZ, antizyme; AZI, antizyme inhibitor; dcAdoMet, decarboxylated (S)-adenosyl-(L)-methionine; diAcSPM, N^1, N^{12} -diacetylspermine; eIF5A, eukaryotic initiation factor 5A; Met, (L)-methionine; MTA, methylthioadenosine; OCT, ornithine carbamoyltransferase; ODC, ornithine decarboxylase; PAO, polyamine oxidase; SMO, spermine oxidase; SPDSy, spermidine synthase; SPMSy, spermine synthase; SSAT, spermidine/spermine N^1 -acetyltransferase.

biosynthesis of PAs starts from ornithine to form PUT, SPD and SPM sequentially (Fig. 3). SPM and SPD can either be interconverted (Fig. 3) or terminally degraded with different enzymes (Fig. 6, page 22). All the enzymes involved in the synthesis and degradation of PAs appear to have multiple interlocking feedback mechanisms. Furthermore, there have been recent reports of splice variants of enzymes involved in the PA metabolism (Casero et al., 2003; Cervelli et al., 2004; Pyronnet et al., 2005) giving rise to several isoenzymes with different properties and this makes the elucidation of the mechanisms controlling PA homeostasis even more challenging.

One can discern how such a sophisticated maintenance system allows fine-tuning of PA levels throughout the diversity of tissues, each with entirely different requirements for PAs. The demand for PAs is intimately linked with the cell-cycle (Oredsson, 2003) and the proliferation rate of the tissue. Dramatic consequences can occur when excess or insufficient amounts of PAs are present. These changes can lead to malignancy (reviewed in detail by Gerner and Meyskens, 2004) or apoptosis i.e. programmed cell death (Nitta et al., 2002). Furthermore, the species-specific requirements for PAs can also have huge variability; in this review PA metabolism in plants or prokaryotes will not be covered in any detail.

2.2.1 Polyamine biosynthesis

The primary precursor of PAs, (*L*)-ornithine, can either be derived from the diet or produced from (*L*)-arginine by mitochondrial arginase II (EC 3.5.3.1). Previously it was thought that arginase II, instead of cytosolic arginase I, was more important. However, now it appears that isoform I is the enzyme predominantly responsible (especially during development). Knock-out mice for arginase I survive only 12 days whereas knockout mice for arginase II have no disturbed phenotype (Cederbaum et al., 2004). These

results raise questions about the exact roles of these two isoenzymes.

Ornithine can be converted to citrulline by ornithine carbamoyltransferase (EC 2.1.3.3) within the urea cycle. However, in the case of the PA biosynthesis, ornithine decarboxylase (ODC, EC 4.1.1.17) is the sole key enzyme producing PUT. ODC is a very fascinating enzyme (extensive reviews by Schipper and Verhofstad, 2002; Shantz and Pegg, 1999). It has a very short half-life (a short as 10 to 20 minutes up to 2 hours) and it has been localized mostly in cytoplasm (inducible form) and occasionally in the nucleus (uninducible form) depending on the cell type. Active ODC uses PL phosphate as its cofactor and is a homodimer where the two active sites are located between the monomers. ODC is inactivated when it forms a heterodimer with antizyme (AZ).

AZ is a unique protein as complete AZ protein expression requires a +1 translational frameshift which is triggered by increasing PA concentrations (Matsufuji et al., 1995). Functional AZ binds ODC protein (Murakami et al., 1985) and alters its configuration, revealing the C-terminal PEST sequence (rich in proline (P), glutamic acid (E), serine (S) and threonine (T)) a sequence which is common in proteins with rapid turnover rates (Rogers et al., 1986). ODC is degraded by 26 S proteasome in an ATP-dependent, but ubiquitin-independent, manner (Murakami et al., 1992). At least three different AZs have been reported; AZ1 is strongly associated with ODC degradation, AZ2 down-regulates PA transport (Zhu et al., 1999) and AZ3 is expressed only in the testis (Tosaka et al., 2000). Growth stimuli can cause the release of ODC from AZ by the so-called antizyme inhibitor, a compound which has higher affinity for AZ than the affinity of AZ for ODC. Interestingly AZ inhibits the degradation of AZI (Bercovich and Kahana, 2004).

The next step in the synthesis of higher PAs which includes decarboxylation is the conversion of (*S*)-adenosyl-(*L*)-methionine (AdoMet) to its

decarboxylated form (dcAdoMet) by adenosylmethionine decarboxylase (AdoMetDC, EC 4.1.1.50). AdoMet, derived from (L)-methionine, is a common methyl group donor in biological methylations (Chiang et al., 1996). Like ODC, AdoMetDC has a short half-life of about 1 hour in most mammalian cells (reviewed by Jänne et al., 1991).

Spermidine synthase (SPDSy, EC 2.5.1.16) uses PUT as an acceptor for the aminopropyl group from dcAdoMet to form SPD. Another similar aminopropyl transfer reaction from dcAdoMet to SPD is catalyzed by spermine synthase (SPMSy, EC 2.5.1.22) to produce SPM. Even though these two enzymes are functionally similar, they represent two distinctly different enzymes and contain only one homologous domain, which most likely is related to the binding of dcAdoMet (Korhonen et al., 1995). SPDSy can be found in all organisms whereas SPMSy exists only in mammalian cells. Both synthases are rather stable (half-lives of about 12 hours) and their turnover appears to be regulated by the availability of their substrates (reviewed by Jänne et al., 1991).

Both of the above synthases also produce 5'-methylthioadenosine (MTA), the significance of which is often neglected. MTA acts as a feedback regulator of SPMSy (strongest), SPDSy and ODC. The crucial salvage of adenine from MTA is catalyzed by an MTA-specific phosphorylase. Interestingly, many malignant cells lack this phosphorylase activity and simply secrete MTA (Nobori et al., 1996). Furthermore, MTA appears to have cell signaling characteristics and can evoke inhibition of protein methylation and/or phosphorylation (reviewed by Avila et al., 2004).

SPD appears to be the key compound in the studies of PA-related tissue regeneration and SPM is considered more or less to be a storage pool for SPD (Alhonen et al., 2002). However, SPM can act as a free radical scavenger (Ha et al., 1998) and

appears to be a regulator of mitochondrial Ca^{2+} transport (Salvi and Toninello, 2004). On the other hand, both SPM and SPD can stabilize soluble DNA as effectively and probably have an impact on the DNA packaging (Saminathan et al., 2002). Surprisingly, SPMSy deficient Gy-mice (virtually without any SPM but with compensatory high SPD contents in all organs) grow to full adulthood in spite of some neuronal defects and compromised male fertility. The growth rate of fibroblasts derived from the Gy-mice is normal (Mackintosh and Pegg, 2000). Therefore, SPD and SPM seem to have multiple and different roles in maintaining cellular function, but may be interchangeable to some extent.

During deoxyhypusine synthesis, the aminobutyl moiety of SPD is transferred to the intermediate deoxyhypusal-residue (Park et al., 1981). Deoxyhypusine is further hydrolyzed to hypusine which is an integral part of eukaryotic initiation factor 5A (eIF5A) and essential for eukaryotic cell proliferation (Park et al., 1993). Furthermore, the accumulation of unmodified, hypusine-lacking eIF5A leads to apoptosis (Jin et al., 2003). Since eIF5A has quite a long biological half-life, it is sometimes difficult to differentiate between a low amount of SPD and loss of eIF5A as the cause of cytostatic effects.

Under physiological conditions, all the above reactions are practically irreversible and the two decarboxylases are considered as the rate-controlling enzymes in PA biosynthesis (reviewed by Shantz and Pegg, 1999). The regulation of ODC is complex and occurs at the levels of transcription, translation and protein degradation. PAs also have a strong feedback effect in the regulation of both decarboxylases: the ODC activity is down-regulated by all PAs whereas AdoMetDC is positively controlled by the amount of PUT. The encoding regions of both ODC and AdoMetDC genes contain multiple regulatory elements that recognize different anabolic and

catabolic inducers such as growth factors, tumor promoters and hormones.

2.2.2 Polyamine interconversion

In mammals the interconversion of SPM and SPD to SPD and PUT, respectively, involves successive acetylation by SSAT and oxidation by PAO. In addition, SPM can also be converted directly to SPD by the recently discovered SMO which was initially mistaken for PAO (Wang et al., 2001a).

Acetylation is considered as the rate-limiting step in the interconversion of higher PAs due to the fact that SSAT is inducible by a huge number of different stimuli and is localized to cytosol (reviewed by Casero and Pegg, 1993; Seiler, 1987). A 250-fold SSAT induction was first detected in the rat liver after CCl_4 treatment (Matsui and Pegg, 1980). The basal level of SSAT is very low and it has one of the shortest half-lives of all enzymes (Matsui et al., 1981). However, regulation of SSAT seems to include many mechanisms from transcription through to protein degradation. The SSAT gene is preceded by a TATA-less promoter which contains several elements such as the PA responsive element (PRE) (Tomitori et al., 2002) and cis-elements for regulation. SSAT has an MATEE sequence (Coleman et al., 1995) which may partly be responsible for its rapid turnover, acting similarly to PEST sequence in the case of ODC. The natural substrates for SSAT are SPD and SPM producing N^1 -acetyl-SPD (AcSPD, Fig. 3) and N^1 -acetyl-SPM (AcSPM, Fig. 3), respectively (Ragione and Pegg, 1983). SSAT also acetylates AcSPM further to N^1, N^{12} -diacetyl-SPM (diAcSPM, Fig. 3) (Vujcic et al., 2000).

PAO has been studied from many sources (Suzuki et al., 1984; Tsukada et al., 1988) and the enzyme was first purified from the rat liver (Hölttä, 1977). It seems that purified rat liver PAO has a M_w of 60'000, is sensitive to sulfhydryl and carbonyl group reagents, has an optimum pH of

10 for oxidation of PAs in the presence of molecular oxygen and may require Fe^{2+} ions (Hölttä, 1977). PAO has been localized to both cytosol and peroxisomes in the rat liver (Van den Munckhof et al., 1995) and PAO activity can be found virtually in all vertebrate tissues (Seiler, 1995). Mammalian PAO has high affinity towards acetylated PAs; AcSPD, AcSPM and diAcSPM being excellent substrates; however, N^8 -acetyl-SPD is not a substrate (Seiler et al., 1980; Suzuki et al., 1981). Interestingly, two PAO isozymes with slightly different enzymatic properties were found in the L1210 cell line (Libby and Porter, 1987). The mammalian PAO attacks secondary N^4 of the substrate from the *exo*-side whereas maize PAO attacks the same atom from the *endo*-side; moreover, the maize PAO does not act on acetylated PAs and actually may be part of a distinct oxidase sub-family. Both mammalian and plant PAO require O_2 and utilize tightly but not covalently bound flavin adenine dinucleotide (FAD) as the cofactor (Fig. 5).

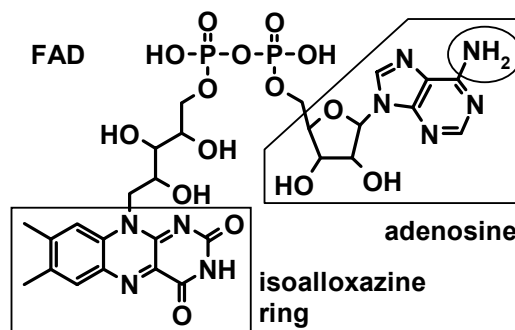


Figure 4 Flavin adenine dinucleotide (FAD), the cofactor of polyamine oxidase.

The FAD cofactor includes an adenosine-residue (with one free amine, circled in Fig. 4) a common feature of other cofactors. A flexible carbon chain (containing two phosphate groups) forms a bridge between the adenosine-residue and the isoalloxazine ring. The oxidized isoalloxazine ring is a good electron sink and readily accepts electrons from the substrate (i.e. it is reduced). The reduced ring structure reduces

molecular O₂ to form one molecule of H₂O₂ per carbon–nitrogen bond cleaved and the aromatic ring structure is oxidized. The free oxidized isoalloxazine ring is planar while the reduced form is bent at the N⁵–N¹⁰ axis (Fig. 5) and this feature may be important in the control of the redox state of the enzyme (Silverman, 2000).

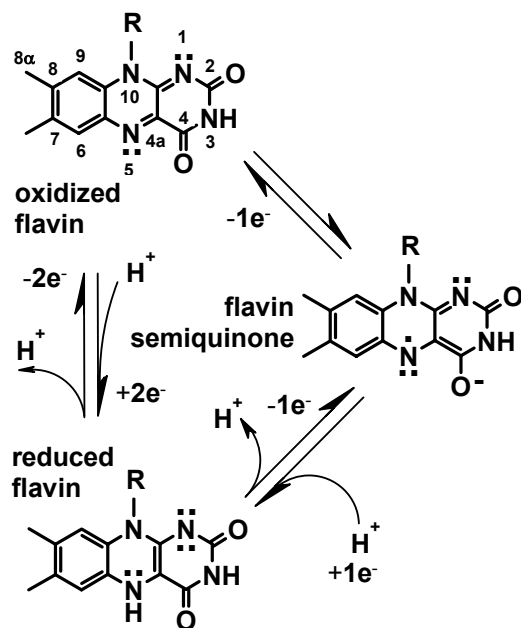


Figure 5 Three redox states of flavin according to Silverman (2000)

Crystallized maize PAO contains 470 amino acids (AAs) and has a 30 Å long U-shaped catalytic tunnel which appears to be optimal for catalysis of linear PAs and the innermost section of this tunnel is located in front of flavin (Binda et al., 1999). The active site is embedded with aromatic side chains and many carbonyl groups that can form hydrogen bonds with the substrate. The larger end of the catalytic tunnel is lined with several acidic AA-residues which may help to steer protonated PAs into the active site and may permit the passage of bulky substrates. No charged groups are detected in the catalytic tunnel probably allowing for a varied nitrogen–carbon organization in the substrate (Binda et al.,

2001). General features of the maize PAO (Cona et al., 2004) appear similar to both the barley PAO (Cervelli et al., 2001) and the mouse SMO (Cervelli et al., 2003).

Recently different mammalian PAOs have been cloned by two groups (Wu et al., 2003; Vujcic et al., 2003) but currently no crystallized mammalian PAOs are available nor has their regulation been described. The human PAO codes for a protein with 511 AAs with M_w of 55'500 and the mouse PAO contains 504 AAs with M_w of 55'000.

When we reported the degradation of SPM to SPD in SSAT-deficient cells (Niiranen et al., 2002), it was evident that a new player controlling the PA homeostasis had been discovered. Shortly thereafter the properties of recombinant SMO from multiple sources were described (Cervelli et al., 2003; Vujcic et al., 2002). Four different splice variants of human SMO with slightly different properties have been reported (Murray-Stewart et al., 2002). However, from nine mouse SMO splice variants, the one with the highest activity was cytoplasmic while another, less active, SMO variant was found in both nucleus and cytoplasm after transfection into a murine neuroblastoma cell line (Cervelli et al., 2004). There is a strong indication that SMO utilizes FAD as a cofactor since its sequence contains flavin-binding domains (Cervelli et al., 2004). SMO strongly favors SPM over its acetylated derivatives and does not use SPD as a substrate at all. SMO attacks the same carbon–nitrogen bond as the mammalian PAO and similarly produces H₂O₂. It seems that the H₂O₂ specifically produced by SMO leads to DNA damage and mitochondrial membrane depolarization as signs of apoptosis (Amendola et al., 2005; Chaturvedi et al., 2004; Xu et al., 2004). Presently, the only crystal structure of an SMO-like protein has been that obtained from yeast (Huang et al., 2005). The

mammalian SMO appears to be slightly larger than PAO with 555 AAs and M_w of 62'000.

2.2.3 Terminal degradation of polyamines

Amine oxidase studies have a long history and one of the first extensive reviews dates back to 1950s (Blaschko, 1952). In mammals, there are several other amino oxidases in addition to PAO and SMO. These oxidases can be divided into two subgroups according to their cofactors (flavin or copper-containing oxidases).

Monoamine oxidase (MAO, EC 1.4.3.4) has two variants (A and B) which have over 70 % sequence similarity. MAOs are located in the mitochondria and use covalently bound (via 8α -carbon) flavin mononucleotide (FMN, Fig. 7) as their cofactor (Binda et al., 2002a). However, the significant difference to PAO is that MAOs exclusively attack primary amines.

The overall structure of crystallized human MAO B resembles only slightly the maize PAO; however, the active sites in both oxidases

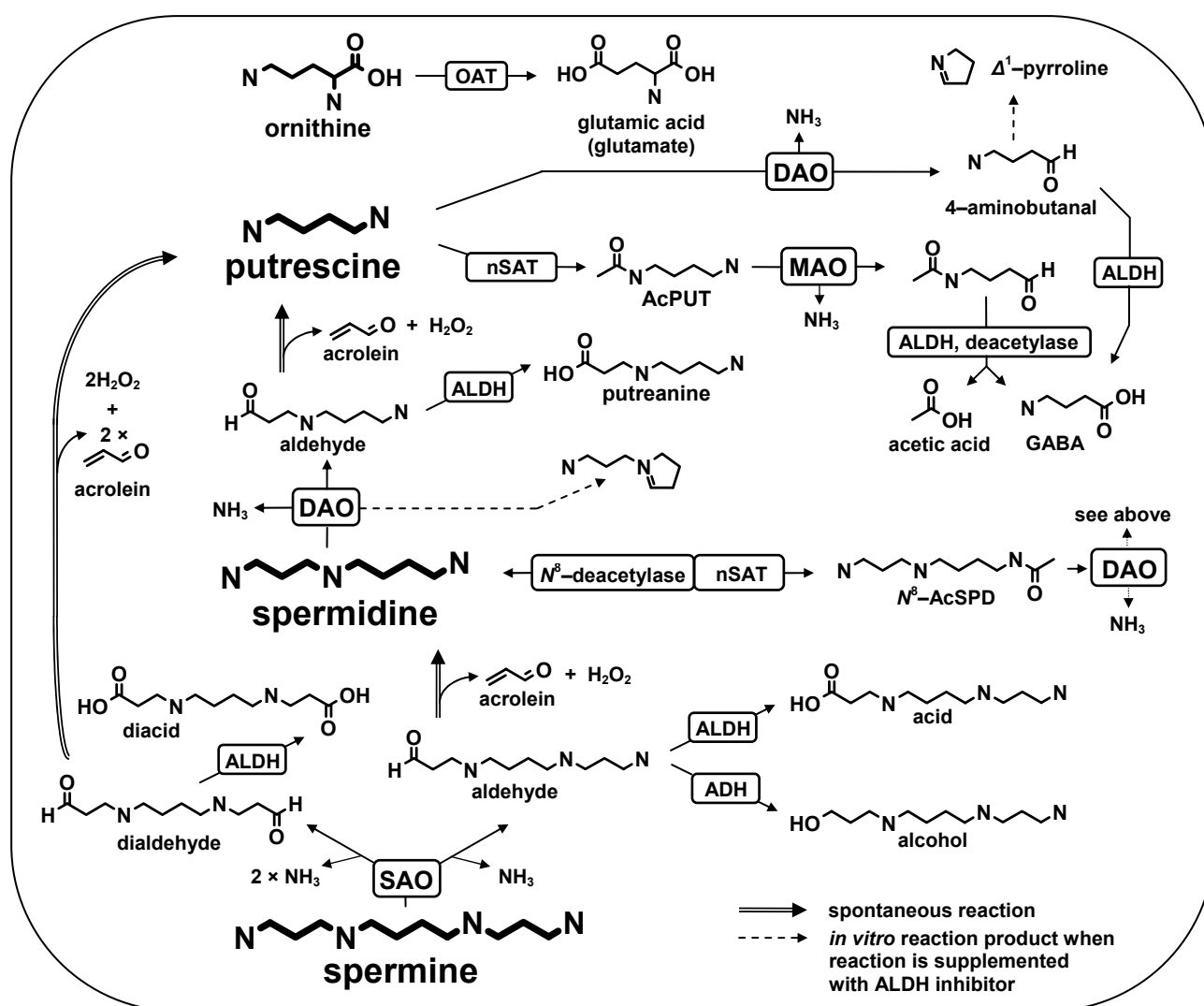


Figure 6 Terminal degradation of polyamines in mammalian cells

AcPUT, acetylputrescine; ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; SAO, serum amineoxidase; DAO, diamine oxidase; GABA, γ -aminobutyric acid; MAO, monoamine oxidase; N^8 -AcSPD, N^8 -acetylspermidine; nSAT, nuclear spermidine N^8 -acetyltransferase; OAT, ornithine aminotransferase.

