REVIEW ARTICLE

A perspective of polyamine metabolism

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Polyamines are essential for the growth and function of normal cells. They interact with various macromolecules, both electrostatically and covalently and, as a consequence, have a variety of cellular effects. The complexity of polyamine metabolism and the multitude of compensatory mechanisms that are invoked to maintain polyamine homoeostasis argue that these amines are critical to cell survival. The regulation of polyamine content within cells occurs at several levels, including transcription and translation. In addition, novel features such as the +1 frameshift required for antizyme production and the rapid turnover of several of the enzymes involved in the pathway make the regulation of polyamine metabolism a fascinating subject. The link between polyamine content and human disease is unequivocal, and significant success has been obtained in the treatment of a number of parasitic infections. Targeting the polyamine pathway as a means of treating cancer has met with limited success, although the development of drugs such as DFMO (α-difluoromethylornithine), a rationally designed anticancer agent, has revolutionized our understanding of polyamine function in cell growth and provided 'proof of concept' that influencing polyamine metabolism and content within tumour cells will prevent tumour growth. The more recent development of the polyamine analogues has been pivotal in advancing our understanding of the necessity to deplete all three polyamines to induce apoptosis in tumour cells. The current thinking is that the polyamine inhibitors/analogues may also be useful agents in the chemoprevention of cancer and, in this area, we may yet see a revival of DFMO. The future will be in adopting a functional genomics approach to identifying polyamine-regulated genes linked to either carcinogenesis or apoptosis.

Key words: apoptosis, cancer, cell growth, putrescine, spermidine, spermine.

INTRODUCTION

The initial discovery of the polyamines dates back to 1678 when Antonie van Leeuwenhoek isolated some 'three-sided' crystals from human semen [1]. However, it was not until 1924 that the empirical formula of the crystals was deduced [2], and it was a further 2 years before the products were synthesized chemically [3]. The names spermidine and spermine therefore reflect the original discovery. Putrescine (1,4-diaminobutane) was first isolated from *Vibrio cholerae*, but it derives its common name from the large quantities found in putrefying flesh [4]. From these inauspicious beginnings it is therefore perhaps surprising that, today, polyamines should be considered critical regulators of cell growth, differentiation and cell death. In the last 30 years there has been a steady rise in the number of publications per annum focussing on polyamines, with approx. 1600 papers published in 2000.

Polyamines are found in all living species, except two orders of Archaea, Methanobacteriales and Halobacteriales [5]. This conservation across evolution is a positive feature in that it argues for their importance in cell survival, but it may also be a drawback in that it implies a lack of specific function [6].

POLYAMINES AS CATIONS

At physiological pH, polyamines carry a positive charge on each nitrogen atom and it has been suggested that polyamines are simply 'supercations', equivalent to one or two calcium or magnesium molecules. However, the charge on the polyamines is distributed along the entire length of the carbon chain, making them unique and distinct from the point charges of the cellular bivalent cations.

Their positive charge enables polyamines to interact electrostatically with polyanionic macromolecules within the cell. Spermidine and spermine can bridge the major and minor grooves of DNA, acting as a clamp holding together either two different molecules or two distant parts of the same molecule [7]. Structural studies indicate that the polyamines interact with individual rather than multiple DNA molecules [8]. Selectivity of polyamine binding to secondary structures of DNA has been suggested from crystallographic studies with polyamines having a preference for pyrimidine residues, particularly thymidine, although this may be influenced by the neighbouring nucleotides and the nature of the secondary structure [9]. Polyamine analogues such as bis(ethyl)homospermine ('BEHSpm'; 'BE-4-4-4') have been shown to alter the DNA–nuclear matrix interaction, suggesting that not only do polyamines alter the structure of DNA, but they also influence its function [10]. In the nucleosome, polyamine depletion results in partial unwinding of DNA and unmasking of sequences previously buried in the particle. These newly revealed sequences are potential binding sites for factors regulating transcription [11]. This, together with the fact that polyamines favour the formation of triplex DNA at neutral pH, may provide a mechanism whereby polyamines regulate the transcription of growth regulatory genes such as c-*myc* [12–14].

Abbreviations used: AZ, antizyme; AZI, AZ inhibitor; cdk, cyclin-dependent kinase; CHENSpm, N^1-ethyl-N^4-[(cycloheptyl)methyl]-4,8-diazaundecane; dcSAM, decarboxylated *S*-adenosylmethionine; DFMO, α-difluoromethylornithine; MDL 72527, N^1,N^4-bis(buta-2,3-dienyl)butane-1,4-diamine; MGBG, methyglyoxal bis(guanylhydrazone); Nrf-2, nuclear factor-E2-related factor 2; ODC, ornithine decarboxylase; PAO, polyamine oxidase; PRE, polyamine response element; SAMDC, *S*-adenosylmethionine decarboxylase; SMO, spermine oxidase; SSAT, spermidine/spermine N^1-acetyltransferase; UTR, untranslated region.

