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Perspective

Terminally Alkylated Polyamine Analogues as Chemotherapeutic Agents

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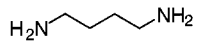
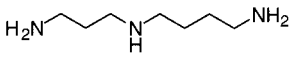
Introduction

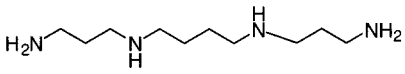
The polyamines spermidine (1,8-diamino-4-azaoctane, **2**) and spermine (1,12-diamino-4,9-diazadodecane, **3**), as well as the precursor molecule putrescine (1,4-diaminobutane, **1**) (Figure 1), are polycationic compounds



putrescine, **1**



spermidine, **2**



spermine, **3**

Figure 1. Structures of the natural polyamines: putrescine (**1**), spermidine (**2**), and spermine (**3**).

which are found in significant amounts in nearly every prokaryotic and eukaryotic cell type. Despite the ubiquitous nature of these compounds, the precise roles that polyamines play in cellular physiology are still being defined, with new avenues for research arising continuously. As a result, there are active research programs focusing on polyamine metabolism in an extremely diverse set of disciplines. The pathways for polyamine metabolism have been elucidated for a relatively small number of organisms. There are important interspecies differences in polyamine metabolism, especially between eukaryotic cells, plants, and some bacteria and protozoa. In some prokaryotes, only putrescine and spermidine are synthesized, while in other cases, such as certain thermophilic bacteria, polyamines with chains longer than spermine are found. In some parasitic organisms, there are additional enzymes which are not present in the host cell and, as such, provide a target for the design of specific antiparasitic agents. However, the enzymes involved in human and mammalian polyamine metabolism are reasonably similar, and inhibitors targeted to these enzymes rely on the observation that polyamine metabolism is accelerated and polyamines are required in higher quantities, in target cell types. The diversity of biological research in the polyamine field is the subject of an excellent book by Seymour Cohen.[1] This Perspective will deal with the use of polyamine analogues as chemotherapeutic agents, i.e., the use of synthetic polyamine analogues as anticancer or antiinfective agents. The reader should be aware of additional areas of polyamine research (polyamines as modulators of the NMDA receptor, polyamine-based venoms, polyamines as potential carriers for drug delivery, polyamines used in boron-neutron capture therapy, etc.) which are beyond the scope of this review.

The polyamine pathway represents an important target for chemotherapeutic intervention, since depletion of polyamines results in the disruption of a variety