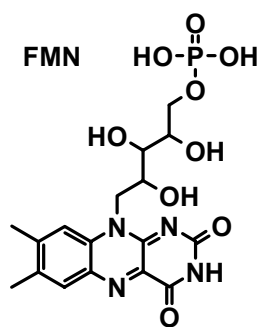


Figure 7 Flavin mono nucleotide (FMN), the cofactor of monoamine oxidases.

are lined by many aromatic and aliphatic residues forming a highly apolar environment for substrates (Binda et al., 2002b). The only apparent structural difference between MAO A and B is a 50 AA-long C-terminal segment in MAO B that binds it to the outer mitochondrial membrane. Both MAO A and B take part in the biogenic monoamine neurotransmitter metabolism but have a lesser role in the catabolism of acetylated amines. MAO inhibitors are of clinical importance in the treatment of depression (MAO A inhibitors e.g. moclobemide) and Parkinson's disease (MAO-B inhibitors e.g. selegiline, reviewed by Agostinelli et al., 2004).

Diamine oxidase (DAO, EC 1.4.3.6) was originally known as histaminase. It requires copper and utilizes a post-translationally altered tyrosine residue, 2,4,5-trihydroxyphenylalanine quinone (Dooley, 1999), as its cofactor (TPQ, Fig. 8) which is an absolute requirement for catalytic activity (Brazeau et al., 2004). DAO can be

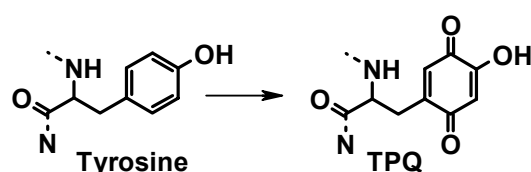


Figure 8 Post-translationally modified tyrosine-residue in the active site of diamine oxidase. TPQ, 2,4,5-trihydroxyphenylalanine quinone.

detected in many mammalian organs but shows high activity in rapidly proliferating tissues like intestinal mucosa. However, in brain, virtually no DAO activity is detectable. The best substrates for DAO are histamine, PUT and SPD. Cyclic amines can also be formed as the DAO-reaction products *in vitro* or in the presence of aldehyde metabolizing enzyme inhibitors (Sessa and Perin, 1994). Increased DAO activities have been associated with tumor promotion (Kusche et al., 1988).

Other amine oxidases similar to DAO have also been detected from different animal sources (like bovine serum, pig and equine plasmas). They are here collectively named as serum amine oxidases (SAOs) and have the same cofactor requirements as DAO. These SAOs use both SPM and SPD as substrates and attack the primary amino groups. The reaction products are ammonia and aldehydes that degrade spontaneously giving rise to another very reactive aldehyde, acrolein (Lee and Sayre, 1998). Numerous other copper-containing amine oxidases have also been identified from a wide variety of species (including micro-organisms and plants); they are all dimers and exhibit very similar properties (extensively reviewed by Agostinelli et al., 2004). To make the tale of amine degradation even more complicated, there is evidence that polyphenolic compounds from plants also have oxidative deamination activities (possibly via their quinone forming properties) in the presence of copper (Akagawa and Suyama, 2001).

There is a nuclear SPD acetyltransferase that can use both PUT and SPD (only from the aminobutyl moiety) as substrates but, in contrast to SSAT, is not inducible. This nuclear acetyltransferase also appears to be linked with histone acetylation and it is suggested that transporting SPD out of the nucleus could be involved in the cell growth regulation via histone

acetylation (Desiderio et al., 1992; Desiderio, 1992). N^8 -acetyl-SPD is a poor substrate for DAO but can be returned into circulation by N^8 -acetylspermidine deacetylase (EC 3.5.1.48).

Ornithine can also be converted to glutamate by ornithine specific aminotransferase, OAT (EC 2.6.1.13). PUT is a precursor for γ -aminobutyric acid, GABA. Both glutamate and GABA are important neurotransmitters.

2.3 POLYAMINE ANALOGUES

The PAs have central roles in many cellular processes (Wallace et al., 2003). As the population ages, the incidence of many diseases will increase and many of these diseases, e.g. cancer, are strongly associated with distorted PA homeostasis (Thomas and Thomas, 2003). Therefore, understanding PAs is important if we wish to understand the exact mechanisms of cellular functions under extreme conditions.

There are two alternative ways to alter PA levels i.e. the inhibition of their biosynthesis or enhancement of their degradation. Both options are possible with compounds that either mimic the natural substrates or affect the activities of the enzymes involved in the regulation of PA homeostasis. It is not rare that such artificial compounds have more than one effect.

There are at least three possibilities to alter natural PAs: 1) substituents at one or both ends of the PA, 2) substituents along the backbone of the parent PA or 3) altering the original PA backbone length or replacing atom(s) within it. Figure 9 shows schemes for SPD and SPM analogues. In prokaryotes, the PA backbone may

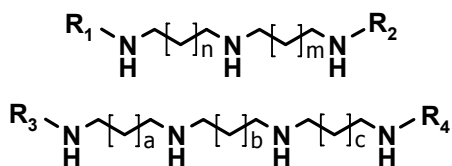


Figure 9 Schemes for SPD and SPM analogues.

contain up to 6 nitrogen atoms (Terui et al., 2005). If $n=m=a=b=c=1$ or 2 , the derivatives are called nor-PAs or homo-PAs, respectively. R_1 , R_2 , R_3 and R_4 can be almost any moiety ($-\text{CH}_3$, $-\text{CH}_2\text{CH}_3$ etc.) and often the above approaches are combined. It appears that the charge distribution is decisive in the recognition and subsequent fate of each analogue (Bergeron et al., 1995a). Here, no PA derivatives particularly related to MAOs, DAO or SAOs will be reviewed.

2.3.1 Analogues affecting polyamine synthesis

The initial promise of blocking the PA biosynthesis as cancer treatment has not been realized – it seems that the inhibition of PA synthesis results in multiple compensatory effects. Nevertheless, a few such inhibitory drugs are shortly described here for their scientific value.

ODC is a logical target for drugs designed to interfere with PA biosynthesis as PUT is the

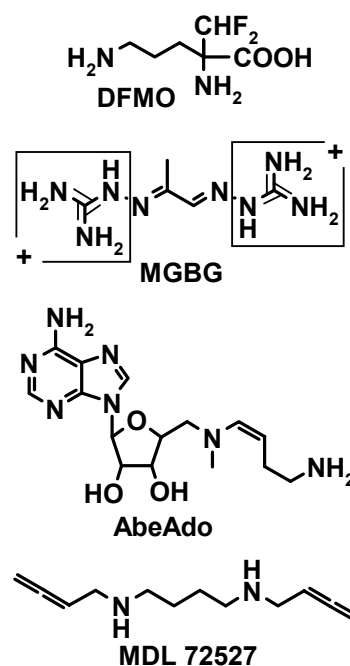


Figure 10 Some PA metabolism inhibitors. The resonance structures of MGBG are marked with boxes. DFMO, ODC inhibitor; MGBG; AdoMetDC inhibitor; AbeAdo; AdoMetDC inhibitor; MDL 72527, PAO inhibitor.

precursor for all higher PAs. Irreversible ODC inhibitors, such as α -difluoromethylornithine, (DFMO, Fig. 10) were originally developed as anticancer drugs. DFMO decreases ODC activity and depletes PUT time-dependently. It is usually cytostatic and its effects are reversed with PA replenishment (extensively reviewed by Seiler, 2003a). Even though native ODC uses exclusively (*L*)-ornithine, the different enantiomers of DFMO appear to have minimal differences when used to inhibit recombinant ODC (Qu et al., 2003). An earlier study with differently methylated PUT analogues showed only slight inhibition of ODC, comparable to that of PUT (Ruiz et al., 1986). DFMO is quite well tolerated by humans but causes some troublesome complications (such as loss of hearing). Fortunately these side effects are reversible. Presently DFMO is used as an anti-parasitic drug in developing countries and has been tried as a cancer chemopreventive agent when combined with other drugs.

The selective AdoMetDC inhibitors such as methylglyoxal-bis(guanylhydrazone), (MGBG, it contains two resonance structures (guanyls), Fig. 10) deplete higher PAs and result in the induction of ODC and the accumulation of PUT. Unfortunately, in humans, MGBG proved to

be too toxic to permit clinical use. In rats, MGBG treatment increases PAO activity in liver and spleen but decreases it in thymus (Feroli and Armanni, 2003; Feroli et al., 2004). Enzyme activated AdoMetDC inhibitor 5'-{[(*Z*)-4-amino-2-butenyl] methylamino}-5'-deoxyadenosine, (AbeAdo, Fig. 10) has been successfully used in the treatment of African trypanosomas (Bitonti et al., 1990). It is important to note that the use of AdoMetDC inhibitors results in the accumulation of unmodified eIF5A. Inhibition of either SPDSy or SPMSy appears to have only a limited effect *in vitro* as usually new PA homeostasis is reached rather rapidly and the treated cells divide only at a somewhat reduced rate (reviewed by Seiler, 2003a).

2.3.2 Analogues affecting polyamine catabolism

Different SPM derivatives (Fig. 11) have proven to be valuable tools in PA catabolism-related studies (extensively reviewed by Seiler, 2003b). Bis-ethylated SPM analogues (DESPM and DENSPM, Fig. 11) are very powerful SSAT inducers (Casero et al., 1989) and deplete natural PAs effectively (Libby et al., 1989). It seems that SPM derivatives stabilize the SSAT protein preventing its ubiquitination (necessary for many short-lived enzymes targeted to proteosomal degradation) by conformational changes (Coleman and Pegg, 2001). Furthermore, these SPM analogues also down-regulate both ODC and AdoMetDC (Chang et al., 1992). Due to these properties, DENSPM is currently in clinical trials for treatment of several cancer types (Hahm et al., 2002).

Only in DENSPM-responsive cells, are there significant expressions of polyamine-modulated factor-1 (PMF-1) and Nf-E2 related transcription factor (Nrf-2, Casero et al., 2003). Nrf-2 binds to PRE of SSAT and transcription cofactor, PMF-1 (with no DNA-binding domain) binds to Nrf-2 via

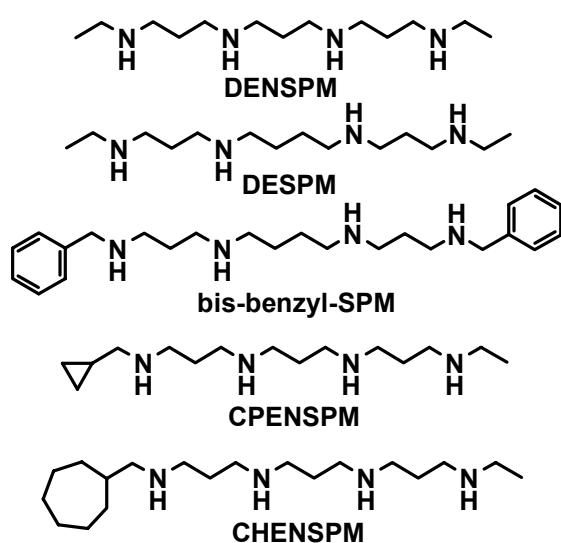


Figure 11 Some differently *N*-alkylated SPM analogues.

a unique leuzine–zipper–coil (Wang et al., 2001b) to modulate SSAT transcription. DENSPM treatment significantly increases the expression of PMF–1 and the extremely rapid induction of SSAT suggests that some PMF–1 binds to Nrf–2 even without external stimuli.

DENSPM is proposed to be degraded in several steps *in vivo* (Bergeron et al., 2000; Bergeron et al., 1995b) and it seems that PAO is the enzyme responsible for its degradation (Vujcic et al., 2003). Mechanistic studies with DESPM shows that reduction of flavin is the rate–controlling step in the substrate degradation (Royo and Fitzpatrick, 2005).

Only berenil and pentamidine have been described being as effective SSAT inhibitors (Libby and Porter, 1992). However, new methods are being used more frequently and SSAT suppression by small interfering RNA directly revealed a link between SSAT induction by DENSPM and apoptosis (Chen et al., 2003).

Both CHENSPM and CPENSPM deplete PAs but only CPENSPM can induce SSAT in 24 h while CHENSPM increases DNA fragmentation, alters cell morphology and induces G₂/M cell cycle arrest (Nairn et al., 2000). Furthermore, both analogues have very different effects on tubulin polymerization; CHENSPM enhances this process while CPENSPM slows the polymerization as compared to SPM (Webb et al., 1999). *In vitro* exposure to CPENSPM causes increased transcription of hSMO, mRNA stabilization, *de novo* synthesis of protein and massive SMO activity (Devereux et al., 2003; Wang et al., 2005). Furthermore, the SMO promoter appears to be sensitive to some PA analogues, pointing to a complicated transcriptional regulation.

It seems that differences in terminal alkyl groups are important for the observed effects of SPM mimetics. Terminally bis–methylated SPM analogues are only cytostatic whereas bis–ethyl– and bis–propyl–SPM derivatives are cytotoxic (Kramer et al., 1997).

In mice a single injection of MDL 72527 (N^1, N^4 –bis(2,3–butadienyl)–1,4–butanediamine, 20 mg/kg, Fig. 10, p. 24) rapidly inhibits PAO and results in an accumulation of AcSPD (Bolkenius et al., 1985). However, within two days, the PAO activity reappears slowly in liver and kidney. Long–term treatment with the drug does not appear to cause any toxic effects. It seems that MDL 72527 reacts with mammalian PAO differently than other substrates and binds covalently to N^5 of isoalloxazine ring, thus inactivating the enzyme (Wu et al., 2005). SMO, on the other hand, is less susceptible than PAO to inhibition by MDL 72527 exposure (Vujcic et al., 2002).

2.3.3 α -Methylated polyamine analogues

From the late 1970s onwards, differently alkylated PAs were synthesized in order to inhibit PA synthesis, and in the mid–1980s, several structural PA derivatives with dimethylated carbons were introduced (Nagarajan and Ganem, 1986). SPD containing dimethylated C¹, C², C³ or C⁵ carbons (Fig. 12) is resistant to acetylation. Similarly, bis– α – and bis– γ –dimethylated SPM analogues are not acetylated. However, C⁸–dimethyl–SPD is acetylated by SSAT as efficiently as SPD. None of the SPD mimetics are substrates for SPMSy. All the mentioned SPD and SPM analogues can support cell growth when natural PAs are depleted by DFMO (Nagarajan et al., 1988).

The first study with MeSPD, MeSPM and Me₂SPM showed that a single methyl group in α –position is enough to protect against acetylation.

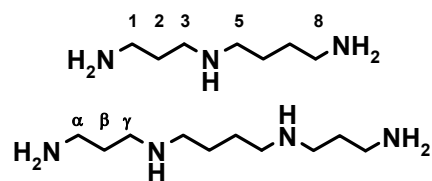


Figure 12 Possible numbers or names of carbons in SPD and SPM.

Furthermore, the analogues restore cell growth in the presence of DFMO similarly to dimethylated PA derivatives. All of the studied analogues deplete natural PAs effectively; MeSPD is not degraded *in vitro* but slow turnover into MeSPM is detected and Me₂SPM is not entirely stable (Lakanen et al., 1992). Natural PAs; SPD and SPM (via interconversion to SPD), and MeSPD rescue the L1210 cells from AdoMetDC inhibitor AbeAdo-induced hypusine depletion whereas Me₂SPM does not (Byers et al., 1994). Interestingly, in CHO cells, AbeAdo treatment results in high PUT contents whereas the DFMO treatment depletes PUT neither effect being modified by extensive uptake of MeSPD or Me₂SPM. The amount of PUT does not affect the uptake of higher PAs, indicating that there are two alternate PA transport systems in these cells. Furthermore, a study in CHO-MG cells lacking the PA uptake system suggests that PA-uptake is essential to maintain the cellular PA levels in this CHO cell line (Byers et al., 1994).

Many PA analogues cause extensive SSAT induction with simultaneous ODC suppression and cell growth arrest. Nevertheless, Me₂SPM can maintain the cell growth even though the SSAT activity increases significantly (and the ODC activity remains unaltered) in a human lung carcinoma cell line NCI H157 (Yang et al., 1995). It seems that cells can survive very high SSAT activities when supplemented with a growth-supporting PA mimetic. Furthermore, MeSPD, MeSPM and Me₂SPM are as effective in inducing the conversion of right-handed B-DNA to left-handed Z-DNA (Varnado et al., 2000) as their unmethylated parent molecules.

2.3.4 Polyamine transport system and polyamine analogues

Acetylation may be a recognition signal for "unnecessary" PAs as acetylated PAs are usually exported out of the cells (Seiler, 1987). However,

there are significant species-specific differences in excretion. In human urine, most of the PAs are in the acetylated form (10-fold difference) but in the rat urine mostly PUT is detected, indicating that acetylation is not required for excretion in the rat. On the other hand, PA-depleted cells (i.e. after DFMO treatment) import extracellular PAs very effectively. However, the PA uptake in cells is not dependent on the export and generally the intracellular PA contents appear to have the opposite effect on uptake (reviewed in detail by Seiler and Moulinoux, 1996).

In spite of extensive efforts, no PA specific transporter has been cloned. Different PA transporter systems seem to exist depending on the cell type. In some cells there is one system for PUT whereas other PAs use a separate transport system. In some other cases, all PAs appear to use the same system for transmembrane movement. Since the stringency of these transporter systems seems to be rather low, PAs have been evaluated as possible vehicles for selective drug delivery into malignant cells that often require substantial PA uptake to maintain growth.

PAs appear to be good carriers for drug delivery, as even anthracene (an intercalator that prevents topoisomerases from resealing DNA breaks during DNA synthesis and transcription;

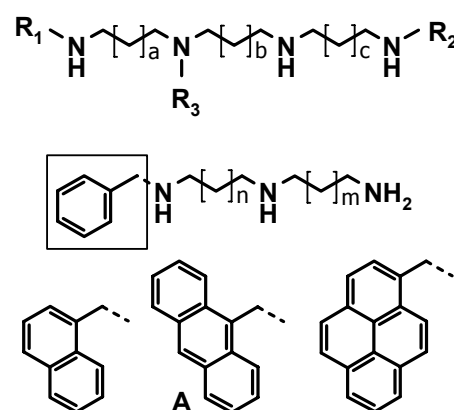


Figure 13 Schemes for PA analogues with ring structure containing conjugates. A, anthracene.

compound A in Fig. 13) conjugated homo-SPD ($n=m=2$) is readily imported into cells (Wang et al., 2003a). Anthracene-conjugated SPM analogues ($R_1=A$, $R_2=H$ or aminopropyl, $R_3=H$) appear to be even better vehicles. Interestingly, a SPM analogue with anthracene in the side-chain ($R_3=-CH_2CH_2CH_2-A$) is also taken up effectively (Phanstiel et al., 2000). However, homo-SPD containing four interlocked 6-carbon ring structure conjugate is too bulky (compound on right in Fig. 13). As expected, the SPD derivatives compete with SPD but in the L1210 cell-line anthracene-SPM analogues seem to use some other transport system than SPD. Also longer SPM derivatives (a, b, c, $n \geq 1$, $R_2=$ aminopropyl etc.) appear to be potential tools for studying transmembrane trafficking (Phanstiel et al., 2000). Studies with CHO and CHO-MG cell lines confirmed that PA transport system is used for the uptake of these analogues (Wang et al., 2003b).