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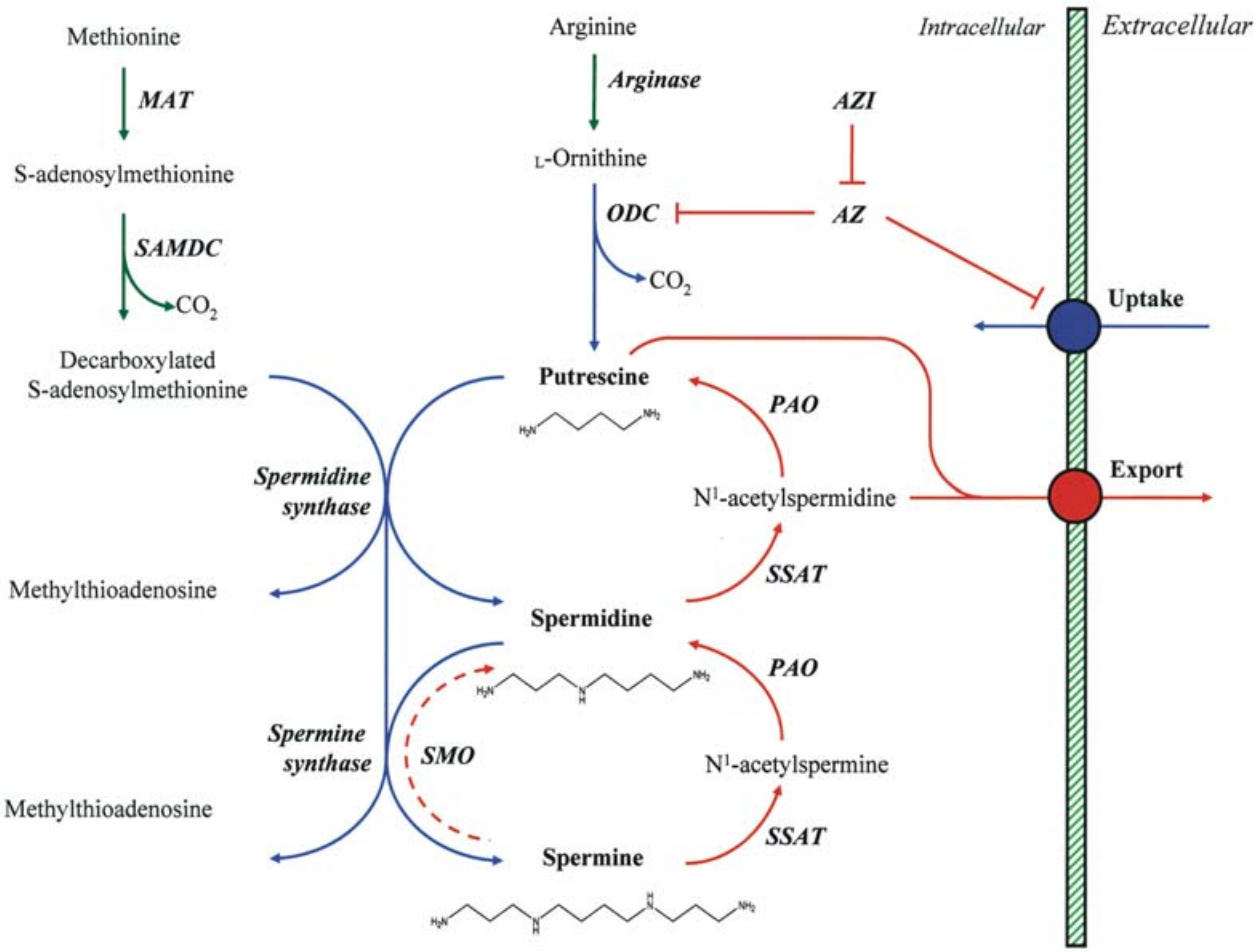


Scheme 1 Pathways of polyamine metabolism

Further abbreviation: *MAT*, methionine adenosyltransferase.

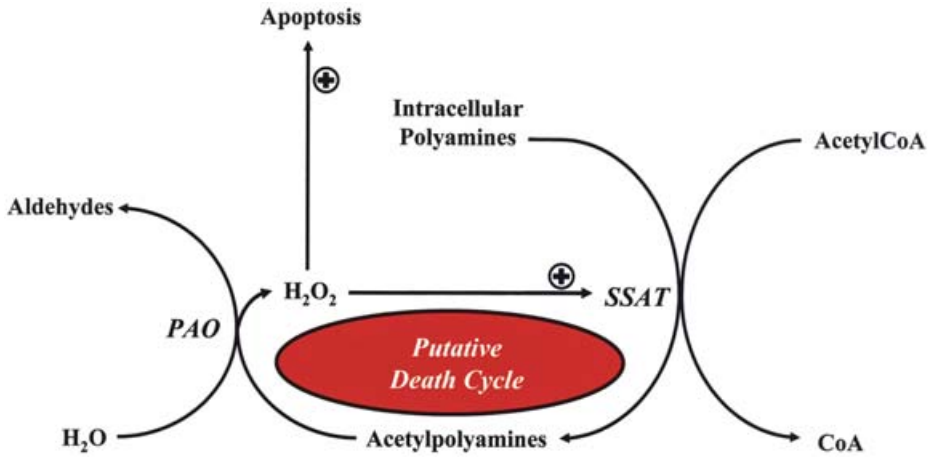
In addition to interracting with DNA and RNA, polyamines can also interact with acidic phospholipids in membranes [15]. In general, spermidine and spermine increase the rigidity of the membrane by forming complexes with phospholipids and proteins. They may also have an antioxidant role, preventing lipid peroxidation [16]. Polyamines have been implicated in the regulation of several membrane-bound enzymes, including adenylate cyclase [17], tissue transglutaminase [18] and some ion channels such as NMDA (*N*-methyl-D-aspartate), KIR (inwardly rectifying K^+) and voltage-activated Ca^{2+} channels [19–21].

If, however, charge is the defining feature of the polyamines, then surely one polycation would be sufficient? The most obvious choice would be spermine, as it has the greatest charge, longest length and most flexibility. The sheer complexity of the regulation and metabolism used by the polyamines argues that they, or their associated enzyme activities, have other critical functions within the cell not based solely on direct charge–charge interactions.

POLYAMINE METABOLISM

In eukaryotic cells, the three polyamines are synthesized from L-arginine (via L-ornithine) and L-methionine by a series of six interdependent enzyme reactions (Scheme 1). Putrescine is formed from the decarboxylation of ornithine, by ODC (ornithine decarboxylase; EC 4.1.1.17), and this combines with dcSAM (decarboxylated *S*-adenosylmethionine) formed by SAMDC (*S*-adenosylmethionine decarboxylase; EC 4.1.1.50), to produce spermidine via spermidine synthase (EC 2.5.1.16), and spermine through a second aminopropyltransferase reaction involving spermine synthase (EC 2.5.1.22). The synthases are stable enzymes that are expressed constitutively with little recorded inducibility [22]. Both enzymes are active as homodimers: spermidine synthase has a subunit molecular mass of 36 kDa, whereas spermine synthase consists of two subunits of 44 kDa. Unlike the decarboxylases, both enzymes are regulated by the availability of their substrates, with the K_m values resembling closely the tissue concentrations for dcSAM and putrescine or spermidine.

SSAT (spermidine/spermine N^1-acetyltransferase; EC 2.3.1.57) is the first step in the retroconversion process, using acetyl-CoA to form N^1-acetylspermidine and spermine. The N^1-acetyl derivatives are then the preferred substrates of FAD-dependent PAO (polyamine oxidase; EC 1.5.3.11), producing spermidine and putrescine respectively [23]. The intermediate products of polyamine catabolism, N^1-acetylspermidine and N^1-acetylspermine, are found only rarely in normal cells, mainly because these are the major polyamines exported from the cell [24]. Acetylpolyamines are, however, found in high concentrations in cancer cells, providing a link between alterations in polyamine metabolism and



Scheme 2 Polyamine metabolism and the potential for cell death

Metabolism of polyamines via SSAT and PAO produces H_2O_2. H_2O_2 induces both SSAT and cell death, thus causing a positive cell-death-signal-generating cycle.

carcinogenesis [25,26]. Oxidation of acetylated polyamines results in the production of stoichiometric amounts of 3-acetamidopropanol and H_2O_2, both of which have been shown to result in toxicity and cell death [27,28]. Effectively, this means that the metabolism of the higher polyamines could generate a self-sustaining cell death cycle (Scheme 2). SSAT and PAO work in concert to acetylate and oxidize the polyamines, generating H_2O_2 on each oxidation. H_2O_2 is an inducer of SSAT activity, thereby perpetuating the cycling. The high local concentrations of H_2O_2 produced could then lead to oxidative stress and cell death [29].