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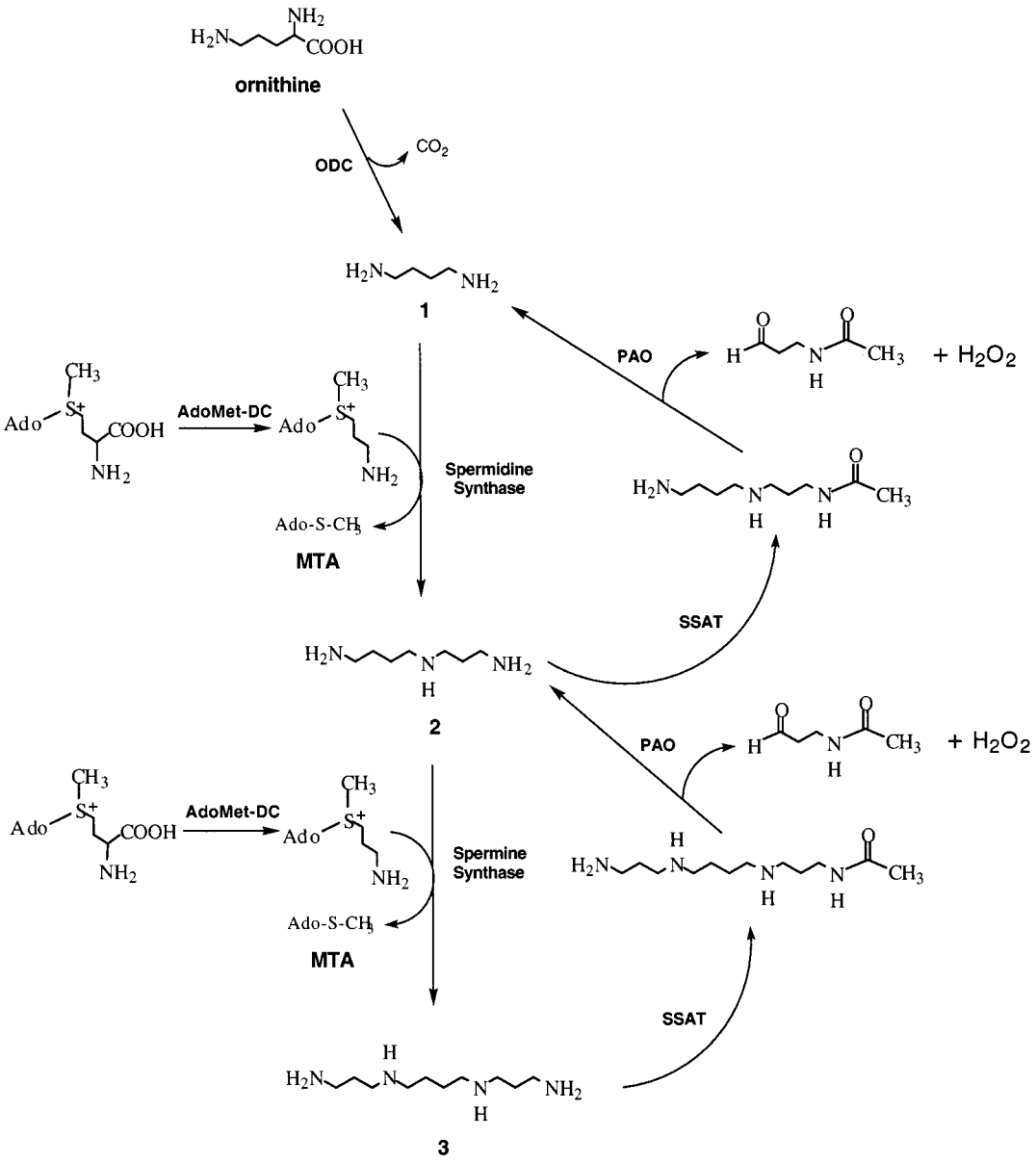


Figure 2. Biosynthesis of mammalian polyamines.

of cellular functions and may in specific cases result in cytotoxicity.[2] Inhibitors of the polyamine pathway, therefore, have traditionally been developed as potential antitumor and/or antiparasitic agents. Such inhibitors also play a critical role as research tools to elucidate the cellular functions of the naturally occurring polyamines, especially if these agents are specific for a single enzyme in the pathway. Inhibitors have now been developed for the enzymes in the polyamine biosynthetic pathway, ornithine decarboxylase (ODC),[3] *S*-adenosylmethionine decarboxylase (AdoMet-DC),[3−14] and for the aminopropyltransferases spermidine synthase[15] and spermine synthase.[16] The interruption of the polyamine metabolic pathway by these inhibitors leads to a variety of responses ranging from cessation of cell growth to overt cytotoxicity.[17,18] The range of these activities appears to be both agent- and cell-type-specific. On the basis of this knowledge, we and others have focused on the development of agents which interfere with the polyamine pathway as a means of antineoplastic and antiparasitic intervention.