2.4 TRANSGENIC RODENTS WITH ALTERED POLYAMINE METABOLISM PRODUCED IN THE UNIVERSITY OF KUOPIO

Animals over-expressing human ODC, rat AdoMetDC, human SPDSy and mouse SSAT have been generated. Transgenic animals carrying mouse MT promoter-driven human ODC and mouse SSAT are also available. In the ODC transgenic rodents, ODC is expressed in all tissues with the greatest activities (20 to 80-fold increases) being detected in testis, brain, muscle and heart (Halmekytö et al., 1991). Interestingly, the accumulated PUT is not converted to higher PAs (Halmekytö et al., 1993) and no activation of PA catabolism or urinary excretion is evident in the ODC over-expressing rodents, indicating that some other mechanisms can prevent excessive accumulation of higher PAs. Both the ODC transgenic male mice and rats suffer from greatly decreased fertility but show no signs of generally increased tumorigenesis (Alhonen et al., 1995)

even though elevated ODC activity has been linked with transformation (Auvinen et al., 1992). However, the ODC transgenic mice are more sensitive than their syngenic littermates to chemically induced skin papillomas and there is evidence that ODC is linked with skin tumorigenesis though the actual mechanisms are unknown.

The AdoMetDC and SPDSy transgenic animals express the transgenes only moderately (up to 5-fold) and do not show any apparent phenotypic alterations. Furthermore, both the AdoMetDC/ODC (Heljasvaara et al., 1997) and the SPDSy/ODC (Kauppinen et al., 1993) hybrid mice appear normal and PA pools in these animals remain practically unchanged.

Activated PA catabolism is clearly evident in both the SSAT over-expressing mice and the MT-SSAT transgenic rodents. The SSAT over-expression greatly affects the transgenic mice; PUT and AcSPD become greatly accumulated while higher PAs are depleted. Interestingly, PUT accumulation (be it from ODC or SSAT over-expression) is tolerated in brain and may even be neuroprotective (Kaasinen et al., 2000; Lukkarinen et al., 1999). Surprisingly, the low degree of basal SSAT elevation results in female infertility, skin wrinkling and hair loss (Pietilä et al., 1997). Unexpectedly, the MT-ODC/MT-SSAT hybrid mice show signs of even more severe PA catabolism as the accumulating PUT is not converted to SPD (Suppola et al., 2001). Furthermore, MT-SSAT transgenic mice are extremely sensitive to continuous DENSPM treatment as indicated by the high mortality rate in five days associated with ultrastructural abnormalities (i.e. mitochondrial swelling) in the liver (Alhonen et al., 1999; Suppola et al., 1999). Here, some characteristics of different tissues of the MT-SSAT transgenic mice and rat are briefly described (for an extensive review see Jänne et al., 2004).

LIVER: Liver is the organ processing most of the toxic substances and as such is responsible for maintaining balanced homeostasis of many vital functions. The regeneration potential of liver is essential as any damage may be life-threatening. The initiation of the liver regeneration after partial hepatectomy is directly dependent on hepatic PA contents in rat (Alhonen et al., 2002). Partial hepatectomy is characterized with severe SPD depletion in the MT-SSAT rats and SPD level appears to be critical for early liver regeneration. The hepatic recovery processes are initiated as effectively in MeSPD pretreated MT-SSAT rats as in their syngenic littermates without MeSPD treatment but not in transgenic rats without SPD analogue supplementation (Räsänen et al., 2002).

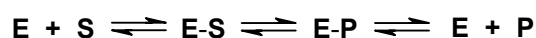
PANCREAS: Pancreas with its endocrine and exocrine portions is another tissue with multiple functions. The endocrine portion is involved in sugar metabolism while the exocrine portion excretes digestive enzymes into the duodenum. Under normal conditions, autodigestion is prevented as the digestive enzymes are mostly produced in inactive forms and stored in the zymogen granules. However, in acute pancreatitis (common causes are alcohol and drug abuse) premature activation of the digestive enzymes (most notably trypsinogen) seems to cause inflammation and pancreatic tissue injury. Zinc administration in MT-SSAT transgenic rat induces acute pancreatitis (Alhonen et al., 2000) whereas DENSPM treatment alone is not sufficient to cause the inflammation. However, combined treatment with DENSPM/MDL 72527 also induces acute pancreatitis indicating that the loss of SPD, not the H₂O₂ production, is the key factor in the development of the disease in these animals. The pretreatment with MeSPD prevents the SSAT induction mediated development of pancreatitis and confirms the crucial importance of SPD in these animals.

SKIN: The skin of the MT-SSAT rat wrinkles with age and the animals start losing hair at the age of 6 to 8 weeks, similar to the MT-SSAT transgenic mice. The keratinocyte differentiation in the MT-SSAT mice is clearly abnormal (Pietilä et al., 2005) and suggests that the availability of PUT is linked to correct keratinocyte maturation. As a proof of the significance of PUT, DFMO treatment of transgenic mice restores hair growth to some extent. However, no studies on the MT-SSAT transgenic rat skin have been carried out.

2.5 OPTIMIZING ENZYME ACTION

By definition, enzymes are biological catalysts that accelerate chemical reactions by many orders of magnitude (acceleration ranges for example from 10⁶ for carbonic anhydrase to 10¹⁷ for alkaline phosphatase compared to the non-enzymatic reaction). The rate of acceleration is expressed as k_{cat} and it stands for the maximum number of substrate molecules converted to product molecules per active site per second (for example, the k_{cat} value for papain is 10 while for catalase it is 10⁷). The size of enzymes varies from fairly small (M_w of few thousands g/mol) to extremely large molecules (M_w of millions g/mol and composed of multiple sub-units). Enzymes may have high substrate specificities that depend on environmental factors. Interestingly RNA can also catalyze reactions (reviewed by Lorsch and Szostak, 1996). The enzymatic reaction can be presented as free substrate (S) forming a complex with enzyme (E-S), conversion of substrate to product in the active centre (E-P) from which the product is released (Scheme 1).

The enzymes are composed of hundreds or thousands of covalently bound AAs which fold in according to the nature of AAs. Also other



Scheme 1 Basic scheme for enzymatic reaction from free substrate to released product.

non-covalent bonds maintain the three dimensional structure of the proteins intact; hydrogen bonds are essential for α -helix and β -sheet integrity but electrostatic interactions between the side-chains of AAs are also important. Ionic, ion-dipole and dipole-dipole interactions as well as charge transfer complexes are present in proteins. The essential phenomena in many enzymatic reactions are hydrophobic interactions i.e. organized water molecules are pushed away from the hydrophobic surfaces of the enzyme and the substrate as they approach each other (entropy increases). Only an accumulation of these rather weak non-covalent interactions permits the substrate binding. The mechanisms of the enzymatic catalysis include approximation (two substrates are close to each other), nucleophilic (covalent) catalysis (enzyme binds substrate covalently forming a more reactive intermediate), acid-base catalysis (proton transfer), electrostatic catalysis (the ionic charge stabilizes transition state), desolvation (H_2O removal destabilizes the charged group of the substrate in a lower dielectric constant environment) and strain which is characterized by conformational distortion(s) in the enzyme and/or the substrate (reviewed in an excellent book by Silverman, 2000).

2.5.1 Stereospecificity of enzymes

Proteins are extremely chiral compounds, thus it is no surprise that enzymes express a great degree of selectivity towards the different isomers of the substrates. The stereoselectivity of the enzymatic reaction arises from the configuration of the active site which depends on the specific functional groups of AAs. One isomer of the substrate simply may fit poorly or not at all into the active site whereas a product of the enzymatic reaction with an unfavorable configuration may be released from the enzyme very slowly or not at all (enzyme activated inhibitors). The production of enantiomerically pure compounds is imperative in

pharmaceutical industry as many drugs are more active in one configuration and the "wrong" enantiomer may be inactive, inhibitory or even toxic.

2.5.2 Forced evolution of enzymes

Enzymes have tremendous potential for use as practical catalysts in the pharmaceutical industry. Currently there are not many industrially used enzymes and these are mostly limited to some rather simple enzymes like lipases, esterases, catalases, peroxidases and hydroxylases (reviewed by Zaks and Dodds, 1997). During the last few decades, several approaches have been introduced to increase the utility of the enzymes in the chemical industry. Improving the stability of the enzymes and altering the substrate specificities are of obvious interest.

Directed evolution is a method with which new enzymes are developed and screened for greatly improved performance (Chirumamilla et al., 2001). The procedure involves mutation of the gene of the target enzyme, expression of the new gene(s) and screening of the freshly-synthesized enzymes for the desired properties. When small regions of the enzymes are targeted, site-directed mutagenesis with degenerate oligonucleotides is a simple method (Hermes et al., 1990). Another effective method is error prone PCR where the target gene is amplified in varying concentrations of Mn^{2+} . However, the most successful method is DNA shuffling where the gene of interest is cleaved into many random fragments, purified and recombined in a PCR like process without exogenous primers. After terminal primer addition in the final step of extension, full-length sequences are amplified and cloned (Cramer et al., 1998). The greatest advantage is the fact that wild type genes can be pooled from the same family of enzymes (possibly from different organisms) and amplified under mutagenic conditions (Stemmer, 1994). The described

methods have produced some very useful industrial applications such as enzymes that can utilize benzene, toluene, PCB and related biphenyl compounds as their substrate (Kumamaru et al., 1998). There are many successful applications i.e. a peroxidase (used in oxidation of dyes) that is stable in alkaline (pH 8.5–10) and highly oxidative conditions, proteases and phospholipases with greatly improved thermal stabilities and a subtilisin (protease) mutant that does not require calcium and is 100-fold more stable in strongly chelating conditions than the wild-type enzyme (reviewed by Chirumamilla et al., 2001).

2.5.3 Allosteric regulation

Enzymatic reactions are equilibrium reactions and the products often have feedback effects. A product may compete for the active site with the substrate (negative feedback) or interact with the enzyme protein at some other site (allosteric site). Binding of the regulator to the allosteric site may induce positive or negative conformational changes in the enzyme (Kern and Zuiderweg, 2003) i.e. the substrates may access the active site more or less easily, respectively. The affinity of G418 to ODC is rather low ($K_i=8$ mM at pH 7.0 with (L)-ornithine) but clearly causes conformational alterations to ODC protein (Jackson et al., 2003). It appears that allosteric ligands are often small hydrophobic molecules (mostly inhibitors) that may bind on the surface but also deep into the core of the enzyme or between the dimerization interfaces of the subunits of the enzyme stabilizing one conformation (reviewed by Hardy and Wells, 2004).

2.5.4 Other factors affecting enzymatic reactions

The pharmaceutical industry aims for cost-effective methods. However, protein–protein interactions become more likely when the protein

concentration is increased. This phenomenon exhibits similar characteristics to allosteric regulation (Goh et al., 2004). Protein immobilization is a way to counter many negative effects and the method also enhances the use of enzymes in continuous processes. The stabilities, substrate specificities and activities of the enzymes used may improve after immobilization. Also the separation of the products as well as recycling and disposal of the enzymes is straightforward (reviewed by Cao et al., 2003).

2.5.5 Enzymes in organic solvents

As long as the use of enzymes is limited to aqueous media, the bioconversion of many organic chemicals is limited. The solubility of many potential compounds is very low in water and the recovery of the products may be difficult. Furthermore, common organic reagents degrade in water, unwanted side reactions are frequent and thermodynamic equilibria of many processes are unfavorable. Thus, the use of an organic solvent would circumvent many of the problems associated with water-based reaction media and the concept of “medium engineering” has surfaced after the appearance of some interesting findings of enzyme behavior in non-aqueous solvents (reviewed by Klivanov, 1997).

The major parameter in aqueous media is pH but this has no significance in organic solvents. However, enzymes have “pH memory” i.e. the catalytic activity reflects the pH of the aqueous solvent from which the enzymes were collected. Proteins are also very rigid in non-aqueous media because intra-protein interactions are far stronger when there are no H₂O-molecules present. Structural inflexibility also means that enzymes have “ligand memory” and the active site retains the configuration of the ligand with which it was lyophilized. This structural stability is especially valuable when enantiomeric selectivity of the enzyme is sought (reviewed by Klivanov, 2001).

Enzymes often express totally unpredictable properties in organic media. Such qualities include extreme thermostability and dramatic substrate specificity alterations: enantiomer selectivity may change between different organic solvents; different functional groups are targeted etc. The absence of hydrogen bonds may even permit for reverse reactions to occur e.g. ester formation from alcohols and acids (reviewed by Carrea and Riva, 2000).

Unfortunately, enzymes tend to be far less active in organic media. However, some solutions are available: hydrogen bond forming solvents (glycerol, ethylene glycol) may be used to "relax" proteins and even small amounts of water (< 3 %) can be added into the reaction media. Lyophilization appears to be major reason for the low activities of enzymes in organic media and thus lyoprotectants like poly(ethyleneglycol), crown ethers, sugars and even salts have been used during the enzyme preparation (reviewed by Klibanov, 2001).

3 AIMS OF THE STUDY

The PA derivatives with a single methyl group in α -position (MeSPD, MeSPM and Me₂SPM) were first introduced in the early 1990s. However, the synthetic protocols proposed at that time were inefficient and appropriate only for milligram-scale production. Accordingly, these compounds have previously been studied only *in vitro*.

This study was initiated when it was shown that MeSPD can fulfill extremely well the cellular role of natural SPD during severe PA depletion (Räsänen et al., 2002). However, a good supply of these substances was necessary for extensive *in vitro* and *in vivo* studies. Furthermore, to obtain a thorough understanding of the fates of these PA analogues in the cellular environment and their actual relevance to the PA homeostasis, multiple simultaneous approaches were required.

The major goals in this study were:

- ▶ Establish an immortalized fibroblast cell line from the MT–SSAT transgenic rat to be used as a tool to test hypotheses before *in vivo* studies.
- ▶ Examine the stabilities of all three α -methylated PA derivatives in rat liver extracts and as substrates for recombinant oxidases (SMO and PAO) *in vitro*.
- ▶ Study the fates and significances of the PA derivatives in the MT–SSAT transgenic animals *in vivo*.
- ▶ Study the significance of substrate chirality in PAO-mediated oxidation.

4 MATERIALS AND METHODS

4.1 POLYAMINE ANALOGUES AND OTHER CHEMICALS (I–III)

The synthesis of the α -methylated PA derivatives is described in detail in **II**. The enantiomers of MeSpd and AcMeSpd (1-amino-8-acetamido-5-azanonane) were prepared as presented earlier (Grigorenko et al., 2005) and in **II**, respectively. The two isomers of MeSPM and the diastereomers of Me₂SPM were also synthesized according to the principles presented in **II** and described in greater detail in **III**. The purities of all α -methylated PA analogues and their different enantiomers were checked with ¹H- and ¹³C-NMRs (Avance DRX Spectrometer at 500.13 MHz) and all proved to be >99.5 % pure. DENSPM, DESPM and bis-benzyl-SPM (Fig. 10, page 24) were synthesized principally as described earlier (Rehse et al., 1990) and proved to be >98 % pure according to ¹H- and ¹³C-NMR. All compounds were further studied with the HPLC and again showed excellent purities. MDL 72527 was a generous gift from Hoechst-Roussel.

Aminoxy-ethyl-putrescine (AOE-PUT) and its acetyl and oxime derivatives were synthesized as referred to in **III**, lyophilized and dissolved in 100 mM stock solutions in water. All proved to be >95 % pure according to both NMR and HPLC studies. Preformed Schiff bases of SPM with PL (PL=SPM) was prepared as described in **III**.

The short PA analogues (2-2-SPD and 2-3-2-SPM, Fig. 14) and their bis-substituted derivatives were prepared ($R_1=R_2=R_3=R_4=$ -ethyl or -acetyl groups) as described earlier (Saramäki, 2004).

All other chemicals were purchased from Sigma-Aldrich and Fluka. Freshly distilled carbonyl compounds were checked by ¹H-NMR, dissolved as 100 mM stock solutions in ethanol and stored in the refrigerator prior to studies. Hydrochloride

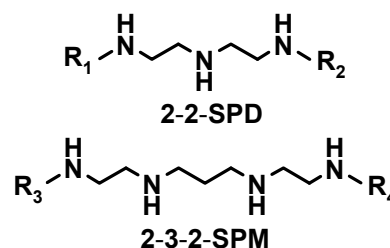


Figure 14 Schemes for short SPD and SPM derivatives.

salt of PL was dissolved in H₂O and stored in -20 °C. [6-³H]Thymidine (specific radioactivity 18 μ Ci/mmol) for DNA-synthesis studies was obtained from Perkin Elmer Life Sciences.

4.2 TRANSGENIC ANIMALS (I, II)

The generation of the MT-SSAT transgenic rats and mice has been described earlier in detail (Alhonen et al., 2000; Suppola et al., 1999). Both animal lines were produced by the standard pronuclear injection technique using the same transgene construct; the mouse MT-promoter driven mouse SSAT. The animals were housed in the National Laboratory Animal Centre of Kuopio University under controlled temperature (20 \pm 2 °C), humidity (50–70 %) and lighting (12 h/12 h) and fed *ad libitum*.

Different α -methylated PA analogues and MDL 72527 were dissolved in saline and administered i.p. according to Table I. After sacrifice, the tissue pieces were frozen in liquid nitrogen and homogenized in standard buffer (25 mM Tris-HCl pH 7.4 with 0.1 mM EDTA and 1 mM dithiothreitol, DTT). The 20 μ l aliquots of the homogenates, supplemented with 180 μ l of 5 % w/v sulphosalicylic acid in 10 μ M 1,7-diaminoheptane, were used for the PA assays. The homogenates were centrifuged (at +4 °C and

Table I Different polyamine analogue doses and treatment timetables for animal studies

ANIMAL	TREATMENT	DOSAGE	TIME BEFORE SACRIFICE	PUBL.
<i>RAT</i> <i>Liver regeneration studies</i>				
MT–SSAT	MeSPD	5 mg/kg	20 h + 4 h	I
transgenic	MeSPD	25 mg/kg	20 h + 4 h	I
	Me ₂ SPM	25 mg/kg	20 h + 4 h	I
<i>RAT</i> <i>Analogue stability / toxicity studies</i>				
MT–SSAT	MeSPD	25 mg/kg	22 h + 16 h	I + II
transgenic	MeSPM	25 mg/kg	22 h + 16 h	I + II
	Me ₂ SPM	25 mg/kg	22 h + 16 h	I + II
	MDL 72527 with or without analogue treatment	50 mg/kg	40 h + 24 h	I
<i>MOUSE</i> <i>Analogue stability / toxicity studies</i>				
MT–SSAT	MeSPD	50–500 mg/kg	24 h	II
transgenic and	MeSPM	12.5–50 mg/kg	24 h	II
syngenic mice	Me ₂ SPM	12.5–50 mg/kg	24 h	II

13'000 × g for 30 min) and the supernatant fractions were used for the enzyme assays.

In the liver regeneration experiments, the rats were sedated with a 0.1 ml dose of fluanisone (Hypnorm, Janssen) and fentanyl citrate mixture (10 mg/ml and 0.315 mg/ml; respectively). The anesthetic was a mixture of midazolam (Dormicum, Roche), fluanisone and fentanyl citrate (1.25 mg/ml, 2.5 mg/ml and 0.07875 mg/ml; respectively). The anesthetic was administered at 0.2 ml/100 g dose. The Institutional Animal Care and Use Committee of the University of Kuopio and the Provincial Government approved the animal experiments.

4.3 IMMORTALIZED MT–SSAT TRANSGENIC RAT FIBROBLASTS (I, III)

The 13 days old MT–SSAT transgenic rat fetuses were used as the sources for the fibroblasts. The

fibroblast processing was performed as described earlier (Mackintosh and Pegg, 2000). Transfection was performed with the same commercial kit LIPOFECTAMINE™ plus; the same plasmid expressing SV40 small *t* and large *T* antigens (a kind gift from Dr. M. J. Tevethia, the Department of Microbiology and Immunology, Pennsylvania State University College of Medicine, Hershey, PA, USA) was also used. Thirty one different cell populations out of 240 were identified with the aid of the quantitative PCR and appropriate cell populations were selected for further studies according to their growth rates, external appearance and SSAT expression.