The reactions forming polyamines were, until recently, considered essentially irreversible, with a separate retroconversion pathway to recycle spermidine and putrescine from spermine and spermidine respectively (Scheme 1). However, towards the end of 2002, an oxidase was cloned that converts spermine back into spermidine without the need for an acetylation step [132]. This enzyme has now been termed 'spermine oxidase' (SMO) [30], and we wait with interest to learn more of its function and regulation. These pathways of synthesis and breakdown are highly regulated, and several of the enzymes involved are subject to control at many levels, including transcription, post-transcription, translation and post-translation (see below).

TRANSPORT OF POLYAMINES

Although *de novo* synthesis is the major route to the production of polyamines in mammalian cells, transport into and out of the cell also contributes to polyamine homoeostasis [31]. Preformed polyamines are derived either from the diet (we all consume large quantities of polyamines everyday) [32] or from the intestinal flora [33]. Despite the fact that we do eat significant quantities of polyamines, the bioavailability of these polyamines in man is not known. In order to establish whether these dietary amines are beneficial or, indeed, detrimental, their bioavailability in man must be assessed properly. The contribution to the total polyamine pool from gut metabolism is less clear, but, at present, it seems unlikely that microbially derived polyamines are a major contributor to the total body content.

Despite a sustained effort over the last decade, the mammalian polyamine transporter has not yet been cloned. It has been suggested that the polyamine transporter is carrier-mediated, energy-dependent and saturable [34,35]. However, recent evidence points to endocytosis as an alternative mechanism of polyamine internalization [36]. Although some cells have a single carrier for all three polyamines, most cell types appear to have two classes of carrier: one with a preference for putrescine and one for spermidine and spermine [37]. The evidence for the separate transporters comes from competition and substrate-specificity studies and from their dependence, or not, on sodium [38]. The polyamine uptake system transports molecules as diverse as paraquat [39], MGBG [methyglyoxal bis(guanylhydrazone)] [40] and polyamine analogues [41,42]. This lack of specificity has been used to advantage in the design of potential inhibitors of polyamine metabolism and in the targeting of cytotoxic drugs to DNA [43].

Polyamines can also be transported out of cells [24]. Export is a selective process that is regulated by the growth status of the cell, being switched on by a decrease in cell growth rate and switched off in response to a growth stimulus [44,45]. For example, in cultured cells, polyamine export increases in response to contact inhibition of growth, decreases in serum or nutrients [44,46], treatment with antiproliferative drugs [47] and viral infection [48]. On the other hand, export is decreased by initiation of cell growth (e.g. by addition of fresh serum) [44]. In most cases, the major polyamines exported from the cell are N^1-acetylspermidine and putrescine [45]. This is in contrast with the normal intracellular polyamine pool, where the predominant polyamine, at least in human cells, is spermine. This evidence indicates that export is a selective and regulated process, with metabolism required before efflux. Thus the enzymes involved in polyamine catabolism and the outward transporter should be regulated by the same signals.

Preliminary evidence from our laboratory using selective uptake inhibitors indicates that the inward and outward transporters are separate and distinct, since the inhibitors of uptake had no effect on polyamine export (H. M. Wallace, A. J. Mackarel, A. V. Fraser and R. A. Fearn, unpublished work).

Specific inhibitors, directed to almost every step in the pathway, have been synthesized and developed (Table 1) – the only exception is the outward export process, for which there is, as yet, no inhibitor. These agents have tended to be single enzyme inhibitors and, in general, have been found to deplete only two out of the three polyamines. Although useful experimental tools

Table 1 Known inhibitors of polyamine metabolism

Enzyme	Inhibitor		Reference
	Abbreviation	Systematic name	
SAMDC	AbeAdo	5′-{[(*Z*)-4-Amino-2-butenyl]methylamino}-5′-deoxyadenosine	[49]
	AdoMac	*S*-[5′-Deoxy-5′-adenosyl]-1-ammonio-4-[methylsulphonio]-2-cyclopentene	[50]
	AdoMao	*S*-[5′-Deoxy-5′-adenosyl]-1-aminoxy-4-[methylsulphonio]-2-cyclopentene	[51]
	AMA	*S*-[5′-Deoxy-5′-adenosyl]methylthioethylhydroxylamine	[52]
	APA	1-Amino-oxy-3-aminopropane	[53]
	CGP 39937	(2,2-Bipyridine)-6,6′-dicarboximidamide	[54]
	CGP 48664	4-Amidinoindanon-1-(2′-amidino)hydrazone	[54]
	EGBG	Ethylglyoxal bis(guanylhydrazone)	[55]
	MGBG	Methylglyoxal bis(guanylhydrazone)	[56]
	MAOEA	5′-Deoxy-5′-[(2-aminooxyethyl)methylamino] adenosine	[57]
	MHZPA	5′-Deoxy-5′-[(3-hydrazinopropyl)methylamino] adenosine	[57]
ODC	AEO	α-Ethylornithine	[58]
	AHO	α-Hydrazino-ornithine	[59]
	APA	1-Amino-oxy-3-aminopropane	[53]
	AVO	α-Vinylornithine	[58]
	AHMPA	($\pm$)-5-Amino-2-hydrazine-2-methylpentanoic acid	[60]
	DAB	1,4-Diaminobutane	[61]
	DAP	1,3-Diaminopropane	[62]
	DAPOH	1,3-Diaminopropan-2-ol	[63]
	DFMO	α-Difluoromethylornithine	[64]
	DL-HAVA	DL-α-Hydrazino-δ-aminovaleric acid	[65]
	MAP	(2*R*,5*R*)-6-Heptyne-2,5-diamine	[66]
	MFMP	DL-α-Monofluoromethylputrescine	[67]
	V-MFMO	(*E*)-2-Fluoromethyldehydro-ornithine	[68]
	α-MO	α-Methylornithine	[69]
Spermidine synthase	AdoDATO	*S*-Adenosyl-1,8-diamino-3-thio-octane	[70]
	APA	1-Amino-oxy-3-aminopropane	[53]
	DCHA	Dicyclohexylamine sulphate	[71]
	4MCHA	*trans*-4-Methylcyclohexylamine	[72]
Spermine synthase	AdoDATAD	*S*-Adenosyl-1,12-diamino-3-thio-9-azadodecane	[73]
	AP-APA	1-Amino-oxy-3-*N*-(3-aminopropyl)aminopropane	[74]
	APCHA	*N*-(3-Aminopropyl)cyclohexylamine	[72]
	AOE-PU	*N*-(2-Amino-oxyethyl)-1,4-diaminobutane	[74]
SSAT	Berenil	1,3-Tris-(4′-amidinophenyl)triazine	[75]
	Pentamidine	*p,p′*-(Pentamethylenedioxy)dibenzamidine	[75]
PAO	MDL 72527	N^1,N^4-bis(buta-2,3-dienyl)butane-1,4-diamine	[76]
	Pentamidine	*p,p′*-(Pentamethylenedioxy)dibenzamidine	[75]
SMO	N(1)OSSpm	N^1-(n-octanesulphonyl)spermine	[30]
Polyamine transport	AOSPM	11-[(Amino)oxy]-4,9-diaza-1-aminoundecane	[77]
	ORI 1202	N^1-Spermyl-L-lysinamide	[78]

in defining the role of the polyamines in a number of cellular processes, the inhibitors, with the notable exception of α-difluoromethylornithine (DFMO), have proven to be of limited use in the treatment and/or prevention of disease.