Polyamine Biochemistry

The biosynthesis and catabolism of the polyamines putrescine (**1**), spermidine (**2**), and spermine (**3**) are carefully controlled processes in all eukaryotic cell types. Although the precise roles which the polyamines play in cell physiology are not well-defined, the requirement for the polyamines in mammalian cell growth appears to be absolute.[18,19] The mammalian polyamine biosynthetic pathway is shown in Figure 2.[19,20] Ornithine is converted to putrescine (**1**) by the enzyme ODC. This enzyme is a typical pyridoxal phosphate-requiring amino acid decarboxylase and has been studied extensively. ODC is one of the control points in the pathway, producing a product which is committed to polyamine biosynthesis. The synthesis and degradation of ODC are controlled by a number of factors, and the enzyme itself has a half-life of about 10 min.[19,20] Mammalian ODC, a dimeric enzyme with a molecular weight of about 80 kDa, is a highly unstable protein, and cellular levels of ODC depend on rates of synthesis and degradation.[19,21]

In mammalian cells, the degradation of ODC is facilitated by a specific ODC-antizyme,[22] a protein which also appears to down-regulate polyamine transport.[23] For this reason, competitive inhibitors of ODC have proven to be of limited value, since the synthesis of new protein occurs very rapidly. The catalytic mechanism of ODC involves formation of a Schiff base between the amino group of ornithine and the pyridoxal phosphate cofactor, which is tightly bound to ODC.[21] The most useful inhibitor of ODC to date, α-difluoromethylornithine (DFMO), takes advantage of this aspect of the mechanism.[3] Formation of a Schiff base between DFMO and ODC results in the generation of a latent electrophile, and ODC is rapidly and irreversibly deactivated. The discovery of DFMO has provided an enormous stimulus to the field of mammalian polyamine biology. In addition, DFMO has been marketed as a treatment for *Pneumocystis carinii* secondary infections in AIDS/ARC patients, and has been shown to be somewhat effective in curing infections of *Trypanosoma brucei rhodesiense* in limited clinical trials.[24,25]

The committed intermediate putrescine is next converted to spermidine (**2**) via an aminopropyltransferase known as spermidine synthase. A second closely related but distinct aminopropyltransferase, spermine synthase, then adds an additional aminopropyl group to spermidine (**2**) to yield spermine (**3**), the longest polyamine occurring in mammalian systems. The byproduct for the spermidine and spermine synthase reactions is 5′-methylthioadenosine (MTA), generated from the cosubstrate, AdoMet-DC. MTA is a potent product inhibitor for the aminopropyltransfer process; in mammalian systems, MTA is rapidly hydrolyzed by the enzyme MTA phosphorylase, and the components are converted to adenosine and methionine via salvage pathways. Selective inhibition of the individual aminopropyltransferases has proven to be a significant problem, due to the similarity of the reactions catalyzed by the two enzymes. Mammalian spermidine synthase and spermine synthase each consist of two subunits (M_r = 35 kDa each or 44 kDa each, respectively) and require no cofactors.[26] Both mammalian enzymes have been isolated and purified to homogeneity. A variety of MTA analogues have been shown to act as inhibitors for the aminopropyltransferases, but these agents generally cannot distinguish between the two enzymes. By far the most significant advance in the selective inhibition of the aminopropyltransferases is the development of *S*-adenosyl-1,8-diamino-3-thiooctane (AdoDATO)[15] and *S*-adenosyl-1,12-diamino-3-thio-9-azadodecane (AdoDATAD)[16] which are multisubstrate analogue inhibitors for spermidine synthase and spermine synthase, respectively. AdoDATO inhibits spermidine synthase with an IC_{50} of 50 nM while having no significant effect on spermine synthase. Conversely, AdoDATAD inhibits spermine synthase selectively (IC_{50} = 20 nM) while leaving spermidine synthase unaffected. Unfortunately, AdoDATAD appears to be rapidly metabolized in L1210 cells by polyamine oxidase (PAO), resulting in the formation of AdoDATO and other cytotoxic metabolites.[27]