In different experiments, the immortalized fibroblasts were grown in DMEM (Invitrogen) supplemented with heat-inactivated 10 % FBS, gentamycin (50 µg/ml; Gibco) and with or without geneticin (G418) supplementation (500 µg/ml;

Sigma). The freshly plated cells were allowed to adhere for 24 h before fresh medium and the drugs were supplemented. After the incubation, the cells were washed once with PBS, detached with trypsin and counted. The PA concentrations were measured from the supernatant fractions after sulphosalicylic acid precipitation.

4.4 RECOMBINANT PROTEINS (I, III)

The production of the plasmids containing cDNAs coding the human SMO and human PAO ORFs with 6 \times His at the N-terminus is explained in detail in **I**. These plasmids were provided by Dr. Vujcic, Grace Cancer Drug Center, Roswell Park Cancer Institute, Buffalo, New York 14263, USA. Bacteria containing plasmids were cultured and collected according to Qiagen Qiaexpressionist™ manual and the proteins were purified under native conditions using Ni-NTA His Bind Resin (Novagen) according to the manufacturer's instructions. PAO was further purified with affinity chromatography and the procedure is explained in detail in **I**. As the last step proved to be ineffective, in **II** and **III** it was not performed. The production of the plasmid containing the recombinant human SSAT cDNA was performed by Dr. Anne Uimari and the exact procedure is described in **I**. Similarly to the oxidases, hSSAT was also purified with Ni-NTA Resin under native conditions.

Both hSMO and hSSAT were very pure according to SDS-PAGE while hPAO was estimated to be 80 % pure. However, the contaminating bacterial protein in hPAO preparation did not have any PAO-like activity. The protein concentrations were measured by using the Bio-Rad Protein Assay (Bio-Rad, CA, USA). The kinetic studies of the oxidizing enzymes were performed mostly in duplicate with three to six different 10 up to 1000 μ M substrate concentrations (**I-III**) and a few in triplicate (**III**). The hSMO and hPAO reactions were carried out in a total volume of 180 μ l 100 mM glycine-

NaOH at pH 9.5 (**I-III**), 100 mM alanine-NaOH at pH 9.5 (**III**), 50 mM borate at pH 9.3 (**III**), 50 mM phosphate at pH 7.4 or pH 9.5 buffers (**III**); all buffers contained 5 mM DTT as well. Different freshly distilled 5 mM aldehydes (**I, III**) were used to decrease the K_m values and to increase the reaction velocities of hPAO (Hölttä, 1977). The effects of different structurally related 5 mM ketones (**III**) on the metabolism of the different isomers of MeSPD were similarly tested with hPAO. The reactions were allowed to proceed from 10 to 60 min at +37 °C before stopping the reactions with sulphosalicylic acid precipitation. The reaction mixtures without hPAO were routinely checked to exclude any enzyme independent degradation of substrates. The kinetic studies of hSSAT were performed with four different 100 up to 1000 μ M SPD or SPM substrate concentrations. Similarly, four different 100 up to 1000 μ M MeSPD or Me₂SPM concentrations were used as competitive inhibitors.

4.5 LIVER EXTRACTS (I, III)

Wild-type Wistar rats were sacrificed and livers were processed as described in **4.2**. All supernatants were combined and eluted at +4 °C in the standard buffer (**4.2, I**) or the borate buffer (**4.4, III**) with high-salt supplementation (500 mM NaCl) to remove the natural PAs. The elutions were performed with Amicon Ultra-15 centrifugal filter devices (Millipore) with nominal M_w limit of 30,000. The resulting liver eluates were desalted thrice in the same columns with non-salt buffer to remove excess salt. The final eluates were pooled and the protein concentrations measured with the aid of the Bio-Rad protein assay.

The reaction buffers, 100 mM glycine-NaOH at pH 9.5 (**I**) or 50 mM borate at pH 9.3 (**III**), were used to study the metabolism of the natural PAs and the α -methylated derivatives. An appropriate amount of the liver extract was used

in the total reaction volume of 180 μ l. The reactions were initiated with the addition of the PAs or their analogues; in the case of MDL 72527 (250 μ M), extracts were pre-incubated for 10 min to inactivate PAO before substrate addition. Different 5 mM aldehydes were used similarly as in **4.4** to enhance the activity of PAO. The reaction tubes were incubated for 60 (**I**) or 120 (**III**) min in +37 °C water-bath and stopped as in **4.2**.

4.6 ANALYTICAL METHODS

The treatment of the transgenic rats before partial hepatectomy and the determination of DNA synthesis rate were performed as described earlier (Räsänen et al., 2002). The ODC activities (**I**) were measured as described by Jänne and Williams-Ashman (1971). The SSAT activities (**I–III**) were measured essentially as described by Bernacki et al. (1995). Furthermore, the tissue PAO activities (**I**) were measured principally as described by Kumazawa et al. (1990) except that radioactive N^1, N^{11} -diacetylnorspermine was used as the substrate. Alanine aminotransferase and α -amylase were determined from heparinized blood plasma of the mice using an analyzer system Microlab 200 from Merck.

The concentrations of the PAs and analogues were determined with the aid of HPLC (**I–III**) essentially as described by Hyvönen et al. (1992). Separation of (*R*)- and (*S*)-isomers of MeSPD (**III**) was performed with Chiral-HPLC (Wheik-O 1 (*R,R*) 25 cm * 4.6 mm column and isocratic run 0–75 min with a flow-rate of 0.55 ml/min 70 % EtOH) after dansylation and treatment as described earlier (Porter et al., 1985).

4.7 STATISTICAL METHODS

Where applicable, the data is expressed as means \pm SD and analyzed by two-tailed Student's test. For multiple comparisons, one-way analysis of variance with the Dunnett's *post hoc* test was

used with the aid of a software package, GraphPad Prism 3.0 (GraphPad Software Inc., San Diego, USA).

5 RESULTS

5.1 METABOLIC STABILITY OF α -METHYLATED POLYAMINE ANALOGUES (I)

The recombinant protein studies showed that MeSPD similarly to SPD was not a substrate for either hPAO or hSMO. However, both MeSPM and Me₂SPM were degraded by both oxidases. Surprisingly, the activity of hPAO seemed to increase with the number of methyl substituents, whereas the activity of hSMO clearly decreased with the same substrates when compared to SPM as a substrate. As expected, the activity of hPAO was enhanced by aldehyde supplementation. Furthermore, MeSPD and Me₂SPM were not substrates but competitive inhibitors of hSSAT when natural PAs were used as substrates, confirming that the α -methylation protects the compound from acetylation but not from oxidation. The fates of the analogues in the liver extract studies (corresponding to the cellular environment) supported the results achieved with the recombinant proteins.

The studies with the immortalized MT-SSAT fibroblasts verified that both SPM derivatives were not metabolically totally stable. However, Me₂SPM was clearly more resistant to degradation than MeSPM. Furthermore, MeSPD appeared to be metabolically stable and was not converted to MeSPM. The PA analogues accumulated efficiently in the cells, induced the SSAT activity to some extent and effectively substituted for the natural PAs. As expected, MeSPM was the least tolerated analogue due to its more extensive degradation.

MeSPD did not appear to undergo any metabolism *in vivo*. In line with the *in vitro* studies, both SPM analogues were degraded in the liver of the MT-SSAT transgenic rat. Pretreatment of the transgenic rats with a low MeSPD dose (2×5 mg/kg) did not restore the early liver regeneration after partial hepatectomy.

However, transgenic rats pretreated with Me₂SPM (2×25 mg/kg) and higher MeSPD (2×25 mg/kg) dose did not suffer from delayed liver regeneration after surgical removal of two thirds of the liver. In fact, Me₂SPM appeared to be even more effective than MeSPD on a molar basis. MeSPD detected in the liver of Me₂SPM treated animals was only half of MeSPD detected in the liver of rats given the lower MeSPD dose. The salvaging effect of Me₂SPM was verified *in vitro* revealing that Me₂SPM reversed the growth inhibition by DFMO in the MT-SSAT fibroblasts.

5.2 EFFICIENT SYNTHESIS PROTOCOLS FOR α -METHYLATED POLYAMINE ANALOGUES AND THEIR *IN VIVO* AND *IN VITRO* STUDIES (II)

Straightforward synthetic protocols for each α -methylated PA derivative were developed. Commercially available ethyl 3-aminobutyrate was used as the starting material for MeSPD, MeSPM and Me₂SPM syntheses. The mesylated derivative of benzyl chloroformate protected 3-aminobutanol was the key intermediate for the preparation of all PA mimetics. MeSPD, MeSPM and Me₂SPM were prepared in five, seven and seven steps, respectively. The synthesis yields were satisfactory: 40–60 % of the starting material was converted to the target compound. Furthermore, (*R*)- and (*S*)-alaninols were used as the starting material for the production of the two isomers of AcMeSPD. The preparations of both AcMeSPD enantiomers in seven steps were successful and about 50 % yields for both (*R*)- and (*S*)-AcMeSPD were achieved.

The toxicity of the three α -methylated PA derivatives was extensively tested with syngenic and the MT-SSAT transgenic mice and rats. It can be concluded that all the analogues were well tolerated by mice and SPD was depleted in a

dose-dependent manner. α -Amylase and alanine aminotransferase (ALAT) activities (as determined from a heparinized blood plasma) remained virtually unaltered and the insignificantly increased SSAT activities also did not appear to have any deleterious effects. The transgenic rats tolerated only 2×25 mg/kg doses of the drugs.

The fibroblast studies showed effective depletion of natural PAs in both control and the SSAT over-expressing cell lines. In both cell lines, 1 mM MeSPD resulted in total loss of PUT and 90–95 % reduction of SPD in 48 h whereas SPM levels remained at about two thirds when compared to untreated cells. One millimolar Me₂SPM supplementation in both cell lines was even more effective resulting in a total loss of PUT and 90–95 % depletions of both SPD and SPM. At concentrations higher than 10 μ M, MeSPM was toxic to both cell lines. Supplementation of 1 mM aminoguanidine (AG) prevented the degradation of MeSPM effectively indicating that the unmethylated end of the SPM derivative is readily targeted by AG-sensitive oxidases. The SSAT activities remained unaffected in the control cell line and increased only slightly in transgenic cells as a result of exposure to the PA analogues. When comparing the uptake of the three PA derivatives, it seemed that the maximal uptake of these compounds was achievable with rather low concentrations in the studied cell lines. Ten micromolar MeSPM supplementation resulted in accumulation of MeSPD (as a degradation product of MeSPM) to about two thirds of the level achieved with PA derivatives in 1 mM MeSPD or Me₂SPM treated cells. In the presence of AG, the accumulation of MeSPM (combined with the minimal amount of MeSPD) was only marginally lower (about 50–60 % of detected analogues in 1 mM MeSPD/Me₂SPM treated cells). However, *in vivo*, the drug accumulation was dose-dependent.

Racemic AcMeSPD was a substrate ($K_m = 100$ μ M, $k_{cat} = 1.3$ s⁻¹) of hPAO suggesting that

acetylation is essential for the degradation of MeSPD and acetylation should greatly improve the degradation of both MeSPM and Me₂SPM by hPAO. The important finding was that (*R*)-AcMeSpd ($K_m = 95$ μ M, $k_{cat} = 9.0$ s⁻¹) was almost as good a substrate as AcSpd ($K_m = 14$ μ M, $k_{cat} = 8.5$ s⁻¹) for hPAO. (*S*)-AcMeSpd ($K_m = 170$ μ M, $k_{cat} = 1.2$ s⁻¹), however, appeared to be rather poor a substrate suggesting that hPAO has hidden potency for stereospecificity and may readily use (*R*)-enantiomer of AcMeSPD.

5.3 STEREOSPECIFICALLY FLEXIBLE POLYAMINE OXIDASE (III)

Seventeen aldehydes were tested for their ability to enhance hPAO-mediated degradation of racemic, (*R*)- and (*S*)-MeSPD (Fig. 16). The structures of the aldehydes greatly influenced the degradation rates of the substrates. For example, aliphatic aldehydes (Fig. 16, line E) appeared to be less effective than aromatic aldehydes (Fig. 16, lines A–D). The aldehyde sensitive site of hPAO appears to be restricted in its crucial dimensions as the position of carboxaldehyde group in structures with two aromatic rings is critical (Fig. 16, line A). Furthermore, nitrogen in the *p*-position to the carboxaldehyde group appears to be essential for effective degradation of (*S*)-MeSPD (Fig. 16, line B). PL with two additional hydroxyl groups (Fig. 16, line B on right) greatly increased the oxidation of (*S*)-isomer which was generally an unfavored substrate with the tested aldehydes. Furthermore, the position of the hydroxyl group in the three different hydroxyl-BAs was critical to the substrate degradation and only the *m*-position allowed any marked degradation of (*R*)-MeSPD (Fig. 16, line C). Cyclohexane carboxaldehyde also allowed efficient degradation of both racemic and the (*R*)-enantiomer of MeSPD. The position of the nitro group did not appear to have any major effect on the oxidation

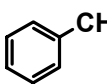
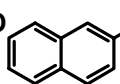
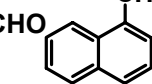

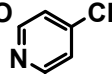
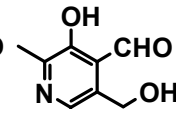
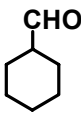
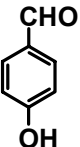
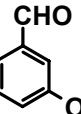
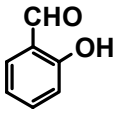
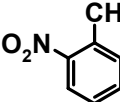
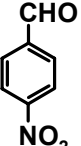
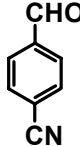
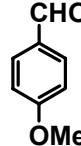
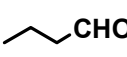
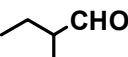

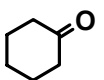
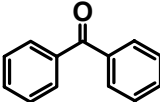

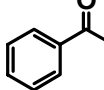
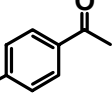
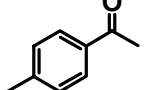
A				
				
racemic	12.7	5.8	nd.	
(<i>R</i>)-MeSPD	46.2	25.4	nd.	
(<i>S</i>)-MeSPD	2.1	0.7	nd.	
B				
				
racemic	18.0	11.6	5.1	
(<i>R</i>)-MeSPD	40.4	14.6	nd.	
(<i>S</i>)-MeSPD	4.7	8.2	14.1	
C				
				
racemic	8.6	1.2	6.7	nd.
(<i>R</i>)-MeSPD	28.0	1.7	16.1	0.9
(<i>S</i>)-MeSPD	1.3	0.7	2.0	nd.
D				
				
racemic	11.0	8.7	9.2	6.3
(<i>R</i>)-MeSPD	37.3	23.0	34.5	20.0
(<i>S</i>)-MeSPD	3.5	2.7	2.2	1.1
E				
				
racemic	2.9	3.0	0.4	
(<i>R</i>)-MeSPD	7.0	6.7	0.6	
(<i>S</i>)-MeSPD	0.8	0.7	0.5	
F				
				
				

Figure 16, lines A–E The percentages of hPAO-mediated oxidations of 200 μ M racemic, (*R*)- and (*S*)-MeSPD in the presence of 5 mM aldehydes in 1 hour at +37 °C in 100 mM glycine–NaOH at pH 9.5. **Line F** Structurally related ketones that proved ineffective. nd., not detected.

rates (Fig. 16, line D). Moreover, electron-repulsing ($-\text{OCH}_3$) or electron-withdrawing ($-\text{CN}$) substituents opposite to the carboxaldehyde group had no marked differences on the oxidation rates of the two MeSPD isomers (Fig. 16, line D). The structurally related and tested ketones proved ineffective as hPAO enhancers (Fig. 16, line F).

Based on the screening studies with different aldehydes it seemed possible to enrich different isomers of MeSPD from racemic MeSPD by hPAO in the presence of guide-molecules (Fig. 17). BA supplementation in the reaction mixture was expected to result in the predominant degradation of (*R*)-MeSPD whereas PL should have evoked an opposite affect i.e. the degradation of (*S*)-MeSPD. Moreover, pyridine 4-carboxaldehyde (P4CA) appeared to be an unselective enhancer for the degradations of both MeSPD enantiomers in the presence of hPAO (Fig. 16, aldehyde in the middle of line B). Chiral-HPLC analysis after 5 mM BA and PL supplemented reactions revealed that this indeed had occurred (Fig. 18). No reduction of reaction products was performed. Therefore, it is not clear whether 3-aminobutanals retain their configuration or if they are racemized during the reaction. Nonetheless, the 3-aminobutanal formed is labile in the aqueous environment and degrades rapidly. The rat liver extract studies showed that native PAO is also prone to stereospecific steering by BA and PL.

The key aldehydes were also studied with the three diastereomers of Me₂SPM and the two enantiomers of MeSPM. BA increased the reaction rates of hPAO with all chiral substrates whereas PL supplementation clearly enhanced the hPAO-mediated degradation of only (*S,S*)-Me₂SPM and did not enhance the use of the methylated terminus of (*R*)-MeSPM. The kinetic studies revealed that successful 5 mM BA or PL enhanced

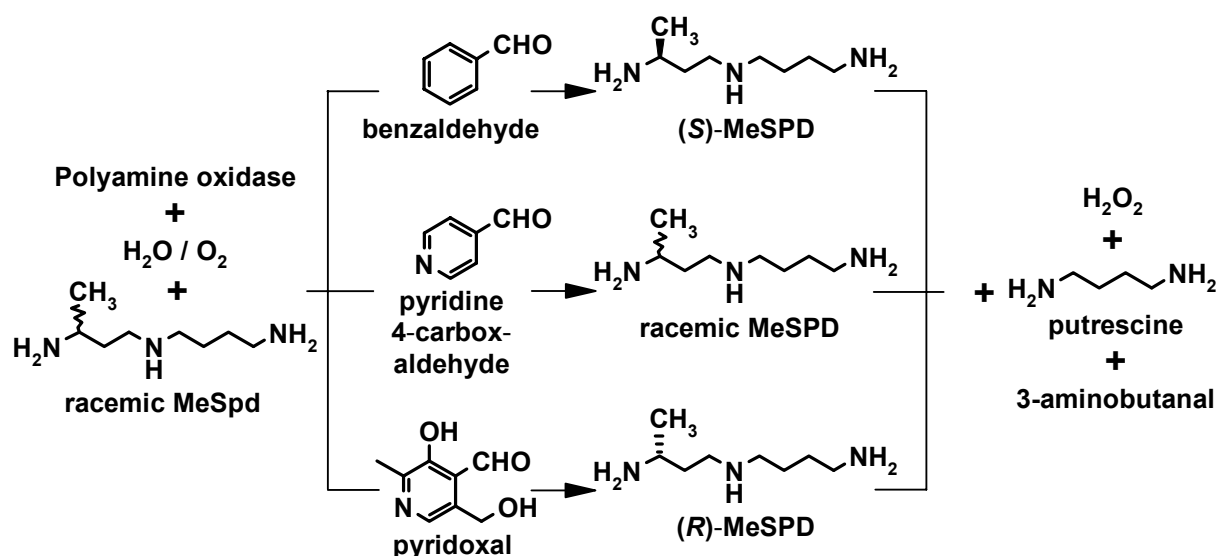


Figure 17 Scheme for stereospecific degradation of MeSPD by PAO in the presence of aromatic aldehydes

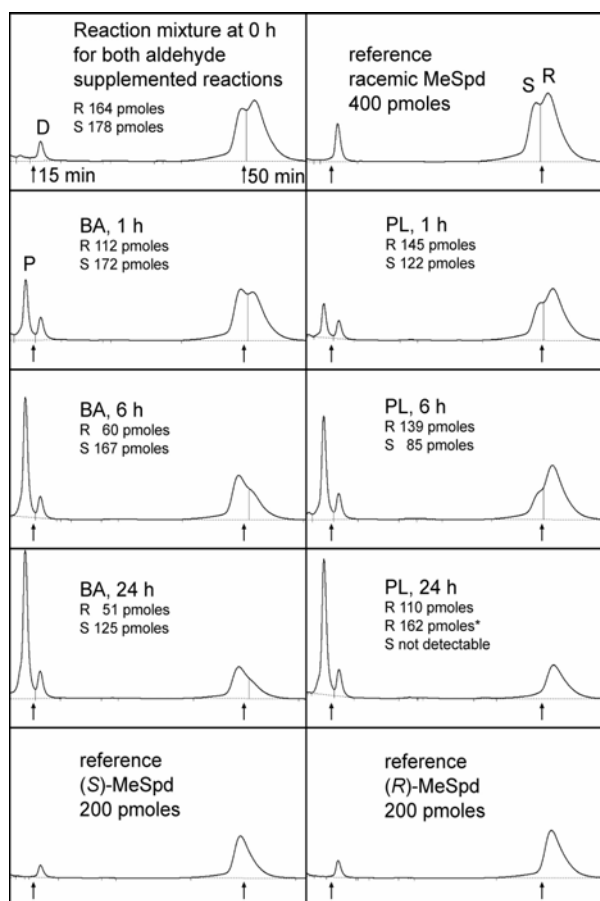


Figure 18 Chiral-HPLC analysis of 5 mM BA or PL supplemented hPAO with 200 μ M MeSPD as the substrate in 100 mM glycine-NaOH at pH 9.5 during 24 h. D, 1,7-diaminoheptane; P, PUT; R, (R)-MeSPD; S, (S)-MeSPD.

hPAO reactions exhibited increased k_{cat} values and almost invariably reduced K_m values (Table II). In the presence of 5 mM P4CA, the affinities of the substrates to hPAO were poorer (especially with SPM mimetics) than with 5 mM BA and PL.