REGULATION OF METABOLISM

What, then, are the key regulatory points in the metabolism of the polyamines? The critical enzymes have historically been ODC and SSAT, although recent evidence also points to an increasingly important role for PAO. ODC and SSAT are considered to be rate-limiting for biosynthesis and catabolism respectively, because of their early and rapid responses to stimuli. However, 'rate-limiting' is inappropriate, and 'regulatory' is a more correct term. Recently, PAO has also been found to be inducible, and therefore provides an additional point of regulation in the retroconversion pathway [79].

ODC

ODC is in itself an interesting enzyme with several novel regulatory features. It is a highly inducible, cytosolic, subunit enzyme that responds to a range of trophic stimuli [80]. It has a short half-life (10 min–1 h) compared with many mammalian enzymes whose half-lives are more often expressed in days [81]. ODC requires pyridoxal phosphate as a cofactor, and thiol-group-reducing agents are necessary for enzyme activity, possibly owing to the high number of cysteine residues in the protein.

ODC contains two PEST (proline-, glutamate-, serine- and threonine-rich) regions that are rich in proline, glutamic acid, aspartic acid, serine and threonine [82]. The PEST region located at the C-terminus of ODC is essential for the degradation of the enzyme, and truncations and mutations in this region result in stabilization of the enzyme [83]. ODC activity is dependent upon the formation of a dimer with the active site, occurring at the interface between the two subunits [84]. Residues at the active site critical to ODC activity include Lys^{169} and His^{197} [85].

ODC expression is also regulated by transcription, stability and the efficiency of translation of the mRNA. At a transcriptional level, ODC expression can be regulated by oncogenes. The *hODC* gene contains three CACGTG regions: one at the 5′ promoter region and two others in intron 1 [86] that bind the protein product of the *c-myc* oncogene [87]. Overexpression of c-*myc* and other oncogenes such as *v-mos* [88], Ha-*ras* [89] and *c-fos* [90] can lead to overexpression and induction of ODC and, ultimately, carcinogenesis. ODC mRNA has long 5′ and 3′ UTRs (untranslated regions) and, whereas neither region seems to be involved in polyamine-mediated feedback control of ODC activity [91], the 3′ UTR may have a role in regulation under special circumstances, such as hypotonic shock [92].

Increases in ODC activity are one of the early changes observed in cells stimulated to grow and these increases precede changes in DNA synthesis by several hours, making ODC an 'immediate early' response gene [93]. ODC is subject to both positive and negative feedback regulation by polyamines: high polyamine concentrations decrease, and low polyamine concentrations increase, activity. The feedback regulation appears to be a mixture of post-transcriptional regulation and the induction of a unique ODC-specific inhibitor termed 'antizyme' (AZ) [94].

AZ

AZ is a small (23 kDa) regulatory protein that is induced by increased intracellular concentrations of polyamines that trigger a +1 translational frameshift on the AZ mRNA, allowing the complete AZ protein to be expressed [95]. AZ binds to ODC [96] and the AZ–ODC complex is degraded by the 26 S proteasome. Unusually, the degradation of ODC by this proteasome occurs in an ATP-dependent, but ubiquitin-independent, manner [97,98]. The majority of proteins degraded by the 26 S proteasome use ubiquitin to target the molecule for degradation [99,100]. ODC can be released from AZ by another unique protein, called 'antizyme inhibitor' (AZI), which liberates ODC in the presence of growth stimuli by virtue of having a higher affinity for AZ than for ODC [101]. Additionally, AZ can alter polyamine homoeostasis by down-regulating polyamine uptake independent of the effects on ODC (Scheme 1) [102–104]. It may be that AZ binding to ODC causes a conformational change in ODC, resulting in exposure of its C-terminus, so targeting it for degradation [105].

Currently, three forms of AZ have been identified and characterized, with each having a specific role in polyamine metabolism [106]. AZ 1 is strongly associated with the degradation of ODC. AZ 2 has been shown to have a low ability to induce ODC degradation, and has shown to have more involvement in the negative regulation of polyamine transport [102,107]. The expression of AZ 3 is limited to testis germ cells, where its expression occurs at a particular stage of spermatogenesis [108,109]. A putative fourth AZ is currently being investigated [106].

SSAT

SSAT is a cytosolic enzyme originally identified as a homodimer [110], but now believed to be a homotetramer of molecular mass about 80 kDa [111,112]. It acetylates specifically at primary amino groups, with no reports of acetylation at secondary amino groups. Spermidine and spermine, but not putrescine, are substrates for the enzyme. In our hands, spermidine is approximately three times more efficient as a substrate of the SSAT enzyme than is spermine (C. S. Coleman and H. M. Wallace, unpublished work). Acetylation occurs by a Bi Bi kinetic model, with the substrate binding to the active site first and the acetylated product being released last. The acetyl-CoA binding site is proposed to be in a conserved region of 20 amino acids beginning with Arg^{101} and consisting of the sequence RGFGIGS [113]. Arginine at positions 142 and 143 are also required for acetyl-CoA binding [114].

Spermidine is asymmetric, and two products can be formed: N^1-acetylspermidine, where the acetyl group is attached to the aminopropyl group; and N^8-acetylspermidine, with the acetyl group is attached to the aminobutyl group. The N^8-specific acetyltransferase is a separate nuclear enzyme with a substrate specificity that includes histones [115]. This enzyme is not inducible, and the N^8-acetylspermidine is either deacetylated or excreted. N^8-Acetylspermidine is not a substrate for PAO.

Acetylated polyamines have a decreased positive charge relative to free polyamines, and therefore, as with acetylated histones, will have a decreased affinity for DNA and RNA, thus weakening or preventing binding to intracellular sites. N^1-Acetylpolyamines are the preferred substrates for PAO and are also the major excretory products from cells [45]. Thus competition exists between oxidation and export, and this appears to be regulated by growth status. If polyamines are required for cell growth, then recycling of spermine to putrescine occurs. If, however, cell growth is restricted, then the acetyl derivatives are exported from the cell, resulting in a net loss of polyamines [116].