The aminopropyl donor for both aminopropyltransferases is decarboxylated AdoMet (dc-AdoMet), produced from AdoMet by the action of AdoMet-DC. AdoMet-DC, like ODC, is a highly regulated enzyme in mammalian cells and also serves as a regulatory point in the pathway.[19,20] However, unlike ODC, AdoMet-DC belongs to a small class of proteins known as pyruvoyl enzymes.[28] All of the known forms of AdoMet-DC contain a covalently bound pyruvate prosthetic group that is required for activity, although the individual AdoMet-DC isozymes differ in their subunit structure and cation requirements. Because of this feature, AdoMet-DC is inhibited by $NaBH_4$[29] and $NaCNBH_3$.[30] The latter inhibits only in the presence of substrate, and during $NaCNBH_3$ reduction, the enzyme forms a covalently bound product, indicating that a Schiff base is formed during the enzymatic reaction. The *Escherichia coli* form of AdoMet-DC is first synthesized as a proenzyme, which is then cleaved posttranslationally into two smaller polypeptide subunits which are both components of the purified enzyme. The purified enzyme is now thought to be a tetramer consisting of 2α (M_r = 19 kDa) and 2β (M_r = 14 kDa) subunits. The smaller β subunit has a free amino terminus, while the amino terminus of the larger α subunit is blocked by the pyruvoyl group. AdoMet-DC from *E. coli* has been shown to require Mg^{2+} for activity.[31] Mammalian AdoMet-DC, which is activated by putrescine rather than Mg^{2+}, is also formed from a proenzyme (M_r = 38 kDa), which is cleaved and processed to the mature decarboxylase. The active form contains two pairs of subunits (M_r = 32 and 6 kDa), the larger of which contains the pyruvate prosthetic group.[32] The primary sequences and cDNA libraries for AdoMet-DC from a variety of sources have been established,[33] and a crystal structure for human AdoMet-DC has recently been reported.[34] The antileukemic agent methylglyoxal bis(guanylhydrazone) (MGBG, **4**) (Figure 3) is a potent competitive inhibitor of the putrescine-activated mammalian enzyme, with a K_i value of less than 1 μM.[4] However, MGBG is of limited use as a chemotherapeutic agent due to a wide variety of other effects on cells (induction of severe mitochondrial damage, interference with polyamine transport, etc.). Interestingly, one of the newest developments in the area of AdoMet-DC inhibitors has been the discovery

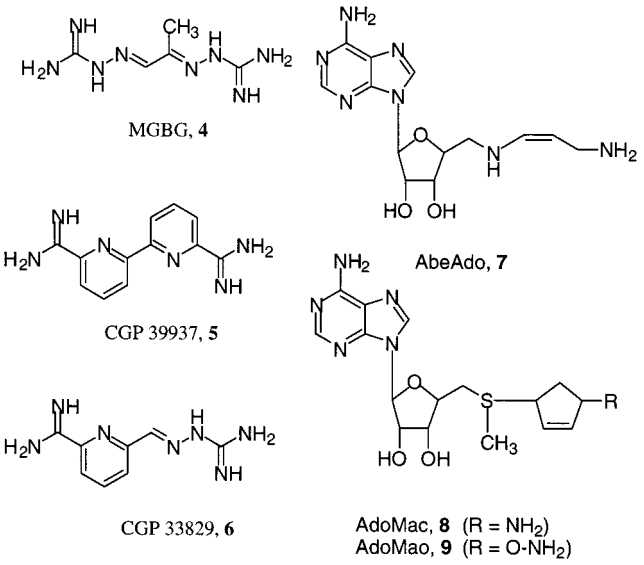


Figure 3. Reversible and irreversible inhibitors of AdoMet-DC.