Increasing the aldehyde concentration clearly increased the hPAO-mediated oxidation rates when both SPM and Me₂SPM were used as substrates in both glycine and borate buffers at high pH. However, the reaction rates were not dependent on the order of PA, aldehyde and hPAO addition. Aldehyde enhanced PAO-mediated reaction is clearly characterized by a complex equilibrium between aldehyde, PA and the enzyme. Kinetic studies showed that, in the presence of 5 mM BA or PL, the affinities of PAs to hPAO were slightly increased. However, when the enzyme was saturated (>90 %) with high substrate concentration (K_m for SPM = 47 μ M), it was possible to estimate the affinities of aldehydes to the hPAO-PA adduct. A 1 mM SPM concentration was used with BA, P4CA or PL concentrations increasing from 0.1 to 5 mM but very low affinities were observed. Both BA and P4CA increased the reaction rates up to 5 mM concentration in the glycine buffer but PL

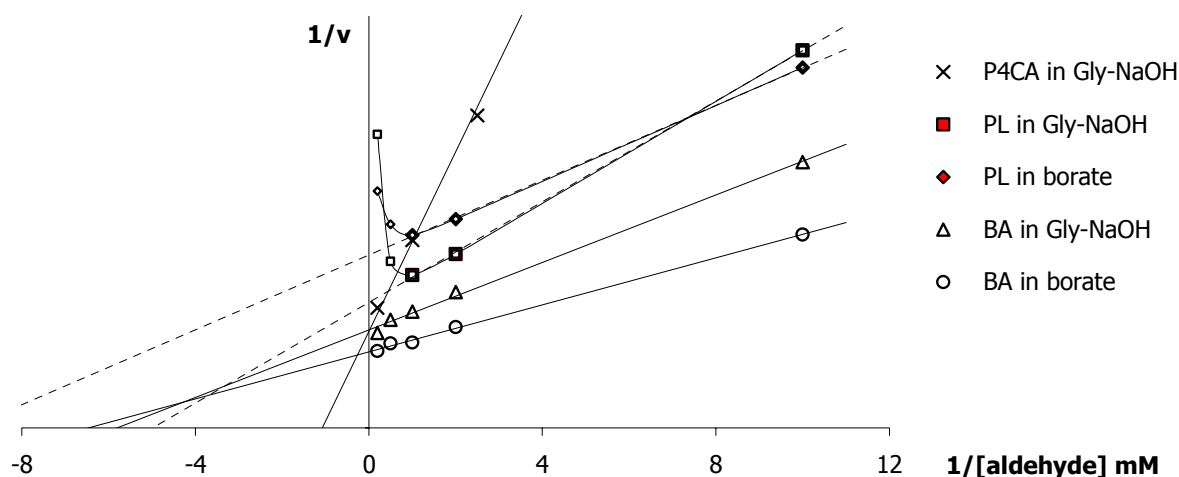


Figure 19 Lineweaver–Burk plotting with increasing aldehydes and 1 mM SPM as a substrate for hPAO. The reactions were carried out in 100 mM glycine–NaOH at pH 9.5 or 50 mM borate at pH 9.3 for 25 min with 0.1 μ g of the protein.

appeared to behave inconsistently (Fig. 19) and the reaction rate enhancement was greatest at 1 mM PL. The phenomenon with PL was observed when SPM (but not SPD) was used as a substrate for hPAO in both 50 mM borate and 100 mM glycine–NaOH buffers at high pH (Fig. 19). Therefore, the kinetic values for SPM and the diastereomers of Me₂SPM were determined in the presence of both 1 and 5 mM PL in the glycine buffer at pH 9.5 (Table II). The kinetic values of hPAO at constant 1 mM Spm and in the presence of increasing aldehyde supplementation were as follows. In the glycine buffer $K_m = 200 \mu\text{M}$ and $k_{\text{cat}} = 5.1 \text{ s}^{-1}$ for PL, $K_m = 170 \mu\text{M}$ and $k_{\text{cat}} = 6.6 \text{ s}^{-1}$ for BA and $K_m = 930 \mu\text{M}$ and $k_{\text{cat}} = 6.6 \text{ s}^{-1}$ values for P4CA were determined. In the borate buffer $K_m = 110 \mu\text{M}$ and $k_{\text{cat}} = 3.7 \text{ s}^{-1}$ for PL and $K_m = 150 \mu\text{M}$ and $k_{\text{cat}} = 8.4 \text{ s}^{-1}$ for BA were similarly determined. It may be that at the higher concentrations PL interacted with the enzyme. The degradation efficacy of 200 μM SPM by 0.1 μg hPAO (nmoles of SPD produced per μg of enzyme per min) in the presence of 5 mM PL was about 70 % compared to the SPM degraded by 0.5 μg hPAO supplemented with 5 mM PL (results not shown). It is also possible that the aldehyde

Table II Some kinetic values of hPAO in 100 mM glycine–NaOH at pH 9.5. (*R*), (*R*)–MeSPD; (*S*), (*S*)–MeSPD; (*R,R*), (*R,R*)–Me₂SPM; (*S,S*), (*S,S*)–Me₂SPM.

Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{s}^{-1}\text{M}^{-1}$)
AcSPD	14	8.5	610×10^3
SPD + 5 mM BA	9.4	0.85	79×10^3
SPD + 5 mM PL	25	0.49	20×10^3
(<i>R</i>) + 5 mM BA	20	0.68	34×10^3
(<i>R</i>) + 5 mM PL	5.9	0.01	1.7×10^3
(<i>S</i>) + 5 mM BA	14	0.06	4.3×10^3
(<i>S</i>) + 5 mM PL	5.1	0.24	47×10^3
AcSPM	1.1	17	15×10^6
SPM	47	0.4	8.5×10^3
SPM + 5 mM BA	4.5	12	2.6×10^6
SPM + 5 mM PL	4.1	2.9	700×10^3
SPM + 1 mM PL	3.9	2.7	710×10^3
(<i>R,R</i>)-Me ₂ SPM	55	0.12	2.2×10^3
(<i>R,R</i>) + 5 mM BA	13	2.7	210×10^3
(<i>R,R</i>) + 5 mM PL	12	0.08	6.7×10^3
(<i>R,R</i>) + 1 mM PL	21	0.13	6.2×10^3
(<i>S,S</i>)-Me ₂ SPM	16	0.97	61×10^3
(<i>S,S</i>) + 5 mM BA	21	6.1	290×10^3
(<i>S,S</i>) + 5 mM PL	5.7	2.4	420×10^3
(<i>S,S</i>) + 1 mM PL	27	6.0	220×10^3

reacted with the reaction intermediate(s) and/or product(s) and/or perhaps the resulting adduct(s) eluted similarly with SPM during the HPLC analysis. The two hydroxyl groups in PL probably play some role in the observed phenomenon. Whatever the reason, it appears that the aldehydes interact poorly with the targets for hPAO but the hPAO–PA–aldehyde–complex is efficiently processed as the k_{cat} values are rather high for hPAO (for AcSPD the k_{cat} value = 8.5 s^{-1}). Furthermore, preformed PL=SPM adducts were studied as substrates for hPAO to circumvent the low affinities of aldehydes for hPAO. As expected, 1 mM PL=SPM was more effectively degraded by hPAO than 1 mM SPM in the presence of 2 mM PL. The kinetic values for PL=SPM were estimated as $K_m > 1 \text{ mM}$ and $k_{\text{cat}} \sim 20 \text{ s}^{-1}$.

It has been suggested that the Schiff base of aldehyde and PA would be the target for PAO–mediated oxidation (Hölttä, 1977). Initial studies were performed in 100 mM glycine–NaOH and since glycine possesses the potential to form Schiff bases, other buffers were required to rule out possible buffer related artefacts. The general stereoselective degradation of MeSPD by the key aldehyde supplemented hPAO was retained in all three buffer systems studied at high pH (Table III). However, the stringency and efficacies of MeSPD oxidations varied greatly. BA supplemented hPAO–reaction appeared the least stereospecific in borate buffer though the degradation of (*R*)–MeSPD was as effective in both borate and NaH_2PO_4 buffers. PL addition to the substrate mixture exhibited the highest reaction rates with racemic, (*R*)– and (*S*)–MeSPD in borate buffer but, similarly to BA, the stereoselectivity of the reaction was best in glycine buffer. In all buffers, P4CA seemed to be an equally unselective guide molecule for enhanced oxidation by hPAO but it was also very ineffective in borate buffer. Brief studies with 100 mM (*D*)– and (*L*)–alanine–NaOH buffers at pH 9.5

Table III PUT (nmoles) detected in three buffers at pH 9.3–9.5 after 1 h reaction with 1 μg of hPAO and indicated amounts of MeSPD in the presence of the key aldehydes.

	racemic MeSPD	(<i>R</i>) MeSPD	(<i>S</i>) MeSPD	
5 mM BA	72 nmoles	36 nmoles	36 nmoles	(<i>R</i> / <i>S</i>)
NaH₂PO₄	3.8	8.4	0.7	12
Borate	6.7	8.5	1.1	8
Gly-NaOH	6.5	14.6	1.1	14
5 mM PL				(<i>S</i> / <i>R</i>)
NaH₂PO₄	1.2	ND	2.3	---
Borate	5.8	0.4	11.5	31
Gly-NaOH	3.5	0.2	9.0	50
5 mM P4CA				(<i>R</i> / <i>S</i>)
NaH₂PO₄	2.0	1.5	0.9	1.6
Borate	0.4	0.3	0.1	1.9
Gly-NaOH	2.5	2.3	1.5	1.5

detected no differences whatsoever with 100 mM glycine–NaOH on the stereoselectivity of hPAO in 5 mM BA or PL supplemented reactions. It seems that glycine or alanine do not participate in the isomer selective oxidation by hPAO in the presence of aldehydes.

Aldehydes can form Schiff bases only with the primary amino groups of PAs. As terminally *N*-alkylated PA derivatives are substrates for hPAO, the enhanced degradations of such compounds would indicate that aldehydes bind to an allosteric site in hPAO. DENSPM and DESPM were efficiently degraded by hPAO and the reactions evidenced the formations of nor–SPD, SPD, ethyl–nor–SPD, ethyl–SPD and probably also monoethyl–1,3–diaminopropane. Both reactions were inhibited by 20–50 % by either 5 mM BA or PL supplementation. Similarly, the oxidation of bis–benzyl–SPM was inhibited when 5 mM BA was added to the reaction mixture. Furthermore, the

oxidations of AcSPD and AcMeSPD were markedly inhibited by the supplementation with either 5 mM BA or PL. It seems that the primary amino group(s) in the substrate is (are) required for the observed aldehyde stimulus.

Reduced Schiff bases might be targets for hPAO mediated oxidation. However, reduction changes the properties of the nitrogen next to the C=N double bond. The Schiff base nitrogen becomes similar to other nitrogens in the SPD backbone and is protonated at physiological pH. We used AOE-PUT and its stable oxime analogues with acetone and BA (Fig. 20). Acetylated AOE-PUT (AcAOE-PUT) was also studied (Fig. 20). The presence of oxygen next to the Schiff base decreased the reaction rates of all AOE-PUT derivatives clearly when compared to AcSPD and BA-SPD adducts. However, the introduction of a hydrophobic phenyl group into AOE-PUT resulted in about a 100-fold difference in the affinities of AcAOE-PUT and acetone oxime to hPAO (Fig. 20). BA=AOE-PUT appears to be a potent competitive inhibitor for PAO.

Further studies aimed at clarifying the mechanism of aldehyde stimulus on hPAO proved

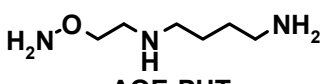
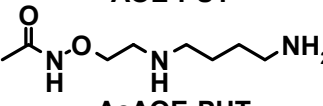
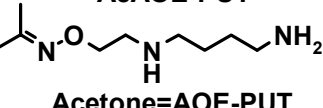
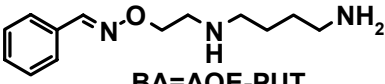
	K_m (μM)	k_{cat} (s^{-1})
 AOE-PUT	very poor substrate	
 AcAOE-PUT	290	0.54
 Acetone=AOE-PUT	380	0.17
 BA=AOE-PUT	3.4	0.04

Figure 20 The kinetic values of hPAO with different AOE-PUT derivatives as substrates in 100 mM glycine-NaOH at pH 9.5.

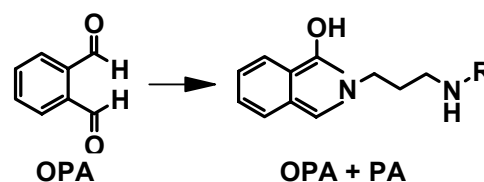


Figure 21 Phthalaldehyde (OPA) attacks primary amino groups.

unsuccessful. No substrate degradation was observed at 50 % concentration of DMSO in the reaction mixture. Phthalaldehyde (OPA, Fig. 21) was used to reduce primary amines of hPAO but it inactivated hPAO during overnight incubation. In fact, already 15 min pre-incubation with the enzyme resulted in hardly detectable product formation (results not shown). However, 15 min pre-incubation of OPA with either SPM or Me₂SPM resulted in marked degradations of both compounds by hPAO (results not shown). The possible stereoselective degradation of OPA-derivatized α -methyl-PAs by hPAO remains to be elucidated.

5.4 OTHER RESULTS

PA homeostasis is highly dependent on the tissue and organism. PAO activity in the rat liver was approximately 10-fold higher than in the mouse liver as evidenced by the degradation rates of AcSPD.

The plasmid used for the immortalization of the rat fibroblasts contained a neomycin selection marker. In the presence of G418, cells recovered faster after plating and DENSPM-induced SSAT activity was about 2-fold higher than without G418 supplementation. The PA transport system in these cells greatly favored Me₂SPM over DENSPM and after a 48 hour simultaneous incubation with both drugs at 20 μM concentrations, a 50-fold difference in their intracellular amounts was observed.

AcSPD and AcMeSPD supplementation (up to 1 mM) had no effect on the growth rates of either syngenic or transgenic immortalized fibroblasts. It

is likely that the uptakes of these compounds are limited. Nevertheless, they might be degraded by SAOs but the presence of these SPD analogues in the growth media did not seem to affect the cells.

Recombinant hPAO did not use the 2–2–SPD or its derivatives as substrates at all. In addition, hPAO supplemented with 5 mM BA did not result in degradation of any of the SPD analogues. Only N^1, N^p -diacetyl–2–3–2–SPM from the three studied SPM analogues, was slightly degraded by hPAO but no kinetic values were measurable. The results are in line with the literature, suggesting that mono–acetylated nor–SPD is the shortest possible substrate for PAO. These compounds were also tested as substrates of hSMO but proved to be inert.

Figure 22 shows the structures of some aldehydes that were also tested as enhancers for hPAO–mediated oxidation of MeSPD. Cinnamaldehyde (aldehyde 1) apparently polymerized when added to the substrate mixture and no substrate degradation was detected. Aldehyde 2, on the other hand, appeared in some way to mediate un–enzymatic degradation of MeSPD. Pyridine 2–carboxaldehyde (aldehyde 3) resulted only in the degradation of (*R*)–MeSPD similarly to most of the other studied aldehydes (Fig. 16).

In addition to PL=Spm adduct studies, kinetic studies with 2:1 (aldehyde [BA and PL] to SPM) substrate mixture as substrates for hPAO were attempted. This approach results in more dynamic reaction conditions than with constant aldehyde and increasing substrate concentrations or vice versa. However, similar results were achieved to those obtained with increasing

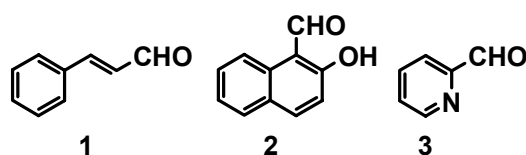


Figure 22 Other tested aldehydes on hPAO–mediated degradation of MeSPD.

aldehyde supplemented reactions in the presence of a constant SPM concentration. Both BA and PL expressed very poor affinities (the K_m values of $\sim 700 \mu\text{M}$ and $>1 \text{ mM}$, respectively, were measured) but the reaction rates were high (the k_{cat} values of $10\text{--}20 \text{ s}^{-1}$ with both aldehydes were estimated).

SPM was the best substrate of hSMO but its analogues were degraded quite differently (Table IV). Five millimolar BA or PL addition to the hSMO reaction mixture with SPM as a substrate appeared to have only a limited effect and only slightly decreased reaction rates were observed (results not shown). Similarly, the oxidation of both racemic MeSPM and Me_2SPM by hSMO appeared unresponsive to the key aldehyde supplementation (Table V). However, different enantiomers of MeSPM and diastereomers of

Table IV The kinetic values of hSMO for SPM and its different analogues as substrates in 100 mM glycine–NaOH at pH 9.5. MeSPM is an asymmetric compound and two sets of values were measured (for methylated and unmethylated terminus).

Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{s}^{-1}\text{M}^{-1}$)
SPM	20	14	700×10^3
racemic			
Me_2SPM	110	1.6	15×10^3
(<i>R,R</i>)-Me_2SPM	25	0.06	2.9×10^3
(<i>R,S</i>)-Me_2SPM	28	0.34	12×10^3
(<i>S,S</i>)-Me_2SPM	53	9.7	180×10^3
racemic			
MeSPM			
(methyl)	31	1.0	32×10^3
(no methyl)	76	2.1	28×10^3
(<i>R</i>)-MeSPM			
(methyl)	2.2	0.19	86×10^3
(no methyl)	3.5	0.20	57×10^3
(<i>S</i>)-MeSPM			
(methyl)	19	2.2	120×10^3
(no methyl)	29	5.1	180×10^3

Table V Nmoles of 500 μ M SPM analogues (corresponding to 90 nmoles at the beginning of the reaction) degraded by hSMO. Five millimolar aldehydes and 30 min reaction times in 100 mM glycine–NaOH at pH 9.5 were used with the indicated protein amounts.

Substrate	hSMO 0.5 μ g	+5 mM BA	+5 mM PL
racemic Me ₂ SPM	12	11	12
(R,R)-Me ₂ SPM	0.6	2.9	1.9
(R,S)-Me ₂ SPM	2.3	7.5	3.7
(S,S)-Me ₂ SPM	36	24	9.4

Substrate	hSMO 0.3 μ g	+5 mM BA	+5 mM PL
racemic MeSPM			
(methyl)	8.9	10	7.8
(no methyl)	9.9	9.0	7.1
(R)-MeSPM			
(methyl)	1.3	4.1	1.3
(no methyl)	0.9	1.2	0.8
(S)-MeSPM			
(methyl)	20	8.2	7.1
(no methyl)	24	9.7	7.0

Me₂SPM were degraded quite differently by hSMO in reaction mixtures containing 5 mM aldehyde (Table V). It seems that hSMO expresses stereoselectivity and the hSMO-mediated oxidations of different enantiomers of α -methylated SPM mimetics are susceptible to aldehydes.