SSAT has several features in common with ODC: it has a short half-life (20–40 min); it is highly regulated at several levels, and is readily inducible. Unlike most proteins that undergo rapid turnover, SSAT does not contain a PEST region [117]. However, the terminal MATEE motif may substitute for a PEST region, which, while lacking proline, does contain serine, threonine and acidic residues [118].

The first observations that SSAT was inducible came from studies with carbon tetrachloride [119] and MGBG [120,121]. Induction of SSAT requires both protein and RNA synthesis [122]. In the case of MGBG, the increase in activity was the result of stabilization of the enzyme protein [121]. Enzyme stabilization has also been reported in response to spermidine and spermine [123]. SSAT, like ODC, is degraded by the 26 S proteasome; however, it is ubiquitin that directs SSAT to the proteasome [118].

The most interesting aspect of SSAT regulation is the response of the enzyme to the polyamine analogues. Several studies have shown induction of SSAT in response to analogues and, in some cases, 'superinduction' of many thousandfold (for a review, see [124]). Superinduction of SSAT is due to a combination of enhanced mRNA transcription, stabilization of the message and the protein, and enhanced translation [125]. A second isoform of SSAT has also been reported [126]. Recently, a polyamine response element (PRE) has been identified in the regulatory region of the human SSAT gene [127]. This *cis* element is associated with the transcription factor Nrf-2 (nuclear factor-E2-related factor 2), which has only been found in cells capable of superinduction of SSAT. Thus Nrf-2, or similar proteins, may be important in SSAT regulation [128].

PAO

Originally it was proposed that PAO was a constitutive enzyme, and that SSAT was the regulating enzyme in polyamine retroconversion. However, PAO activity is increased by growth inhibition [129], in response to the anticancer drug etoposide [130] and in cancer cells when they reach high density [131]. These results provided the initial indications that PAO may also play a role in polyamine homoeostasis.

Wang et al. [132] cloned and characterized a protein, provisionally named PAO-1, with a molecular mass similar to that of

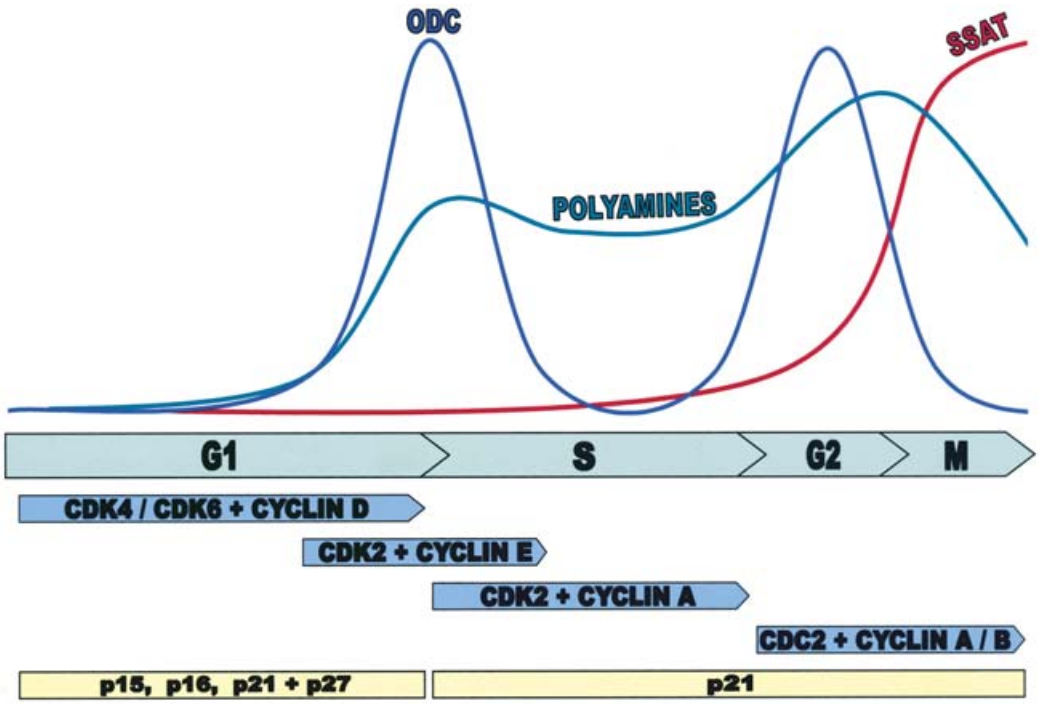


Figure 1 Relationship between polyamine metabolism and cell-cycle-regulatory proteins

Increases in ODC and polyamine concentrations occur in both G_1/S- and G_2-phases of the cell cycle. Alterations in SSAT appear later during M-phase. The changes in expression of the cyclins, cdks and their corresponding inhibitors are specific to each phase of the cell cycle and are shown in block arrows.

the PAO obtained by Holtta in 1977 [133]. However, in these studies, spermine was used as the substrate, leading to controversy as to whether this enzyme is the PAO described previously. A second oxidase, SMO [30,79], has been cloned using similar methodology, again with spermine as the substrate. Further investigations showed that SMO differed by only one amino acid from PAO-1, and, since both enzymes were identified using spermine as a substrate, it seems likely these are both spermine-specific oxidases. Currently, at least four active isoenzymes have been identified and shown to have PAO activity, with each isoenzyme demonstrating a different substrate specificity [134]. There are indications, however, that none of the enzymes purified to date match the profile of the PAO involved in the retroconversion pathway, mainly because the purified protein(s) does not have the preference for acetylated polyamines described in the literature [23,30]. In a separate study [135], embryonic stem cells showed conversion of spermine into spermidine in the absence of SSAT activity, thus suggesting the presence of a SMO-like activity in these embryonic stem cells too.

The use of selective inhibitors of amine oxidases will help to characterize the individual oxidases and the contribution each enzyme/isoform makes to polyamine homoeostasis. Currently, the enzyme nomenclature is confusing, especially between SMO and PAO. The need for clear appropriate naming of the enzymes is essential, and it would seem logical to distinguish between the N^1-acetylspermine-preferring PAO and the spermine-preferring SMO. Regardless, this discovery of multiple inducible enzymes will change the way in which we view the dynamics of polyamine catabolism, with cells no longer requiring the interaction of SSAT to convert spermine into spermidine. It is interesting to re-examine the polyamine export studies in the light of these new discoveries. The possibility exists that spermine is converted into spermidine by SMO, and spermidine is then subject to acetylation prior to export, thus explaining both the preference of SSAT for spermidine as a substrate and the presence of N^1-acetylspermidine and the lack of N^1-acetylspermine in the extracellular environment [45].