of a promising new series of MGBG analogues such as the Ciba-Geigy compounds CGP 39937 (**5**) and CGP 33829 (**6**) (Figure 3).[35,36] All of the analogues showing significant activity are conformationally restricted derivatives of MGBG in which the backbone assumes a partially fixed, *all-trans* conformation. A number of structural analogues of AdoMet have also been developed as potential inhibitors of AdoMet-DC, in which a nucleophilic amine surrogate has been appended to the molecule.[6,8−11,13,14,37−39] The most promising of these, 5′-{[(*Z*)-4-amino-2-butenyl]methylamino}-5′-deoxyadenosine (AbeAdo, **7**) (Figure 3), is a potent enzyme activated inhibitor of AdoMet-DC from *E. coli*.[6] AbeAdo is also a potent inactivator of the rat liver form of AdoMet-DC ($K_i = 0.56\ \mu$M, turnover number = 1.5), and produces a long-lasting, dose-dependent decrease in AdoMet-DC activity in vivo.[40] The *E*-isomer of AbeAdo is 100 times more potent against rat liver AdoMet-DC and 1000 times more potent against *E. coli* AdoMet-DC than the corresponding *Z*-isomer. The conformationally restricted analogue AdoMac (**8**) (Figure 3) was synthesized in all four possible diastereomeric forms and used to probe the stereochemical requirements of AdoMet-DC from *E. coli*[8,9] and the human form of the enzyme.[11] AdoMac also acts as an enzyme-activated inactivator of AdoMet-DC. Interestingly, each form of the enzyme showed a clear preference for one of the four diastereomeric forms, and the preferred diastereomer was different for the two forms of the enzyme. A mixture of the diastereomeric forms of AdoMac inhibited growth in *T. brucei brucei* with an IC_{50} of 5.2 μM. A related analogue, AdoMao (**9**) (Figure 3), inhibited trypanosomal growth with an IC_{50} of 0.9 μM and is currently being evaluated in vivo.

In addition to the enzymes mentioned above, cellular polyamine content is modulated by a pair of acetyltransferases. Spermidine in the cell nucleus is acetylated on the four-carbon end by spermidine-N^8-acetyltransferase, possibly altering the compound's binding affinity for DNA.[41] A specific deacetylase can then reverse this enzymatic acetylation. Cytoplasmic spermidine and spermine serve as substrates for spermidine/spermine-N^1-acetyltransferase (SSAT), resulting in acetylation on the three-carbon end of each molecule (Figure 2).[42] The enzyme is the first and rate-limiting step in the catabolism and interconversion of the polyamines spermidine and spermine. SSAT is characterized by being highly substrate-specific and rapidly inducible and having a very short biological half-life. It has also been shown in several cellular and in vivo systems to be inducible by a variety of stimuli including growth factors, toxic insults, the natural polyamines, and polyamine analogues.[42] Acetylated spermidine or spermine resulting from catalysis by SSAT acts as a substrate for PAO, which catalyzes the formation of 3-acetamidopropionaldehyde and either putrescine or spermidine, respectively (Figure 2). Current information indicates the only fates available to acetylated polyamines are PAO-mediated oxidation or export from the cell. Thus SSAT and PAO together serve as a reverse route, facilitating the interconversion or export of cellular polyamines. It is important to note that the combination of the highly regulated catabolic enzyme SSAT, coupled with the finely controlled synthetic enzymes ODC and AdoMetDC, allows the cell considerable control of intracellular polyamine concentrations.

Under normal conditions, SSAT is present in very low levels in mammalian cells, but it can be induced manyfold by a variety of agents, a fact which led to its discovery in rat liver following treatment with carbon tetrachloride.[43] The structural requirements for interaction with the SSAT active site have not been fully investigated, but some structure−affinity data are available. The natural substrates for the enzyme, spermidine (**2**) and spermine (**3**) (Figure 1), have K_m values of 130 and 34 μM, while norspermidine (1,7-diamino-4-azaheptane) and norspermine (1,11-diamino-4,8-diazaundecane) exhibit K_m values of 9 and 10 μM, respectively.[44] N^1-Acetylspermine is also a substrate ($K_m = 51\ \mu$M), while N^1-acetylspermidine is not, since it does not possess the requisite free aminopropyl moiety. 1,3-Diaminopropane acts as a substrate with a K_m of 460 μM, while putrescine, 1,5-diaminopentane (cadaverine), homospermidine (1,9-diamino-5-azanonane), and histones do not.[43,44] A series of α-methyl[45] and α-*gem*-dimethyl[46] substituted polyamines have been synthesized, but in neither case acted as substrates for SSAT. These observations underscore the fact that a molecule must possess a terminal aminopropyl moiety in order to act as a substrate for SSAT. The reaction catalyzed by SSAT has been characterized as an ordered bimolecular reaction in which spermidine or spermine binds before acetylCoA and the corresponding N^1-acetylpolyamine is the final product to be released.[44] The reaction likely proceeds through a binary complex between the polyamine and acetyl CoA, since it is strongly inhibited by polyamine/acetyl CoA-based multisubstrate adduct inhibitors.[47] The potency of these inhibitors, which varies with the polyamine portion of the multisubstrate adduct, closely follows the substrate affinities mentioned above and thus distinguishes SSAT from enzymes which are known to acetylate histones.[48] The cloning and subsequent study of human SSAT[12,49−52] have catalyzed a rapid increase in the information regarding the regulation of SSAT and its role in the response to various polyamine analogues (see below). More will be said about the efforts to develop agents that specifically inhibit or modulate the activity of SSAT in a later section of this Perspective.