6 DISCUSSION

PAs are essential to sustain normal cellular metabolism both in prokaryotes and eukaryotes. PA homeostasis is a balance between the biosynthesis of these compounds, their degradation, export and uptake. Proliferative stimuli lead to both increased import and enhanced biosynthesis of PAs. On the other hand, the accumulation of PAs not only stimulates PA export and degradation but also promotes inhibitions of both the PA uptake and the PA biosynthesis. As the maintenance of optimal PA concentrations appears to include many interlocking feedback routes, comprehending the mechanisms by which PAs regulate cellular functions has proven elusive.

The PA-mediated proliferation is considered to occur via nucleic acid and protein synthesis. As no direct measurement of cellular localization of PAs is available, the association of PAs can only be measured after disintegrating the cells. In such cell-free studies, most of the cellular PAs appear to be associated with RNA and only a fraction of PAs are free. PAs seem to alter the conformations of different RNAs as both RNA stabilization and destabilization has been observed. As a result, protein synthesis at various stages may be affected. In cell-free DNA, GC-rich regions are targeted by PAs, a phenomenon which may correspond to the situation *in vivo*. PAs also participate in the condensation of histones and the PA export from the nucleus may be one way to enhance transcription. There are also indications that PAs can affect protein phosphorylation (reviewed in detail by Igarashi and Kashiwagi, 2000).

In *E. coli* there is a fairly large group of genes (about 10 % of all studied genes and referred to as "PA modulon") whose initiation codon is preceded by the unusual Shine-Dalgarno sequence. Small alterations to these sequences

result in loss of PA-dependent transcription. In *E. coli* there is also a group of genes that are transcriptionally repressed by PAs. PAs are proposed to "modulate" the levels of many proteins and indirectly maintain optimal conditions of cell functions. It seems that PA-dependent cellular phenomena are slowly being revealed as there is evidence of similar PA-mediated transcriptional control in mammalian cells, too (Yoshida et al., 2004).

The mRNA splicing has lately been investigated in more detail. The phenomenon seems to be associated with extraordinary situations like the response to external stimuli and unregulated cell division. Full SSAT mRNA codes for 171 AAs but a shorter splice variant (responding for 71 AAs) was detected in the presence of DENSPM *in vitro* (unpublished observations) and SSAT splicing appears to be sensitive to environmental stress factors (Kim et al., 2005; Mita et al., 2004). The observed enzyme activity differences between organs may well be due to alternate splicing of mRNA.

PA homeostasis is highly dependent on tissue and organism. In normal rat pancreas the total pool of PAs is 3-fold higher than in liver and the high PA content in pancreas appears to be necessary to maintain the integrity of cellular membranes. On the other hand, the roles of PAs in central nervous system appear to be many-fold and opposite. For example, PUT is thought to be a weak antagonist of *N*-methyl-(*D*)-aspartate receptor whereas both SPD and SPM are agonists of the same receptor and appear to be able to substitute for each other to some extent (Williams, 1997).

Numerous PA derivatives are extensively used as tools to elucidate the cellular functions of PAs. Different PA analogues can be divided into two main classes: terminally *N*-alkylated (R_6

and/or $R_7 = -CH_3, -CH_2CH_3$ etc., Fig. 23) and others with alterations along the backbone of the parent PA. The latter group includes compounds with different lengths of the carbon chains between the nitrogen atoms and alkyl side-chains at carbons or internal nitrogens. The difference between these compounds is that the drugs in the former group do not have free terminal amino groups but the compounds in the latter group do and it may be that this property is the reason for different effects of the PA derivatives in cells.

Even though under physiological conditions all the nitrogens are positively charged in both Me_2SPM and $DENSPM$, the compounds are treated quite differently. Both are competitive inhibitors of SSAT but $DENSPM$ induces SSAT very effectively whereas Me_2SPM results in only moderate SSAT induction. One explanation of the different properties may be that the α -methyl groups do not reach out far enough to induce conformational changes in the SPM analogue sensitive sites whereas the terminal N -ethyl groups of $DENSPM$ may distort the positions of their neighboring groups more effectively. This conformational change in the ligand binding site(s) of the macromolecules, due to the interaction(s) with PA(s) or PA analogue(s), may stabilize the ligand-protein complexes differently and/or exhibit allosteric effects, either of which may explain the observed differences.

Here, the reported biological stabilities of both $MeSPD$ and Me_2SPM were confirmed both *in vivo* and *in vitro*. These PA derivatives appear to be excellent tools with which to elucidate the different cellular roles of SPD and SPM. Furthermore, both $MeSPD$ and Me_2SPM mimic and substitute for natural PAs extremely well: both prevent acute pancreatitis and rescue the early liver regeneration in the MT-SSAT transgenic rat. These compounds do not cause any significant SSAT induction or inflammation *in vivo*. However,

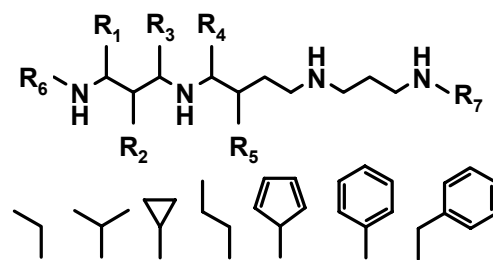


Figure 23 Some possible substituents and their positions at SPM.

only racemic $MeSPD$ and Me_2SPM have been studied in cell cultures and *in vivo*. Enantiomerically pure drugs may very well evoke different effects. The dormant stereoselectivity of PAO has also been revealed due to the chiral nature of these compounds.

A single methyl group at α -position is sufficient to prevent acetylation by SSAT. It would be of interest to study methyl substituents in other positions i.e. R_2 to R_5 (Fig. 23) and the stabilities of such PA derivatives. A single methyl substituent at R_2 -position may be close enough to both N^1 and N^4 to prevent both acetylation by SSAT and degradation by PAO and other amine oxidases. R_3 -substitution might result in a stable compound against PAO-mediated degradation. Furthermore, examination of other singly α -substituted PAs ($R_1 = -CH_2CH_3, -CH_2C_5H_6$ etc., Fig. 23) is necessary to determine the properties of potential substrates for PAO in the presence of aldehydes. Substituents containing functional groups ($-SH, -NH_2, -COOH$ etc.) or atoms other than carbons would also be interesting to investigate. Such PA mimetics might also provide some insight into the mechanisms of hSMO-mediated catalysis.

The aldehyde stimulated degradations of the different enantiomers of α -methylated PAs by hPAO are known to be dependent on the properties of the supplemented aldehyde. However, the studies aimed at understanding the mechanism of aldehyde stimulus on PAO proved

problematic. Aldehydes form Schiff bases with primary amines. However, the possibility that buffer is a mediator in the stereoselective reaction was excluded as the same general stereoselective degradation of MeSPD isomers by the key aldehydes supplemented hPAO was observed in the two AA-free buffers.

The free amino group of FAD (Fig. 4, page 20) and lysine(s) (Fig. 24) in PAO are also possible targets for Schiff base formation. In the active site of maize PAO, a lysine-residue is bridged to N⁵ of the isoalloxazine ring by a water molecule (Binda et al., 1999) and that particular lysine is a promising target for the Schiff base. On the other hand, many cofactors (NAD, NADP and ATP) contain free amino groups similarly to FAD. If covalent modification of cofactors were to result in permanent substrate specificity changes, this might have some industrial applications. To rule out allosteric interaction of aldehyde with the enzyme, reduction of OPA to hPAO was attempted but this resulted in inactivated enzyme. However, binding to the aldehyde-responsive allosteric site in hPAO should enhance the oxidation of terminally *N*-alkylated PAs (compounds like DESPM, DENSPM and bis-benzyl-SPM). Supplementation of aldehydes in the hPAO reaction mixture inhibited the catalysis of these three substrates indicating that there is no positive allosteric site for these aldehydes.

It is plausible that Schiff base with the primary amino groups of the substrate enhances the reaction rates. This is because Schiff base formation results in an uncharged nitrogen and the organization of the molecule may resemble that of AcSPD. However, whether the Schiff base is required for the substrate recognition by PAO or is essential for the catalysis in the active site is harder to elucidate.

As aldehydes are unstable under alkaline conditions increasing aldehyde concentrations

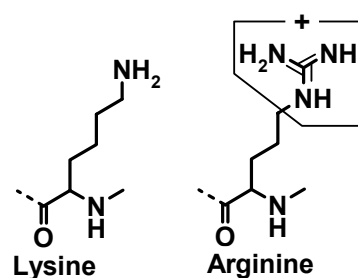


Figure 24 Lysine has a free amino group whereas arginine contains a resonance structure that does not allow Schiff base formation.

were used to allow effective Schiff base formation. The aldehyde concentration appeared to increase the reaction rates of hPAO with both BA and P4CA up to 5 mM. However, the PL-mediated hPAO enhancement appeared greatest at 1 mM PL concentration. It may very well be that PL interacts with the enzyme at higher concentrations and inhibits the reaction. There are also other possibilities e.g. PL may attack the degradation products of the reaction or the resulting compounds may not be detectable with the HPLC analysis system used.

PL forms a hydrogen bond stabilized Schiff base (boxes in Fig. 25) and the preformed PL=SPM adduct was studied as a substrate of hPAO to confirm the degradation of Schiff bases. It seems that the Schiff base enhances the catalysis as the PL=SPM adduct was readily degraded by hPAO. However, the estimated kinetic values of PL=SPM were $K_m > 1$ mM and $k_{cat} \sim 20$ s⁻¹. The reaction rate is exceptionally good for hPAO (the k_{cat} values of AcSPM and AcSPD are 17 s⁻¹ and 8.5 s⁻¹, respectively) but the

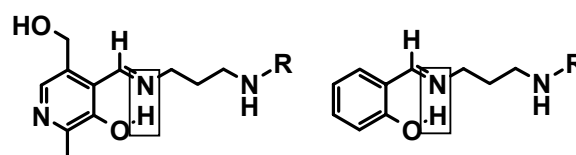


Figure 25 Hydrogen bond stabilized Schiff bases of PL and salicylaldehyde with PAs.

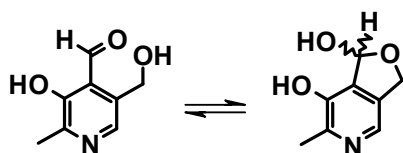


Figure 26 Cyclization of PL results in a chiral carbon.

affinity of the PL=SPM adduct to PAO appears to be very weak. These results might be interpreted to mean that aldehyde reacts with PA in solution and the aldehyde–PA adduct serves as a substrate for hPAO. Furthermore, it could not be confirmed whether chirality of the cyclic form of PL plays any role (Fig. 26). A fraction of PL certainly is in the cyclic conformation in aqueous media.

The observed very weak MeSPD degradation by hPAO in the presence of salicylaldehyde is confusing as this molecule also (similarly to PL) can form a hydrogen bond stabilized Schiff base (Fig. 25, on right). It may be that the Schiff base of salicylaldehyde is ineffective without an additional hydroxyl group to stabilize the intermediate during the catalysis in the active site of hPAO. Another possibility is that salicylaldehyde in some way reacts with the produced PUT and the resulting adducts are not detected by the HPLC analysis (similarly to apparently lowered reaction rates of hPAO in high PL concentration).

Another approach to deduce the significance of the Schiff base was AOE–PUT and its derivatives. Oximes are exact Schiff base mimetics and the carbon=nitrogen bond in oximes is stable. AOE–PUT was a very poor substrate of PAO (similar to SPD) and from the studied molecules the acetyl derivative exhibited the best k_{cat} value (although only $1/15$ th of the k_{cat} value for AcSPD). Preformed Schiff bases of AOE–PUT with BA and acetone exhibited discrepancies: it seems that BA=AOE–PUT has far better affinity (two orders of magnitude) to PAO but a very poor k_{cat} value. The k_{cat} value of acetone=AOE–PUT was only marginally better. However, the k_{cat}/K_m value

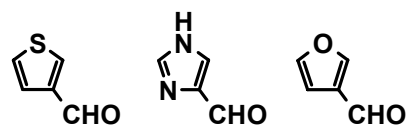


Figure 27 Structures for five atoms containing cyclic aldehydes.

of BA=AOE–PUT was the highest of the studied AOE–PUT derivatives. The results with these SPD derivatives indicate that the aromatic aldehyde–PA adduct is the target for recognition by hPAO. Further investigations to elucidate the mechanism of the observed phenomenon might include studies with the crystallized mammalian PAO and BA=AOE–PUT ($k_{\text{cat}} \sim 1/10$ th of SPM) appears to be interesting ligand to study in that system. At present, mechanistic studies with mammalian PAOs have been only performed with the UV spectroscopy (Royo and Fitzpatrick, 2005).

The underlying mechanism of the aldehyde stimulus for PAO has proved to be elusive and difficult to comprehend. It certainly seems that aldehydes (BA and PL) do not have a positive allosteric site(s) outside the catalytic site of PAO. A six carbon ring structure (aromatic or not) containing Schiff base adduct appears to be essential for the effective MeSPD degradation as the supplementation of hPAO with aliphatic aldehydes resulted in lower reaction rates. In the active site of maize PAO, there are two aromatic AAs (tyrosine and phenylalanine, Binda et al., 1999) that form a sandwich–like structure and the phenyl group of Schiff base adduct might fit between them in hPAO (if a similar structure is present in mammalian PAO). In the future five atom ring structure (Fig. 27) containing aldehydes might prove interesting to investigate.

Aromatic aldehydes might form a cyclic aminal with the aminopropyl moiety of PAs (Ledbetter, 1982; McQuate and Leussing, 1975; Metzler et al., 1980) and this kind of cyclic structure would be energetically favorable (Fig.

28A). The cyclization would fix the α -methyl group closer to the cleavage site and result in another chiral center that might in part explain the stereoselective degradation of MeSPD by PAO. On the other hand, the active site of maize PAO is rather narrow and a compound with two cyclic rings may have difficulty fitting into the catalytic tunnel (Fig. 28B). However, no mammalian PAOs have been crystallized so the configuration of the active site of hPAO can only be speculated. It might even be possible that acetylated PAs are converted to a cyclic form (Fig. 28C) at the active site before undergoing catalysis.

The aromatic aldehydes might react differently from each other in the active site of PAO. The nitrogen opposite to the carboxaldehyde group might bridge to some AA in the active site "locking" aminal in place for the duration of catalysis allowing the use of (*S*)-MeSPD. In the case of PL, additional hydroxyl groups may contribute to the reaction. The degradation patterns of MeSPD in the presence of P4CA, pyridine-3-carboxyaldehyde and pyridine-2-carboxyaldehyde (nitrogens at positions 3, 2 and

active site of PAO. **C** Scheme for cyclic conversion of AcMeSPD at the active site of PAO.

1, respectively, Fig. 23B) by hPAO could be explained by this hypothesis. As the distance between the nitrogen and its counterpart in the active site increases, the less (*S*)-MeSPD degradation that occurs. This "locking" might not happen with the other studied aldehydes and the phenyl ring would rotate into another orientation. That configuration might be energetically more favorable as the enhancement of (*R*)-MeSPD degradation is the dominant observation. As the aldehydes have not been tested extensively with the Me₂SPM diastereomers, one can only speculate about the significance of other chiral carbon(s) of the substrate. Furthermore, the interaction studies with aldehydes and hSMO may be also informative as different enantiomers of α -methylated SPM analogues appear to be degraded with alternating efficacies in the presence of BA and PL.

The pharmaceutical industry is now searching for usable enzymes and their modifications suitable for large-scale applications. Versatile chemical tools are continually being sought and easy-to-prepare, stereoselective, stable enzymes are of interest (Zaks and Dodds, 1997). Another vigorously studied research area is nonaqueous enzymology as water-based reaction buffers can pose problems for many chemical syntheses (Krishna, 2002). Enzymatic polymerization has been confirmed in supercritical fluids, gases, eutectic mixtures, liquid crystals, melts and low-vapor-pressure ionic liquids. Ultimately whole-cell catalysis in a nonaqueous environment may be achieved (extensively reviewed by Klivanov, 2001). Whether PAO (or SMO) has potential as a synthetic chemistry tool remains to be determined.

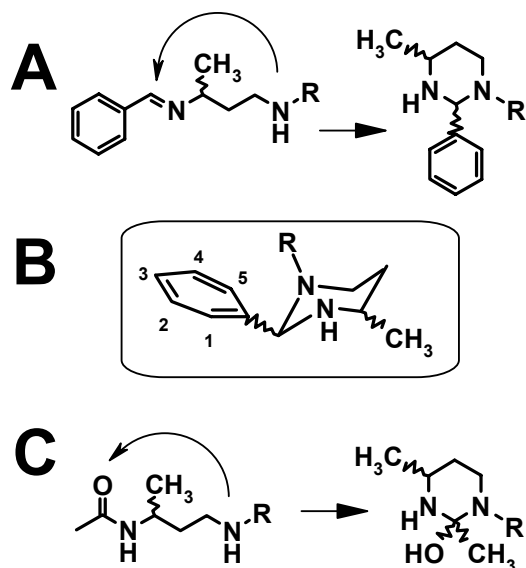


Figure 28 **A** Scheme for cyclic aminal conversion from Schiff base adduct of BA and MeSPD. **B** Cross section of hypothetical cyclic aminal in the

7 SUMMARY

The synthetic routes for preparation of the drugs enabled extensive research with MeSPD, MeSPM and Me₂SPM. The immortalized rat fibroblasts proved to be useful tools to evaluate the fates of the studied PA analogues *in vitro*. The rat liver extract and recombinant protein investigations complemented the cell culture results. *In vivo* studies with the MT–SSAT transgenic rodents were in line with *in vitro* studies.

The presence of a single α -methyl group greatly increases the biological stabilities of the studied PA mimetics. SSAT-mediated acetylation is prevented and hSMO-mediated degradations of both MeSPM and Me₂SPM are clearly diminished. However, hPAO-mediated degradations of both α -methylated SPM derivatives are insignificantly enhanced when compared to SPM. Neither hSMO nor hPAO uses MeSPD as a substrate.

Me₂SPM, similarly to MeSPD, restores the early liver regeneration in the partially hepatectomized MT–SSAT transgenic rat and on a molar basis Me₂SPM appears to be more effective than MeSPD. (*R,R*)–Me₂SPM is the most stable diastereomer against both hSMO- and hPAO-mediated degradations. However, further studies are required to understand the relevance of chirality to the cellular effects of α -methylated PA derivatives.

The dormant stereospecificity of PAO was revealed. The possibility to predetermine the stereoselectivity of PAO-mediated oxidation with guide molecule supplementation was demonstrated. PAO is highly sensitive to aromatic aldehydes and addition of BA to the reaction mixture greatly enhanced the oxidation of (*R*)–MeSPD. PL in turn modified the dominant stereospecificity of PAO and allowed efficient degradation of (*S*)–MeSPD. Furthermore, the present study indicated that the Schiff base between PA and aldehyde is the target of recognition for PAO. However, the exact mechanism for the observed phenomenon remains to be elucidated.