POLYAMINES – MEDIATORS OF CELL GROWTH AND/OR CELL DEATH?

Why have cells developed this complex system of regulation for polyamine metabolism? Historically, it was believed that the role of the polyamines was as intracellular growth factors, increasing the rate of cell growth and differentiation. More recently, it has been shown [136] that polyamines can also regulate the cell-death process known as apoptosis. Thus it now appears that the polyamines are bivalent regulators of cellular function, promoting cell growth or cell death depending on other environmental signals. Under normal circumstances polyamine concentrations regulate their own biosynthesis and prevent overproduction. However, in extreme cases, high exogenous polyamine concentrations can lead to cell death [137,138].

Polyamines and the regulation of cell growth

Normal cell growth is regulated in a cyclical manner by increases and decreases in specific proteins and protein kinases known as cyclins and cyclin-dependent kinases (cdks) [139]. Appropriate activation of the cdks and their partner cyclins is required for continual progression through the cell cycle. The cyclin/cdks exhibit cycle-specific regulation, with cyclins A, B, D and E and their respective cdk partners increasing and decreasing in a regulated and co-ordinated manner during the G_1-, S- and G_2/M-phases of the cell cycle (Figure 1).

Table 2 Cell-cycle changes in response to altered polyamine content

Further abbreviations used: CGP 48664, amidinoindan-1-one 2′-amidinohydrazone; DENSpm, diethylnorspermine; IPENSpm, (*S*)-N^1-(2-methyl-1-butyl)-N^{11}-ethyl-4,8-diazaundecane; MDL 73811, 5′-{[(*Z*)-4-aminobut-2-enyl]methylamino}-5′-deoxyadenosine; CHO, Chinese-hamster ovary.

Cell line	Treatment	Phase of arrest	Effect	Reference
IEC-6	DFMO	G_1	Increased p21, p27 and p53 expression	[146]
Hep-2	DFMO		Increased cyclin A during M phase	[147]
HL-60	DFMO	G_1		[148]
MKN45	DFMO	G_1	Increased p21 expression	[149]
MALME-3M	DFMO/MDL 73811	G_1	Increased p21 expression	[143]
	Methyl, ethyl and propyl spermine analogues	G_1		[152]
SW620	MDL 72527	S		[150]
CHO	CGP 48664		Increased length in S-phase	[151]
CHO	DENSpm		Delay in S-phase	[153]
MDA-MB-468	CHENSpm, IPENSpm	G_2/M		[154]
HL-60	CHENspm	G_1		[155], but see [155a]
	IPENSpm	G_1		[156]
PNT1A/PNT2	Antisense ODC RNA/ antisense AZ cDNA	S		
	SSAT cDNA	G_2/M		[157]
Rodent fibroblasts	Transformed ODC		Increased cyclin D1, D1 kinase and cdk4 expression	
	Transformed SAMDC		Increased cyclin E-dependent kinase and decreased p27 expression	[158]

It has been known for many years that there are also changes in both ODC and polyamine concentrations during the cell cycle [140]. There is an early peak in ODC at G_1-phase, followed by an increase in polyamine content, and a later, second, increase during G_2-phase and prior to mitosis [141]. Thus both polyamines and cyclin/cdks show phased changes through the cell cycle, but the interaction between these two sets of regulatory molecules remains to be defined. One suggestion is that polyamines regulate cyclin degradation [142]. Intracellular polyamine concentrations have been reported to regulate both the up- and down-regulation of important cellular checkpoints within the cell cycle, and this may, in part, explain why their concentrations are controlled throughout the cycle (Figure 1) [143,144].

It is interesting that, whereas the first increase in ODC during the cell cycle (G_1) is mediated by the usual cap-dependent initiation of translation, the second increase occurs when protein synthesis is inhibited. The second increase is cap-independent and is mediated by an internal ribosomal entry site [145]. It has been suggested that putrescine is essential for the cell to enter S-phase, possibly pushing the cell through the G_1 restriction point prior to DNA synthesis. However, it is not just the relative polyamine concentrations that are important in the progression of the cell through the cell cycle – ODC, AZ and SSAT are also up- and down-regulated. ODC and AZ are increased in early S-phase, with a decrease in AZ during mid-S-phase, whereas AZ and SSAT expression are up-regulated in G_2/M-phase (Figure 1) [141].

One would predict from the discussion above that depletion of polyamines would result in cell-growth arrest. The arrest point varies with the drug used, with DFMO, for example, resulting in a G_1-phase block. A summary of these results to date is shown in Table 2.

With such a strong positive relationship to cell growth, it is perhaps not surprising that there has been an increasing effort over the last three decades to link polyamine metabolism to cancer development and to attempt to use inhibitors of polyamine biosynthesis as antiproliferative agents (Table 1). It is now some 30 years since the first observations linked overproduction of polyamines to cancer [159,160], and the number of papers published on this topic continues to rise annually. Despite early promise, the use of polyamine measurements to diagnose cancer proved untenable, owing to a number of false positive results under a variety of conditions. Further research attempted to use urinary polyamine measurements to monitor the response of patients to therapy. Here the relationship predicts that patients in remission will have a urinary polyamine output within the normal range, but that if recurrence of disease takes place, then the output of polyamines will rise [161]. Preliminary studies here in Aberdeen, in collaboration with the Oncology Unit, showed that measurements from a single urine sample, taken at a clinic visit, reflected the values obtained from 24 h collection (H. M. Wallace and A. Hutcheon, unpublished work). To date, there has been no contradictory evidence to this concept, and therefore it is disappointing that more use is not made of this relatively straightforward, non-invasive measurement in monitoring the progress of treated patients.

Intratumour polyamine concentrations are increased in a large number of solid tumours [162–167] compared with control values. Several attempts have been made to correlate polyamine content with prognostic factors, and in most cases there is a positive linkage between higher polyamine content and poorer outcome. In breast cancer a positive correlation exists between tumour polyamine content and recurrence [162], but again, little use has been made of this observation in a clinical setting, despite the

fact that it could help predict those individuals who would benefit from more aggressive therapy.

The high concentrations of polyamines found in cancer cells are the result of several changes in polyamine metabolism. The regulation of ODC, for example, is altered in some tumours, resulting in increased ODC expression. Several studies have confirmed that an increase in ODC activity and the subsequent increase in intracellular polyamine concentrations is an early event in carcinogenesis (reviewed in [168]). Recently, increases in SSAT activity and decreases in PAO activity in breast-cancer compared with normal tissue have also been observed [26].

The mouse model of skin carcinogenesis has been used extensively to examine both the effects of carcinogenic compounds and the relationship between ODC induction and cancer [169]. Phorbol esters, for example, initiate the development of skin tumours accompanied by an increase in ODC activity and polyamine content. Hyperplastic agents, on the other hand, did not increase ODC activity or promote tumour growth [170]. Interestingly, inhibition of ODC by retinoic acid prevented the formation of skin cancers in this model, suggesting a causal role for ODC activity in cancer development [171].