Polyamine Cellular Transport

A final method of controlling intracellular polyamine levels is afforded by one or more specific polyamine transport mechanisms.[53−57] To date, the polyamine transport system in *E. coli* has been the most completely studied, resulting in the isolation of a transporter gene and a series of protein gene products designated PotA−PotF. The PotA protein is a Mg^{2+}- and SH-dependent ATPase which binds the adenine portion of ATP at a domain close to the NH terminus. The PotB and PotC proteins are channel-forming membrane constituents responsible for polyamine transport and also stabilize the PotA protein within the membrane.[58] The PotD protein product is a periplasmic substrate-binding protein which has a higher affinity for spermidine than putrescine.[59] High levels (8 mM) of spermidine appear to produce uptake inhibition of the PotABCD transport system by reducing the ATPase activity of the PotA

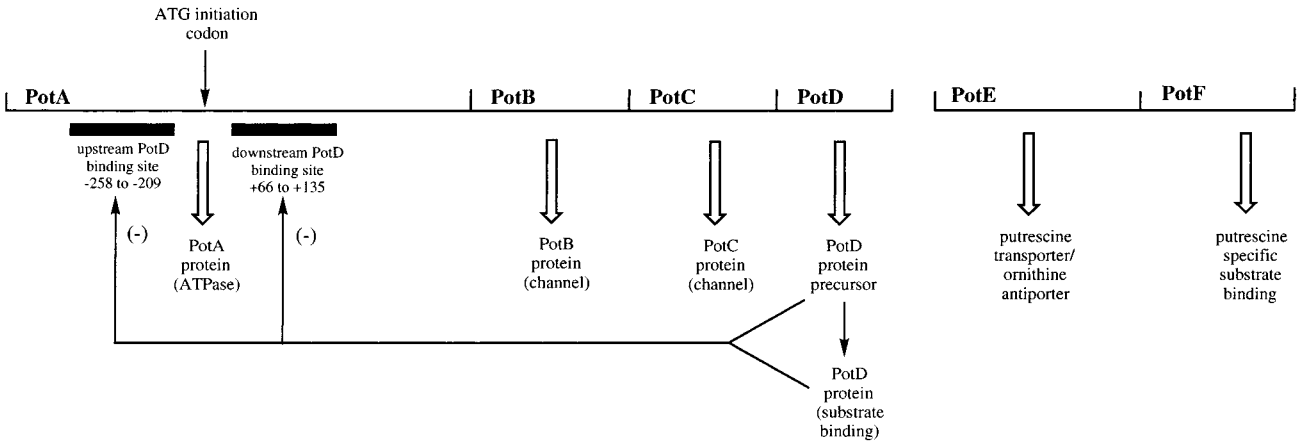


Figure 4. PotABCDEF operon of *E. coli* and control of the PotA−PotD proteins by transcriptional inhibition.