8 REFERENCES

- Agostinelli, E., Arancia, G., Vedova, L. D., Belli, F., Marra, M., Salvi, M., and Toninello, A. (2004). The biological functions of polyamine oxidation products by amine oxidases: perspectives of clinical applications. *Amino Acids* *27*, 347-358.
- Akagawa, M., and Suyama, K. (2001). Amine oxidase-like activity of polyphenols. Mechanism and properties. *Eur J Biochem* *268*, 1953-1963.
- Alhonen, L., Halmekytö, M., Kosma, V.-M., Wahlfors, J., Kauppinen, R., and Jänne, J. (1995). Life-long over-expression of ornithine decarboxylase (ODC) gene in transgenic mice does not lead to generally enhanced tumorigenesis or neuronal degeneration. *Int J Cancer* *63*, 1-3.
- Alhonen, L., Parkkinen, J. J., Keinänen, T., Sinervirta, R., Herzig, K. H., and Jänne, J. (2000). Activation of polyamine catabolism in transgenic rats induces acute pancreatitis. *Proc Natl Acad Sci U S A* *97*, 8290-8295.
- Alhonen, L., Pietilä, M., Halmekytö, M., Kramer, D. L., Jänne, J., and Porter, C. W. (1999). Transgenic mice with activated polyamine catabolism due to overexpression of spermidine/spermine N1-acetyltransferase show enhanced sensitivity to the polyamine analog, N1, N11- diethylnorspermine. *Mol Pharmacol* *55*, 693-698.
- Alhonen, L., Räsänen, T. L., Sinervirta, R., Parkkinen, J. J., Korhonen, V. P., Pietilä, M., and Jänne, J. (2002). Polyamines are required for the initiation of rat liver regeneration. *Biochem J* *362*, 149-153.
- Amendola, R., Bellini, A., Cervelli, M., Degan, P., Marcocci, L., Martini, F., and Mariottini, P. (2005). Direct oxidative DNA damage, apoptosis and radio sensitivity by spermine oxidase activities in mouse neuroblastoma cells. *Biochim Biophys Acta* *1755*, 15-24.
- Auvinen, M., Paasinen, A., Andersson, L. C., and Hölttä, E. (1992). Ornithine decarboxylase activity is critical for cell transformation. *Nature* *360*, 355-358.
- Avila, M. A., Garcia-Trevijano, E. R., Lu, S. C., Corrales, F. J., and Mato, J. M. (2004). Methylthioadenosine. *Int J Biochem Cell Biol* *36*, 2125-2130.
- Bercovich, Z., and Kahana, C. (2004). Degradation of antizyme inhibitor, an ornithine decarboxylase homologous protein, is ubiquitin-dependent and is inhibited by antizyme. *J Biol Chem* *279*, 54097-54102.
- Bergeron, R. J., McManis, J. S., Weimar, W. R., Schreier, K. M., Gao, F., Wu, Q., Ortiz-Ocasio, J., Luchetta, G. R., Porter, C., and Vinson, J. R. T. (1995a). The role of charge in polyamine analogue recognition. *J Med Chem* *38*, 2278-2285.
- Bergeron, R. J., Merriman, R. L., Olson, S. G., Wiegand, J., Bender, J., Streiff, R. R., and Weimar, W. R. (2000). Metabolism and pharmacokinetics of N1,N11-diethylnorspermine in a Cebus apella primate model. *Cancer Res* *60*, 4433-4439.
- Bergeron, R. J., Weimar, W. R., Luchetta, G., Streiff, R. R., Wiegand, J., Perrin, J., Schreier, K. M., Porter, C., Yao, G. W., and Dimova, H. (1995b). Metabolism and pharmacokinetics of N1,N11-diethylnorspermine. *Drug Metab Dispos* *23*, 1117-1125.
- Bernacki, R. J., Oberman, E. J., Seweryniak, K. E., Atwood, A., Bergeron, R. J., and Porter, C. W. (1995). Preclinical antitumor efficacy of the polyamine analogue N¹,N¹¹-diethylnorspermine administered by multiple injections or continuous infusion. *Clin Cancer Res* *1*, 847-857.
- Binda, C., Angelini, R., Federico, R., Ascenzi, P., and Mattevi, A. (2001). Structural bases for inhibitor binding and catalysis in polyamine oxidase. *Biochemistry* *40*, 2766-2776.

- Binda, C., Coda, A., Angelini, R., Federico, R., Ascenzi, P., and Mattevi, A. (1999). A 30 angstrom long U-shaped catalytic tunnel in the crystal structure of polyamine oxidase. *Structure Fold Des* *7*, 265-276.
- Binda, C., Mattevi, A., and Edmondson, D. E. (2002b). Structure-function relationships in flavoenzyme-dependent amine oxidations: a comparison of polyamine oxidase and monoamine oxidase. *J Biol Chem* *277*, 23973-23976.
- Binda, C., Newton-Vinson, P., Hubalek, F., Edmondson, D. E., and Mattevi, A. (2002a). Structure of human monoamine oxidase B, a drug target for the treatment of neurological disorders. *Nat Struct Biol* *9*, 22-26.
- Bitonti, A. J., Byers, T. L., Bush, T. L., Casara, P. J., Bacchi, C. J., Clarkson, A. B., Jr., McCann, P. P., and Sjoerdsma, A. (1990). Cure of *Trypanosoma brucei brucei* and *Trypanosoma brucei rhodesiense* infections in mice with an irreversible inhibitor of S-adenosylmethionine decarboxylase. *Antimicrob Agents Chemother* *34*, 1485-1490.
- Blaschko, H. (1952). Amine oxidase and amine metabolism. *Pharmacol Rev* *4*, 415-458.
- Bolkenius, F. N., Bey, P., and Seiler, N. (1985). Specific inhibition of polyamine oxidase in vivo is a method for the elucidation of its physiological role. *Biochim Biophys Acta* *838*, 69-76.
- Brazeau, B. J., Johnson, B. J., and Wilmot, C. M. (2004). Copper-containing amine oxidases. Biogenesis and catalysis; a structural perspective. *Arch Biochem Biophys* *428*, 22-31.
- Byers, T. L., Lakanen, J. R., Coward, J. K., and Pegg, A. E. (1994). The role of hypusine depletion in cytostasis induced by S-adenosyl-L-methionine decarboxylase inhibition: new evidence provided by 1-methylspermidine and 1,12-dimethylspermine. *Biochem J* *303*, 363-368.
- Cao, L., Langen, L., and Sheldon, R. A. (2003). Immobilised enzymes: carrier-bound or carrier-free? *Curr Opin Biotechnol* *14*, 387-394.
- Carrea, G., and Riva, S. (2000). Properties and Synthetic Applications of Enzymes in Organic Solvents. *Angew Chem Int Ed Engl* *39*, 2226-2254.
- Casero, R. A., Jr., Celano, P., Ervin, S. J., Porter, C. W., Bergeron, R. J., and Libby, P. R. (1989). Differential induction of spermidine/spermine N1-acetyltransferase in human lung cancer cells by the bis(ethyl)polyamine analogues. *Cancer Res* *49*, 3829-3833.
- Casero, R. A., Jr., Wang, Y., Stewart, T. M., Devereux, W., Hacker, A., Wang, Y., Smith, R., and Woster, P. M. (2003). The role of polyamine catabolism in anti-tumour drug response. *Biochem Soc Trans* *31*, 361-365.
- Casero, R. A., Jr., and Pegg, A. E. (1993). Spermidine/spermine N1-acetyltransferase - the turning point in polyamine metabolism. *FASEB J* *7*, 653-661.
- Cederbaum, S. D., Yu, H., Grody, W. W., Kern, R. M., Yoo, P., and Iyer, R. K. (2004). Arginases I and II: do their functions overlap? *Mol Genet Metab* *81*, S38-44.
- Cervelli, M., Bellini, A., Bianchi, M., Marcocci, L., Nocera, S., Polticelli, F., Federico, R., Amendola, R., and Mariottini, P. (2004). Mouse spermine oxidase gene splice variants. Nuclear subcellular localization of a novel active isoform. *Eur J Biochem* *271*, 760-770.
- Cervelli, M., Cona, A., Angelini, R., Polticelli, F., Federico, R., and Mariottini, P. (2001). A barley polyamine oxidase isoform with distinct structural features and subcellular localization. *Eur J Biochem* *268*, 3816-3830.
- Cervelli, M., Polticelli, F., Federico, R., and Mariottini, P. (2003). Heterologous expression and characterization of mouse spermine oxidase. *J Biol Chem* *278*, 5271-5276.

- Chang, B. K., Bergeron, R. J., Porter, C. W., Vinson, J. R., Liang, Y., and Libby, P. R. (1992). Regulatory and antiproliferative effects of N-alkylated polyamine analogues in human and hamster pancreatic adenocarcinoma cell lines. *Cancer Chemother Pharmacol* *30*, 183-188.
- Chaturvedi, R., Cheng, Y., Asim, M., Bussiere, F. I., Xu, H., Gobert, A. P., Hacker, A., Casero, R. A., Jr., and Wilson, K. T. (2004). Induction of polyamine oxidase 1 by *Helicobacter pylori* causes macrophage apoptosis by hydrogen peroxide release and mitochondrial membrane depolarization. *J Biol Chem* *279*, 40161-40173.
- Chen, Y., Kramer, D. L., Jell, J., Vujcic, S., and Porter, C. W. (2003). Small interfering RNA suppression of polyamine analog-induced spermidine/spermine N1-acetyltransferase. *Mol Pharmacol* *64*, 1153-1159.
- Chiang, P. K., Gordon, R. K., Tal, J., Zeng, G. C., Doctor, B. P., Pardhasaradhi, K., and McCann, P. P. (1996). S-adenosylmethionine and methylation. *FASEB J* *10*, 471-480.
- Chirumamilla, R. R., Muralidhar, R., Marchant, R., and Nigam, P. (2001). Improving the quality of industrially important enzymes by directed evolution. *Mol Cell Biochem* *224*, 159-168.
- Coleman, C. S., Huang, H., and Pegg, A. E. (1995). Role of the carboxyl terminal MATEE sequence of spermidine/spermine N 1-acetyltransferase in the activity and stabilization by the polyamine analog N 1,N 12-bis(ethyl)spermine. *Biochemistry* *34*, 13423-13430.
- Coleman, C. S., and Pegg, A. E. (2001). Polyamine analogues inhibit the ubiquitination of spermidine/spermine N1-acetyltransferase and prevent its targeting to the proteasome for degradation. *Biochem J* *358*, 137-145.
- Cona, A., Manetti, F., Leone, R., Corelli, F., Tavladoraki, P., Polticelli, F., and Botta, M. (2004). Molecular basis for the binding of competitive inhibitors of maize polyamine oxidase. *Biochemistry* *43*, 3426-3435.
- Cramer, A., Raillard, S. A., Bermudez, E., and Stemmer, W. P. (1998). DNA shuffling of a family of genes from diverse species accelerates directed evolution. *Nature* *391*, 288-291.
- Desiderio, M., Weibel, M., and Mamont, P. S. (1992). Spermidine nuclear acetylation in rat hepatocytes and in logarithmically growing rat hepatoma cells: Comparison with histone acetylation. *Exp Cell Res* *202*, 501-506.
- Desiderio, M. A. (1992). Opposite responses of nuclear spermidine N8-acetyltransferase and histone acetyltransferase activities to regenerative stimuli in rat liver. *Hepatology* *15*, 928-933.
- Devereux, W., Wang, Y., Stewart, T. M., Hacker, A., Smith, R., Frydman, B., Valasinas, A. L., Reddy, V. K., Marton, L. J., Ward, T. D., *et al.* (2003). Induction of the PAOh1/SMO polyamine oxidase by polyamine analogues in human lung carcinoma cells. *Cancer Chemother Pharmacol* *52*, 383-390.
- Dooley, D. M. (1999). Structure and biogenesis of topaquinone and related cofactors. *J Biol Inorg Chem* *4*, 1-11.
- Fakler, B., Brändle, U., Glowatzki, E., Weidemann, S., Zenner, H.-P., and Ruppertsberg, J. P. (1995). Strong voltage-dependent inward rectification of inward rectifier K⁺ channels is caused by intracellular spermine. *Cell* *80*, 149-154.
- Feroli, M. E., and Armani, A. (2003). Polyamine oxidase activity in rats treated with mitoguazone: specific and permanent decrease in thymus. *Amino Acids* *24*, 187-194.
- Feroli, M. E., Berselli, D., and Caimi, S. (2004). Effect of mitoguazone on polyamine oxidase activity in rat liver. *Toxicol Appl Pharmacol* *201*, 105-111.
- Gerner, E. W., and Meyskens, F. L., Jr. (2004). Polyamines and cancer: old molecules, new understanding. *Nat Rev Cancer* *4*, 781-792.

- Goh, C. S., Milburn, D., and Gerstein, M. (2004). Conformational changes associated with protein-protein interactions. *Curr Opin Struct Biol* *14*, 104-109.
- Grigorenko, N. A., Vepsäläinen, J., Järvinen, A., Keinänen, T. A., Alhonen, L., Jänne, J., and Khomutov, A. R. (2005). Synthesis of (R)- and (S)-isomers of 1-methylspermidine. *Mendeleev Commun* *15*, 142-143.
- Ha, H. C., Sirisoma, N. S., Kuppusamy, P., Zweier, J. L., Woster, P. M., and Casero, R. A., Jr. (1998). The natural polyamine spermine functions directly as a free radical scavenger. *Proc Natl Acad Sci U S A* *95*, 11140-11145.
- Hahm, H. A., Ettinger, D. S., Bowling, K., Hoker, B., Chen, T. L., Zabelina, Y., and Casero, R. A., Jr. (2002). Phase I study of N(1),N(11)-diethylnorspermine in patients with non-small cell lung cancer. *Clin Cancer Res* *8*, 684-690.
- Halmekytö, M., Alhonen, L., Alakujala, L., and Jänne, J. (1993). Transgenic mice over-producing putrescine in their tissues do not convert the diamine into higher polyamines. *Biochem J* *291*, 505-508.
- Halmekytö, M., Alhonen, L., Wahlfors, J., Sinervirta, R., Eloranta, T., and Jänne, J. (1991). Characterization of a transgenic mouse line over-expressing the human ornithine decarboxylase gene. *Biochem J* *278*, 895-898.
- Hamana, K., Niitsu, M., Matsuzaki, S., Samejima, K., Igarashi, Y., and Kodama, T. (1992). Novel linear and branched polyamines in the extremely thermophilic eubacteria *Thermoleophilum*, *Bacillus* and *Hydrogenobacter*. *Biochem J* *284*, 741-747.
- Hardy, J. A., and Wells, J. A. (2004). Searching for new allosteric sites in enzymes. *Curr Opin Struct Biol* *14*, 706-715.
- Heljasvaara, R., Veress, I., Halmekytö, M., Alhonen, L., Jänne, J., Laajala, P., and Pajunen, A. (1997). Transgenic mice over-expressing ornithine and S-adenosylmethionine decarboxylases maintain a physiological polyamine homeostasis in their tissues. *Biochem J* *323*, 457-462.
- Hermes, J. D., Blacklow, S. C., and Knowles, J. R. (1990). Searching sequence space by definably random mutagenesis: improving the catalytic potency of an enzyme. *Proc Natl Acad Sci U S A* *87*, 696-700.
- Huang, Q., Liu, Q., and Hao, Q. (2005). Crystal structures of Fms1 and its complex with spermine reveal substrate specificity. *J Mol Biol* *348*, 951-959.
- Hyvönen, T., Keinänen, T. A., Khomutov, A. R., Khomutov, R. M., and Eloranta, T. O. (1992). Monitoring of the uptake and metabolism of aminoxy analogues of polyamines in cultured cells by high-performance liquid chromatography. *J Chromatogr* *574*, 17-21.
- Hölttä, E. (1977). Oxidation of spermidine and spermine in rat liver: purification and properties of polyamine oxidase. *Biochemistry* *16*, 91-100.
- Igarashi, K., and Kashiwagi, K. (2000). Polyamines: mysterious modulators of cellular functions. *Biochem Biophys Res Commun* *271*, 559-564.
- Jackson, L. K., Goldsmith, E. J., and Phillips, M. A. (2003). X-ray structure determination of *Trypanosoma brucei* ornithine decarboxylase bound to D-ornithine and to G418: insights into substrate binding and ODC conformational flexibility. *J Biol Chem* *278*, 22037-22043.
- Jin, B. F., He, K., Wang, H. X., Wang, J., Zhou, T., Lan, Y., Hu, M. R., Wei, K. H., Yang, S. C., Shen, B. F., and Zhang, X. M. (2003). Proteomic analysis of ubiquitin-proteasome effects: insight into the function of eukaryotic initiation factor 5A. *Oncogene* *22*, 4819-4830.
- Jänne, J., Alhonen, L., and Leinonen, P. (1991). Polyamines: from molecular biology to clinical applications. *Ann Med* *23*, 241-259.

- Jänne, J., Alhonen, L., Pietilä, M., and Keinänen, T. A. (2004). Genetic approaches to the cellular functions of polyamines in mammals. *Eur J Biochem* *271*, 877-894.
- Jänne, J., and Williams-Ashman, H. G. (1971). On the purification of L-ornithine decarboxylase from rat prostate and effects of thiol compounds on the enzyme. *J Biol Chem* *246*, 1725-1732.
- Kaasinen, K., Koistinaho, J., Alhonen, L., and Jänne, J. (2000). Overexpression of spermidine/spermine N-acetyltransferase in transgenic mice protects the animals from kainate-induced toxicity. *Eur J Neurosci* *12*, 540-548.
- Kauppinen, L., Myöhänen, S., Halmekytö, M., Alhonen, L., and Jänne, J. (1993). Transgenic mice overexpressing the human spermidine synthase gene. *Biochem J* *293*, 513-516.
- Kern, D., and Zuiderweg, E. R. (2003). The role of dynamics in allosteric regulation. *Curr Opin Struct Biol* *13*, 748-757.
- Kim, K., Ryu, J. H., Park, J. W., Kim, M. S., and Chun, Y. S. (2005). Induction of a SSAT isoform in response to hypoxia or iron deficiency and its protective effects on cell death. *Biochem Biophys Res Commun* *331*, 78-85.
- Klibanov, A. M. (1997). Why are enzymes less active in organic solvents than in water? *Trends Biotechnol* *15*, 97-101.
- Klibanov, A. M. (2001). Improving enzymes by using them in organic solvents. *Nature* *409*, 241-246.
- Korhonen, V.-P., Halmekytö, M., Kauppinen, L., Myöhänen, S., Wahlfors, J., Keinänen, T., Hyvönen, T., Alhonen, L., Eloranta, T., and Jänne, J. (1995). Molecular cloning of a cDNA encoding human spermine synthase. *DNA Cell Biol* *14*, 841-847.
- Kramer, D. L., FogelPetrovic, M., Diegelman, P., Cooley, J. M., Bernacki, R. J., McManis, J. S., Bergeron, R. J., and Porter, C. W. (1997). Effects of novel spermine analogues on cell cycle progression and apoptosis in MALME-3M human melanoma cells. *Cancer Res* *57*, 5521-5527.
- Kumamaru, T., Suenaga, H., Mitsuoka, M., Watanabe, T., and Furukawa, K. (1998). Enhanced degradation of polychlorinated biphenyls by directed evolution of biphenyl dioxygenase. *Nat Biotechnol* *16*, 663-666.
- Kumazawa, T., Seno, H., and Suzuki, O. (1990). A radioisotopic assay for polyamine oxidase. *Anal Biochem* *188*, 105-108.
- Kusche, J., Menningen, R., Leisten, L., and Krakamp, B. (1988). Large bowel tumor promotion by diamine oxidase inhibition: animal model and clinical aspects. *Adv Exp Med Biol* *250*, 745-752.
- Lakanen, J. R., Coward, J. K., and Pegg, A. E. (1992). alpha-Methyl polyamines: metabolically stable spermidine and spermine mimics capable of supporting growth in cells depleted of polyamines. *J Med Chem* *35*, 724-734.
- Ledbetter, J. W. (1982). Resonance Raman Spectra of the Tautomers of Pyridoxal and Salicylaldehyde Schiff Bases. *J Phys Chem* *86*, 2449-2451.
- Lee, Y., and Sayre, L. M. (1998). Reaffirmation that metabolism of polyamines by bovine plasma amine oxidase occurs strictly at the primary amino termini. *J Biol Chem* *273*, 19490-19494.
- Li, L., Rao, J. N., Guo, X., Liu, L., Santora, R., Bass, B. L., and Wang, J. Y. (2001). Polyamine depletion stabilizes p53 resulting in inhibition of normal intestinal epithelial cell proliferation. *Am J Physiol Cell Physiol* *281*, C941-953.