ODC is a putative proto-oncogene, with several studies showing constitutive activation of ODC during cellular transformation with carcinogens [172], viruses [173] and oncogenes [174]. In addition, deliberate overexpression of *hODC* cDNA led to transformation of NIH 3T3 cells [175] and in nude mice. However increased ODC activity alone (up to 40-fold) is not sufficient to transform cells in the absence of an initiating factor [176]. Combined overexpression of ODC cDNA and a promoter such as c-H-*ras* oncogene and/or PMA is required to transform cells [177,178]. Overall, this evidence indicates that ODC overexpression *per se* does not transform cells, but it is required as part of the initiation of the carcinogenic process. On the other hand, in a mouse skin model of carcinogenesis, O'Brien and colleagues showed that overexpression of ODC was sufficient for tumour development [179]. In these experiments, however, ODC expression levels were very high; it may therefore be that the extent of ODC overexpression is important in commitment to carcinogenesis.

Polyamines and the regulation of cell death

More recent studies have linked polyamines to cell death, particularly the cellular suicide known as apoptosis [180]. The effects of the polyamines are, however, far from simple, with both induction and inhibition of biosynthetic and catabolic enzyme activities being associated with increased and decreased apoptosis [136].

One elegant series of studies by Packham and Cleveland [86] linked increases in ODC activity to apoptosis. Enforced expression of c-Myc lead to increased ODC activity and apoptosis, both of which could be prevented by the ODC inhibitor, DFMO. c-Myc is a regulator of ODC expression, and this study demonstrated that c-Myc and ODC are involved in both cell growth and cell death. It is well known that exogenous polyamines can reverse the growth inhibition caused by DFMO, and in this study a similar reversal was observed for apoptosis. DFMO will delay 2-deoxy-D-ribose-induced apoptosis in HL-60 human promyelogenous leukaemic cells by approx. 24 h, but apoptosis was reinitiated on addition of exogenous polyamines [181]. This suggests that polyamines themselves also regulate apoptosis.

In HL-60 cells, exogenous polyamines prevented DNA fragmentation associated with etoposide-induced apoptotic cell death. This suggests that polyamines are inhibitors of apoptosis. By corollary, polyamine depletion should therefore induce apoptosis. However, the opposite was found, where the treatment of HL-60 cells with DFMO prevented apoptosis (G. S. Lindsay and H. M. Wallace, unpublished work). Similarly, treatment with DFMO decreased the sensitivity of rat/mouse T-cells to apoptosis induced by tumour necrosis factor (TNF), but cell death was also inhibited by exogenous spermine [182]. As DFMO depletes putrescine and spermidine, but can increase spermine content, it may be that spermine is an important regulator of apoptosis. Indeed, replacement of naturally occurring spermine with spermine analogues [e.g. bis(ethyl)norspermine] increases the sensitivity of some cells to apoptosis [182].

All three polyamines prevent cell death in rat cerebellar granule neurons induced by high KCl concentrations [183]. Exogenous spermine again prevented apoptosis, implicating a protective role for polyamines [184]. The protective effects of the polyamines may be due to DNA stabilization [185], protection of DNA from oxidative stress [186] or by inhibition of endonucleases [187].

These apparent contradictions can also be resolved by considering the regulation of polyamine metabolism. Polyamines down-regulate ODC via AZ, and DFMO also inhibits ODC, therefore it seems likely that it is inhibition of ODC that prevents cell death. This would mean that induction of ODC promotes cell death; yet it is clear from the previous discussion that induction of ODC also promotes cell growth. Thus the response of the cell depends on multiple signals for survival or death, and one signal can produce either response, depending on the environment. In support of this, we observed a biphasic effect on ODC in HL-60 cells in response to etoposide, a classic inducer of apoptosis (G. S. Lindsay and H. M. Wallace, unpublished work). There was an early, transient increase (2–4 h) in ODC followed by almost complete inhibition, suggesting that an increase in ODC initiated apoptosis, whereas a decrease was needed to sustain the process.

Despite the protective effects observed, exogenous polyamines can also be toxic in high concentrations. Spermine (2 mM) is toxic to baby-hamster kidney cells in culture. The toxicity was not due to the production of toxic aldehydes, as serum amine oxidases were absent [137]. Aminoguanidine, an amine oxidase inhibitor, prevented some of the observed toxicity, but inhibition of polyamine oxidase within the cells by treatment with MDL 72527 [N^1,N^4-bis(buta-2,3-dienyl)butane-1,4-diamine] potentiated the toxicity [138]. This suggests that spermine *per se* is toxic directly to the cells, although no evidence of apoptosis was observed. On the other hand, in HL-60 cells spermine triggers cytochrome *c* release from mitochondria, initiates caspase 3 activity and causes cell death via apoptosis [188]. Acetylation of spermine suppresses the apoptotic potential, indicating again that it is an interaction of the polyamines themselves and not metabolites. Similar to spermine, putrescine accumulation within cells is also reported to be cytotoxic. DH23A DFMO-resistant cells rapidly accumulate intracellular putrescine in the absence of DFMO. Failure to remove DFMO results in high putrescine inducing cell death [189].

The changes in intracellular polyamine content described above appear to be transient during apoptosis, with polyamine levels mainly decreased in the later stages. Preventing degradation of ODC by the 26 S proteasome inhibits apoptosis, suggesting that, whereas elevation of ODC is essential for apoptosis (as discussed above), degradation of the protein is also required for the completion of cell death [190].

INHIBITORS OF POLYAMINE METABOLISM: DFMO

Polyamines are essential to ensure successful completion of the replication process, with failure to maintain the individual polyamine concentrations leading to cell-cycle arrest, transformation

or cell death (Table 2). Therefore agents that inhibit polyamine biosynthesis will prevent, or at least limit, cell growth. This, together with the fact that polyamine concentrations are increased in cancer cells, has made the polyamine pathway a target suitable for the development of antiproliferative drugs. Inhibitors specifically designed against individual enzymes in the pathway result in polyamine depletion and inhibition of cell growth (Table 1). The biggest problem in utilizing polyamine metabolism as a therapeutic target is, however, the complex regulatory mechanisms that result in compensatory changes in metabolism. These alterations in transport and metabolism act to maintain homoeostasis, and overcome decreases within the intracellular polyamine pools. A good example of this is seen with the effects of DFMO *in vivo*.