protein (Figure 4). This effect is mediated by the PotD protein, which in the presence of 8 mM spermidine binds to two specific sites upstream (−258 to −209 nucleotides) and downstream (+66 to +135 nucleotides) of the ATG initiation codon of the *PotA* gene.[60] At this concentration of spermidine, transcription of the *PotA* gene was reduced by 50% and the expression of PotABCD mRNA was inhibited. The precursor protein to PotD was also found to inhibit transcription of the PotABCD operon, and in vivo, this protein appears to be the major mediator of the observed transcriptional inhibition. The X-ray structure of PotD complexed to spermidine has been determined,[61] and binding of spermidine is mediated by specific amino acid residues, as well as a bound water molecule. The structure and function of the PotE protein has also been studied; uptake of putrescine by PotE is dependent on membrane potential. PotE also functions as a putrescine/ornithine antiporter, but in this case it is not dependent on membrane potential.[62] The PotF protein is a periplasmic putrescine binding protein which has low affinity for spermidine. The crystal structure of PotF bound to putrescine has also been determined.[63] The structure of PotF is similar to that of PotD (35% sequence homology) in that substrate is bound to specific amino acid residues, and a bound water increases substrate affinity through hydrogen bonding. A preliminary SAR study was recently conducted, demonstrating that certain acylated polyamines were capable of inhibiting the transport of polyamines in *E. coli*.[64]

A polyamine transport gene (*YLL028w*) has recently been identified in the yeast strain YW5-1B which codes for a putative polyamine transport protein TPO1.[65] TPO1, a membrane protein containing 586 amino acids, has 12 putative transmembrane segments and 3 glutamic acid resides which may bind polyamines and which are located in positions similar to glutamates found in the PotE protein of *E. coli*. TPO1 appears to be a vacuolar polyamine exporter and is up-regulated by protein kinases, possibly by phosphorylation of serine or threonine in the amino terminal region. Recently, specific polyamine transporters have been detected in *Trypanosoma cruzi* epimastigotes,[66] *Crithidia fasiculata*,[67] and *Leishmania donovanii*.[68]

The process of polyamine transport in mammalian cells is poorly understood, and to date, none of the proteins involved have been isolated and sequenced. Numerous groups are working to elucidate the mechanism(s) of transport and the effects of regulation of transport, in normal and tumor cell lines. Although polyamine transport had been described kinetically,[69] it was not until 1995 that a cell surface polyamine-binding protein was detected by photoaffinity labeling with a norspermine-4-azidosalicylic acid conjugate.[70] Both the L1210 murine leukemia line and the U937 human leukemia line were found to express a glycosylated cell-surface polyamine-binding protein (p118) approximately 50 kDa in size. This finding was later confirmed using a more sophisticated photoprobe, which competes with spermidine for transport with a K_i of 1 μM.[71] In the L1210 line, as well as in the A549 human lung carcinoma line, proteins analogous to p118 were detected, as well as three other polyamine-binding proteins that are potentially involved in transport. Polyamine transport in Chinese hamster ovary (CHO) cells can be inactivated in a time- and concentration-dependent manner by the carbodiimide reagents 1,3-dicyclohexylcarbodiimide (DCC) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), suggesting the involvement of carboxylate moieties in the transport process.[72] CHO cells have also been shown to possess a specific diamine exporter,[73] and a similar exporter has been reported in *Xenopus* oocytes.[74] Recently, a polyamine transport-deficient cell line derived from the NCI H157 non-small-cell lung tumor line (non-SCLC) was isolated and characterized.[75] These cells developed a resistance to polyamine analogue-induced cytotoxicity which is not mediated by the multidrug-resistance (MDR) protein.

A number of factors have been shown to alter the polyamine transport system and, as a result, cellular homeostasis. As was mentioned above, ODC-antizyme has a regulatory effect on polyamine transport in mammalian cells. When FM3A murine cells transfected with the rat antizyme DNA were treated with DFMO, a known inducer of ODC-antizyme, it was determined that ODC-antizyme was a potent negative regulator of polyamine transport.[23] It was subsequently determined that regions 119−144 and 211−216 of the antizyme protein were necessary for down-regulation of polyamine transport.[76] In ZR-75-1 human breast cancer cells, spermidine transport is strongly induced by insulin, estradiol, interleukin-4, and interleukin-3[77] and can also be altered by plasma membrane potential.[78] In the transformed kidney proximal tubule cell line MCT,