- Libby, P. R., Henderson, M., Bergeron, R. J., and Porter, C. W. (1989). Major increases in spermidine/spermine-N1-acetyltransferase activity and their relationship to polyamine depletion and growth inhibition in L1210 cells. *Cancer Res* *49*, 6226-6231.
- Libby, P. R., and Porter, C. W. (1987). Separation of two isozymes of polyamine oxidase from murine L1210 leukemia cells. *Biochem Biophys Res Commun* *144*, 528-535.
- Libby, P. R., and Porter, C. W. (1992). Inhibition of enzymes of polyamine back-conversion by pentamidine and berenil. *Biochem Pharmacol* *44*, 830-832.
- Lorsch, J. R., and Szostak, J. W. (1996). Chance and necessity in the selection of nucleic acid catalysts. *Acc Chem Res* *29*, 103-110.
- Lukkarinen, J. A., Gröhn, O. H., Alhonen, L. I., Jänne, J., and Kauppinen, R. A. (1999). Enhanced ornithine decarboxylase activity is associated with attenuated rate of damage evolution and reduction of infarct volume in transient middle cerebral artery occlusion in the rat. *Brain Res* *826*, 325-329.
- Mackintosh, C. A., and Pegg, A. E. (2000). Effect of spermine synthase deficiency on polyamine biosynthesis and content in mice and embryonic fibroblasts, and the sensitivity of fibroblasts to 1,3-bis-(2-chloroethyl)-N-nitrosourea. *Biochem J* *351*, 439-447.
- Matsufuji, S., Matsufuji, T., Miyazaki, Y., Murakami, Y., Atkins, J. F., Gesteland, R. F., and Hayashi, S.-I. (1995). Autoregulatory frameshifting in decoding mammalian ornithine decarboxylase antizyme. *Cell* *80*, 51-60.
- Matsui, I., and Pegg, A. E. (1980). Increase in acetylation of spermidine in rat liver extracts brought about by treatment with carbon tetrachloride. *Biochem Biophys Res Commun* *92*, 1009-1015.
- Matsui, I., Wiegand, L., and Pegg, A. E. (1981). Properties of spermidine N-acetyltransferase from livers of rats treated with carbon tetrachloride and its role in the conversion of spermidine into putrescine. *J Biol Chem* *256*, 2454-2458.
- McQuate, R. S., and Leussing, D. L. (1975). Kinetic and Equilibrium Studies on the Formation of Zinc(II)-Salicylaldehyde Schiff Bases Derived from Ethylenediamine and 1,3-Diaminopropane. *J Am Chem Soc* *97*, 5117-5125.
- Metzler, C. M., Cahill, A., and Metzler, D. E. (1980). Equilibria and Absorbance Spectra of Schiff Base. *J Am Chem Soc* *102*, 6075-6082.
- Mita, K., Fukuchi, K., Hamana, K., Ichimura, S., and Neno, M. (2004). Accumulation of spermidine/spermine N1-acetyltransferase and alternatively spliced mRNAs as a delayed response of HeLa S3 cells following X-ray irradiation. *Int J Radiat Biol* *80*, 369-375.
- Morgan, J. E., Blankenship, J. W., and Matthews, H. R. (1987). Polyamines and acetylpolyamines increase the stability and alter the conformation of nucleosome core particles. *Biochemistry* *26*, 3643-3649.
- Murakami, Y., Fujita, K., Kameji, T., and Hayashi, S. (1985). Accumulation of ornithine decarboxylase-antizyme complex in HMOA cells. *Biochem J* *225*, 689-697.
- Murakami, Y., Matsufuji, S., Kameji, T., Hayashi, S., Igarashi, K., Tamura, T., Tanaka, K., and Ichihara, A. (1992). Ornithine decarboxylase is degraded by the 26S proteasome without ubiquitination. *Nature* *360*, 597-599.
- Murray-Stewart, T., Wang, Y., Devereux, W., and Casero, R. A., Jr. (2002). Cloning and characterization of multiple human polyamine oxidase splice variants that code for isoenzymes with different biochemical characteristics. *Biochem J* *368*, 673-677.

- Nagarajan, S., and Ganem, B. (1986). Chemistry of naturally occurring polyamines. Nonmetabolizable derivatives of spermine and spermidine. *J Org Chem* *51*.
- Nagarajan, S., Ganem, B., and Pegg, A. E. (1988). Studies of non-metabolizable polyamines that support growth of SV-3T3 cells depleted of natural polyamines by exposure to alpha-difluoromethylornithine. *Biochem J* *254*, 373-378.
- Nairn, L. M., Lindsay, G. S., Woster, P. M., and Wallace, H. M. (2000). Cytotoxicity of novel unsymmetrically substituted inhibitors of polyamine biosynthesis in human cancer cells. *J Cell Physiol* *182*, 209-213.
- Niiranen, K., Pietilä, M., Pirttilä, T. J., Järvinen, A., Halmekytö, M., Korhonen, V. P., Keinänen, T. A., Alhonen, L., and Jänne, J. (2002). Targeted disruption of spermidine/spermine N1-acetyltransferase gene in mouse embryonic stem cells. Effects on polyamine homeostasis and sensitivity to polyamine analogues. *J Biol Chem* *277*, 25323-25328.
- Nitta, T., Igarashi, K., and Yamamoto, N. (2002). Polyamine depletion induces apoptosis through mitochondria-mediated pathway. *Exp Cell Res* *276*, 120-128.
- Nobori, T., Takabayashi, K., Tran, P., Orvis, L., Batova, A., Yu, A. L., and Carson, D. A. (1996). Genomic cloning of methylthioadenosine phosphorylase: a purine metabolic enzyme deficient in multiple different cancers. *Proc Natl Acad Sci U S A* *93*, 6203-6208.
- Oredsson, S. M. (2003). Polyamine dependence of normal cell-cycle progression. *Biochem Soc Trans* *31*, 366-370.
- Park, M. H., Cooper, H. L., and Folk, J. E. (1981). Identification of hypusine, an unusual amino acid, in protein from human lymphocytes and of spermidine as its biosynthetic precursor. *Proc Natl Acad Sci USA* *78*, 2869-2873.
- Park, M. H., Wolff, E. C., and Folk, J. E. (1993). Is hypusine essential for eukaryotic cell proliferation. *TIBS* *18*, 475-479.
- Phanstiel, O. I., Price, H. L., Wang, L., Juusola, J., Kline, M., and Shah, S. M. (2000). The effect of polyamine homologation on the transport and cytotoxicity properties of polyamine-(DNA-intercalator) conjugates. *J Org Chem* *65*, 5590-5599.
- Pietilä, M., Alhonen, L., Halmekytö, M., Kanter, P., Jänne, J., and Porter, C. W. (1997). Activation of polyamine catabolism profoundly alters tissue polyamine pools and affects hair growth and female fertility in transgenic mice overexpressing spermidine / spermine *N*¹-acetyltransferase. *J Biol Chem* *272*, 18746-18751.
- Pietilä, M., Pirinen, E., Keskitalo, S., Juutinen, S., Pasonen-Seppänen, S., Keinänen, T., Alhonen, L., and Jänne, J. (2005). Disturbed keratinocyte differentiation in transgenic mice and organotypic keratinocyte cultures as a result of spermidine/spermine N-acetyltransferase overexpression. *J Invest Dermatol* *124*, 596-601.
- Porter, C. W., Cavanaugh, P. F., Jr., Stolowich, N., Ganis, B., Kelly, E., and Bergeron, R. J. (1985). Biological properties of N4- and N1,N8-spermidine derivatives in cultured L1210 leukemia cells. *Cancer Res* *45*, 2050-2057.
- Pyronnet, S., Pradayrol, L., and Sonenberg, N. (2005). Alternative splicing facilitates internal ribosome entry on the ornithine decarboxylase mRNA. *Cell Mol Life Sci* *62*, 1267-1274.
- Qu, N., Ignatenko, N. A., Yamauchi, P., Stringer, D. E., Levenson, C., Shannon, P., Perrin, S., and Gerner, E. W. (2003). Inhibition of human ornithine decarboxylase activity by enantiomers of difluoromethylornithine. *Biochem J* *375*, 465-470.

- Ragione, F. D., and Pegg, A. E. (1983). Studies of the specificity and kinetics of rat liver spermidine/spermine N1-acetyltransferase. *Biochem J* *213*, 701-706.
- Rehse, K., Puchert, E., and Leissring, S. (1990). [Antiaggregatory and anticoagulant effects of oligoamines. 12. Alkyl- and arylalkyl- derivatives of putrescine, spermidine and spermine]. *Arch Pharm (Weinheim)* *323*, 287-294.
- Rogers, S., Wells, R., and Rechsteiner, M. (1986). Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis. *Science* *234*, 364-368.
- Royo, M., and Fitzpatrick, P. F. (2005). Mechanistic studies of mouse polyamine oxidase with N1,N12-bisethylspermine as a substrate. *Biochemistry* *44*, 7079-7084.
- Ruiz, O., Alonso-Garrido, D. O., Buldain, G., and Frydman, R. B. (1986). Effect of N-alkyl and C-alkylputrescines on the activity of ornithine decarboxylase from rat liver and E.coli. *Biochim Biophys Acta* *873*, 53-61.
- Räsänen, T. L., Alhonen, L., Sinervirta, R., Keinänen, T., Herzig, K. H., Suppola, S., Khomutov, A. R., Vepsäläinen, J., and Jänne, J. (2002). A polyamine analogue prevents acute pancreatitis and restores early liver regeneration in transgenic rats with activated polyamine catabolism. *J Biol Chem* *277*, 39867-39872.
- Salvi, M., and Toninello, A. (2004). Effects of polyamines on mitochondrial Ca(2+) transport. *Biochim Biophys Acta* *1661*, 113-124.
- Saminathan, M., Thomas, T., Shirahata, A., Pillai, C. K., and Thomas, T. J. (2002). Polyamine structural effects on the induction and stabilization of liquid crystalline DNA: potential applications to DNA packaging, gene therapy and polyamine therapeutics. *Nucleic Acids Res* *30*, 3722-3731.
- Saramäki, A. (2004) Synthesis of terminally N-alkylated polyamine analogues and their in vitro studies., M.Sc., University of Kuopio, Kuopio.
- Schipper, R. G., and Verhofstad, A. A. (2002). Distribution patterns of ornithine decarboxylase in cells and tissues: facts, problems, and postulates. *J Histochem Cytochem* *50*, 1143-1160.
- Seiler, N. (1987). Functions of polyamine acetylation. *Can J Physiol Pharmacol* *65*, 2024-2035.
- Seiler, N. (1995). Polyamine oxidase, properties and functions. *Prog Brain Res* *106*, 333-344.
- Seiler, N. (2003a). Thirty years of polyamine-related approaches to cancer therapy. Retrospect and prospect. Part 1. Selective enzyme inhibitors. *Curr Drug Targets* *4*, 537-564.
- Seiler, N. (2003b). Thirty years of polyamine-related approaches to cancer therapy. Retrospect and prospect. Part 2. Structural analogues and derivatives. *Curr Drug Targets* *4*, 565-585.
- Seiler, N., Bolkenius, F. N., Knödgen, B., and Mamont, P. (1980). Polyamine oxidase in rat tissues. *Biochim Biophys Acta* *615*, 480-488.
- Seiler, N. D., J.G., and Moulinoux, J. P. (1996). Polyamine transport in mammalian cells. An update. *Int J Biochem Cell Biol* *28*, 843-861.
- Sessa, A., and Perin, A. (1994). Diamine oxidase in relation to diamine and polyamine metabolism. *Agents Actions* *43*, 69-77.
- Shantz, L. M., and Pegg, A. E. (1999). Translational regulation of ornithine decarboxylase and other enzymes of the polyamine pathway. *Int J Biochem Cell Biol* *31*, 107-122.
- Silverman, R. B. (2000). *The organic chemistry of enzyme catalyzed reactions* (San Diego: Academic Press).

- Stemmer, W. P. (1994). DNA shuffling by random fragmentation and reassembly: in vitro recombination for molecular evolution. *Proc Natl Acad Sci U S A* *91*, 10747-10751.
- Suppola, S., Heikkinen, S., Parkkinen, J. J., Uusi-Oukari, M., Korhonen, V. P., Keinänen, T., Alhonen, L., and Jänne, J. (2001). Concurrent overexpression of ornithine decarboxylase and spermidine/spermine N1-acetyltransferase further accelerates the catabolism of hepatic polyamines in transgenic mice. *Biochem J* *358*, 343-348.
- Suppola, S., Pietilä, M., Parkkinen, J. J., Korhonen, V. P., Alhonen, L., Halmekytö, M., Porter, C. W., and Jänne, J. (1999). Overexpression of spermidine/spermine N1-acetyltransferase under the control of mouse metallothionein I promoter in transgenic mice: evidence for a striking post-transcriptional regulation of transgene expression by a polyamine analogue. *Biochem J* *338*, 311-316.
- Suzuki, O., Matsumoto, T., and Katsumata, Y. (1984). Determination of polyamine oxidase activities in human tissues. *Experientia* *40*, 838-839.
- Suzuki, O., Matsumoto, T., Oya, M., and Katsumata, Y. (1981). Metabolism of acetylpolyamines by monoamine oxidase, diamine oxidase and polyamine oxidase. *Biochim Biophys Acta* *677*, 190-193.
- Terpe, K. (2003). Overview of tag protein fusions: from molecular and biochemical fundamentals to commercial systems. *Appl Microbiol Biotechnol* *60*, 523-533.
- Terui, Y., Ohnuma, M., Hiraga, K., Kawashima, E., and Oshima, T. (2005). Stabilization of nucleic acids by unusual polyamines produced by an extreme thermophile, *Thermus thermophilus*. *Biochem J* *388*, 427-433.
- Thomas, T., and Thomas, T. J. (2003). Polyamine metabolism and cancer. *J Cell Mol Med* *7*, 113-126.
- Tomitori, H., Neno, M., Mita, K., Daino, K., Igarashi, K., and Ichimura, S. (2002). Functional characterization of the human spermidine/spermine N(1)-acetyltransferase gene promoter. *Biochim Biophys Acta* *1579*, 180-184.
- Tosaka, Y., Tanaka, H., Yano, Y., Masai, K., Nozaki, M., Yomogida, K., Otani, S., Nojima, H., and Nishimune, Y. (2000). Identification and characterization of testis specific ornithine decarboxylase antizyme (OAZ-t) gene: expression in haploid germ cells and polyamine-induced frameshifting. *Genes Cells* *5*, 265-276.
- Tsukada, T., Furusako, S., Maekawa, S., Hibasami, H., and Nakashima, K. (1988). Purification by affinity chromatography and characterization of porcine liver cytoplasmic polyamine oxidase. *Int J Biochem* *20*, 695-702.
- Wallace, H. M., Fraser, A. V., and Hughes, A. (2003). A perspective of polyamine metabolism. *Biochem J* *376*, 1-14.
- Van den Munckhof, R. J. M., Denyn, M., Tigchelaar-Gutter, W., Schipper, R. G., Verhofstad, A. A. J., Van Noorden, C. J. F., and Frederiks, W. M. (1995). In situ substrate specificity and ultrastructural localization of polyamine oxidase activity in unfixed rat tissues. *J Histochem Cytochem* *43*, 1155-1162.
- Wang, C., Delcros, J. G., Biggerstaff, J., and Phanstiel, O. I. (2003b). Molecular requirements for targeting the polyamine transport system. Synthesis and biological evaluation of polyamine-anthracene conjugates. *J Med Chem* *46*, 2672-2682.
- Wang, C., Delcros, J. G., Cannon, L., Konate, F., Carias, H., Biggerstaff, J., Gardner, R. A., and Phanstiel, O. I. (2003a). Defining the molecular requirements for the selective delivery of polyamine conjugates into cells containing active polyamine transporters. *J Med Chem* *46*, 5129-5138.
- Wang, Y., Devereux, W., Stewart, T. M., and Casero R. A., Jr. (2001b). Characterization of the interaction between the transcription factors human polyamine modulated factor (PMF-1) and NF-E2-related factor 2

- (Nrf-2) in the transcriptional regulation of the spermidine/spermine N1-acetyltransferase (SSAT) gene. *Biochem J* *355*, 45-49.
- Wang, Y., Devereux, W., Woster, P. M., Stewart, T. M., Hacker, A., and Casero, R. A., Jr. (2001a). Cloning and characterization of a human polyamine oxidase that is inducible by polyamine analogue exposure. *Cancer Res* *61*, 5370-5373.
- Wang, Y., Hacker, A., Murray-Stewart, T., Fleischer, J. G., Woster, P. M., and Casero, R. A., Jr. (2005). Induction of human spermine oxidase SMO(PAOH1) is regulated at the levels of new mRNA synthesis, mRNA stabilization and newly synthesized protein. *Biochem J* *386*, 543-547.
- Varnado, B. L., Voci, C. J., Meyer, L. M., and Coward, J. K. (2000). Circular dichroism and NMR studies of metabolically stable alpha-methylpolyamines: spectral comparison with naturally occurring polyamines. *Bioorg Chem* *28*, 395-408.
- Watanabe, S.-i., Kusama-Eguchi, K., Kobayashi, H., and Igarashi, K. (1991). Estimation of polyamine binding to macromolecules and ATP in bovine lymphocytes and rat liver. *J Biol Chem* *266*, 20803-20809.
- Webb, H. K., Wu, Z., Sirisoma, N., Ha, H. C., Casero, R. A., Jr., and Woster, P. M. (1999). 1-(N-alkylamino)-11-(N-ethylamino)-4,8-diazaundecanes: simple synthetic polyamine analogues that differentially alter tubulin polymerization. *J Med Chem* *42*, 1415-1421.
- Williams, K. (1997). Modulation and block of ion channels: a new biology of polyamines. *Cell Signal* *9*, 1-13.
- Wu, T., Ling, K. Q., Sayre, L. M., and McIntire, W. S. (2005). Inhibition of murine N1-acetylated polyamine oxidase by an acetylenic amine and the allenic amine, MDL 72527. *Biochem Biophys Res Commun* *326*, 483-490.
- Wu, T., Yankovskaya, V., and McIntire, W. S. (2003). Cloning, sequencing, and heterologous expression of the murine peroxisomal flavoprotein, N1-acetylated polyamine oxidase. *J Biol Chem* *278*, 20514-20525.
- Vujcic, S., Diegelman, P., Bacchi, C. J., Kramer, D. L., and Porter, C. W. (2002). Identification and characterization of a novel flavin-containing spermine oxidase of mammalian cell origin. *Biochem J* *367*, 665-675.
- Vujcic, S., Halmekytö, M., Diegelman, P., Gan, G., Kramer, D. L., Jänne, J., and Porter, C. W. (2000). Effects of Conditional Overexpression of Spermidine/Spermine N1- Acetyltransferase on Polyamine Pool Dynamics, Cell Growth, and Sensitivity to Polyamine Analogs. *J Biol Chem* *275*, 38319-38328.
- Vujcic, S., Liang, P., Diegelman, P., Kramer, D. L., and Porter, C. W. (2003). Genomic identification and biochemical characterization of the mammalian polyamine oxidase involved in polyamine back-conversion. *Biochem J* *370*, 19-28.
- Xu, H., Chaturvedi, R., Cheng, Y., Bussiere, F. I., Asim, M., Yao, M. D., Potosky, D., Meltzer, S. J., Rhee, J. G., Kim, S. S., *et al.* (2004). Spermine oxidation induced by *Helicobacter pylori* results in apoptosis and DNA damage: implications for gastric carcinogenesis. *Cancer Res* *64*, 8521-8525.
- Yang, J., Xiao, L., Berkey, K. A., Tamez, P. A., Coward, J. K., and Casero, R. A., Jr. (1995). Significant induction of spermidine/spermine N1-acetyltransferase without cytotoxicity by the growth-supporting polyamine analogue 1,12-dimethylspermine. *J Cell Physiol* *165*, 71-76.
- Yoshida, M., Kashiwagi, K., Shigemasa, A., Taniguchi, S., Yamamoto, K., Makinoshima, H., Ishihama, A., and Igarashi, K. (2004). A unifying model for the role of polyamines in bacterial cell growth, the polyamine modulon. *J Biol Chem* *279*, 46008-46013.
- Zaks, A., and Dodds, D. R. (1997). Application of biocatalysis and biotransformations to the synthesis of pharmaceuticals. *Drug Discov Today* *2*, 513-531.

Zhu, C., Lang, D. W., and Coffino, P. (1999). Antizyme2 is a negative regulator of ornithine decarboxylase and polyamine transport. *J Biol Chem* 274, 26425-26430.