DFMO was the first effective, rationally designed antiproliferative drug aimed at depleting polyamines from cells [64]. Acting as a suicide inhibitor of ODC, DFMO induced growth arrest and decreased the intracellular content of both putrescine and spermidine across a range of cell types (normal and malignant) [64], (for a review, see [191]), and its promise *in vitro* led to the testing of the drug in several *in vivo* models and in clinical trials. However, despite the early promise *in vitro*, subsequent *in vivo* studies failed to demonstrate lasting antitumour effects. DFMO treatment was found to exert a cytostatic, rather than a cytotoxic, effect, mainly due to compensatory increases in the uptake of polyamines from the circulation, maintaining polyamine homoeostasis and negating any depletion of tissue or organ polyamine content [192]. For example, in treatment of acute leukaemia or melanoma, two types of neoplasia thought to be susceptible to DFMO, no clinical response was achieved. These observations were disappointing, but they highlight some of the problems of chemotherapy with DFMO alone. Uptake of DFMO is by diffusion and is therefore unpredictable and slow. DFMO is also rapidly excreted from the body [193]. Consequently, high doses of DFMO are required to maintain the inhibition of ODC. Also, DFMO does not affect spermine content of cells, thus spermine could effectively be recycled and further negate any effect [194]. Compensatory increases in the uptake of polyamines from the diet and circulation and paradoxical increases in the other polyamine metabolic enzymes also contribute to overcoming the inhibitory effects of DFMO. Thus contrary to predictions, DFMO was a disappointment in chemotherapy, with *in vivo* data showing a varied response in human cancers [195]. The single notable success with DFMO in cancer chemotherapy is in the treatment of recurrent gliomas [196].

DFMO did, however, provide the 'proof of concept' that inhibition of polyamine production does prevent the growth of tumour cells. New strategies using the polyamine analogues aim to achieve maximum polyamine depletion by targeting more than one reaction in the polyamine pathway, thus avoiding the limitations observed with DFMO. The question that still needs to be addressed, however, is: 'how great a depletion of polyamine content is required to inhibit cell growth?' This is especially pertinent in view of some recent transgenic model systems that show little impairment of function as a result of life-long alteration in expression of ODC and SAMDC [197]. Similarly, fibroblasts from cells that lack spermine synthase grow at a normal rate in culture and show relatively few altered responses [198].

DFMO has, however, been successful as an anti-parasitic agent where it has been shown to cure acute infections of *Trypanosoma brucei brucei* in mammals [199,200]. It has also shown promise in the cure of African sleeping sickness (caused by *T. brucei gambiense and T. brucei rhodesiense*) [201,202]. DFMO prevents the synthesis of spermidine, which is an essential component of trypanothione, the trypanosome equivalent of glutathione, which protects cells from oxidative stress [203,204]. For a comprehensive review of this area, see [205].

More recently, a resurgence of interest in the use of DFMO in cancer has occurred, this time using DFMO as a chemopreventative rather than a chemotherapeutic agent. DFMO is an attractive drug for cancer prevention as it is relatively non-toxic and therefore can be given long term with few side effects. Meyskens et al. [206] have shown that frequent low doses of DFMO are sufficient to depress polyamine concentrations for prolonged periods, suggesting that the drug may be more effective in chemoprevention. There is potential for DFMO in chemoprevention of tumours in the colon and rectum, where decreases in ODC activity and polyamine content limit tumour formation. Although the exact mechanism is unknown, it is thought that DFMO acts late in tumour progression [207]. Several *in vivo* animal studies have shown that some types of epithelial cancers can be prevented by daily administration of DFMO. These include skin [208], breast [209] and bladder [210] cancers. Chemoprevention regimens are being developed currently in these, and a number of other, human tumours, such as Barrett's oesophagus [211]. These trials show promise, with the few side effects associated with DFMO usage being reversed on discontinuation of treatment [212].

Polyamine analogues

Bearing in mind the limitations of DFMO as a monotherapy, agents were developed that would target more than one reaction in the polyamine pathway. The development of polyamine analogues was pioneered by Porter, Bergeron and colleagues in the 1980s with the generation of the symmetrically substituted analogues such as the bis(ethyl)polyamines [213]. In the 1990s, analogues were further developed by Woster's group, and this led to synthesis of the second generation of unsymmetrically substituted compounds such as CHENSpm {N^1-ethyl-N^4-[(cycloheptyl)-methyl]-4,8-diazaundecane}. More recently, a third generation of polyamine analogues has been developed by the SLIL Biomedical Corporation (Madison, WI, U.S.A.), and these compounds include conformationally restricted, cyclic and oligoamine analogues (for reviews of the analogues, see [214]). The analogues were originally developed as surrogate polyamines to substitute for the natural amines in cell growth. However, it soon became clear that these analogues were growth-inhibitory, which led to the development of an alternative analogue theory.

The concept of polyamine analogues is that, as derivatives of natural polyamines, they are sufficiently similar in structure to the parent compound to allow their recognition and subsequent uptake by the polyamine transporter and to negatively regulate ODC and SAMDC, but are dissimilar enough to be unable to substitute functionally [215]. Thus the analogues will, by their multi-inhibitory approach, not induce the compensatory changes in metabolism such as those seen with DFMO and will, therefore, be more effective in inducing growth arrest and apoptosis.

It has become clear that two categories of analogue exist: the polyamine antimetabolite and the polyamine mimetic. The antimetabolite analogues result in polyamine depletion in conjunction with decreases in cell growth, whereas the polyamine mimetics decrease growth without necessarily producing significant polyamine depletion [156]. A bonus of the use of the analogues is that some 'superinduce' SSAT, a feature that increases their ability to deplete intracellular polyamine content. Although early studies suggested that there was a positive correlation between growth inhibition and SSAT induction, the relationship now appears to be cell-type-specific, with some cells, such as the small-cell-lung-carcinoma and melanoma cells showing a

high level of sensitivity to the analogues [216,217]. Current studies with the analogues include combining them with currently used anticancer drugs, as well as with agents such as DFMO. Preliminary evidence suggests that this is a positive strategy [218].

THE FUTURE?

At present it seems likely that DFMO will find a new lease of life as a cancer chemopreventative agent, either alone or in combination with non-steroidal anti-inflammatory agents. The analogues, too, may have potential as chemopreventative agents, but may be more useful in combination with other cytotoxic drugs, where synergistic effects may be found. Clearly the polyamines can regulate gene expression, and the PRE on SSAT is likely to be the first of many such findings. The advent of the new '. . . omics' technologies will facilitate the identification of other polyamine-regulated genes involved in both cell growth and cell death. The interest in the regulation of polyamine metabolism and function is intense and can, in our opinion, only continue to grow.

We acknowledge the European Social Fund, Grampian University Hospitals NHS Trust Endowments, Sir Samuel Scott of Yews Trust, Tenovus and the University of Aberdeen for financial support of our work.

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Received 1 September 2003/16 September 2003; accepted 18 September 2003
Published as BJ Immediate Publication 18 September 2003, DOI 10.1042/BJ20031327