putrescine uptake was shown to be down-regulated by the administration of nitric oxide donors and by the cytokines TNF-α and IFN-γ.[79] In CHO cells, there appears to be a functional link between polyamine transport and the P-glycoprotein efflux transporter MDR protein.[80] A functioning polyamine transport system may be a requirement for MDR activity, while the expression of functioning P-glycoprotein appears to reduce polyamine transporter activity. In human prostate cancer cell lines, polyamine transport was uniquely insensitive to regulation by polyamines and polyamine analogues.[81] Cell polyamine efflux by the putrescine exporter was enhanced in putrescine-tolerant cells,[82] resulting in the induction of apoptosis.[83]

The identification of polyamine transport systems in mammalian enterocytes began with the recent identification of three specific polyamine binding sites on rabbit intestinal brush border membranes.[84] A similar transport system in rat enterocytes was found to be a Na^+ gradient-dependent polyamine antiporter.[85] This antiporter is saturable and specific for straight-chain polyamines with four or more nitrogens, and the efflux of [^{3}H]spermine could be inhibited by spermine, tetraethylenepentamine, and trientine, a polyamine-like drug used for Wilson's disease. Interestingly, rabbit enterocytes also possess a polyamine transport system on the basolateral surface.[86] This transporter acts as a polyamine importer, does not require a sodium gradient, and presumably provides polyamines from the circulation to assist in rapid enterocyte cell division.

Efforts to synthesize and identify specific polyamine transport inhibitors have recently begun in several laboratories. The structural characteristics of known transport inhibitors (i.e. compounds which deter or compete with [^{3}H]spermine for transport) have been analyzed by QSAR[87] and by comparative molecular field analysis (COMFA),[88] producing a preliminary theoretical model with useful predictive capability. A small number of synthetic polyamine transport inhibitors have recently been described. A polymeric conjugate of spermine was shown to inhibit polyamine transport in MES-SA uterine sarcoma and K562 leukemia cells, as well as in their MDR-positive variants, at a concentration of 20 μM,[89] resulting in cytotoxicity and indicating that interruption of transport is a viable strategy for designing antitumor compounds. The dimeric analogue 2,2′-dithiobis(*N*-ethylspermine-5-carboxamide) (DESC) acts as a potent competitive inhibitor of polyamine transport, with a K_i value of 5 μM in ZR-75-1 human breast cancer cells.[90] Subsequently, a series of related spermidine and norspermidine dimers were described[91] which exhibited enhanced stability and potency with respect to DESC. The polyamine analogue 1,12-diaziridinyl-4,9-diazadodecane (BIS) has been shown to be both an inhibitor and a substrate for the polyamine transporter.[92] BIS appears to produce cytotoxicity by induction of apoptosis in PC-3 and DU-145 androgen-independent prostate cancer cells and also enhances the radiosensitivity of these cells in vitro. Finally, a series of α-methylpolyamines have been described[45] which are taken up by the polyamine transport system in mammalian cells but are not substrates for SSAT or PAO.[93] These analogues have been shown to be valuable as model substrates for studying polyamine transport and the mechanisms of polyamine-mediated cytotoxicity.

Symmetrical, Terminally Alkylated Polyamine Analogues

In the mid-1980s, a few research groups began to describe polyamine analogues as potential antitumor agents. Initially, these analogues were structurally similar to the natural polyamines, in that they had terminal primary amine groups, with variations in the length of the intermediate carbon chains. Edwards and co-workers synthesized a series of di- and triamines related to spermidine and a series of tetraamines derived from 1,8-diaminooctane, and these analogues were evaluated for antitumor activity in an L1210 cultured cell system.[94] In the series of di- and triamines, substitution of alkyl groups at the terminal nitrogens, or replacing the central nitrogens with other heteroatoms, failed to produce spermidine analogues with antitumor effects superior to that of norspermidine. However, tetramines with the general structure **10**, shown in Figure 5, generally showed improved antitumor activity. The most active compound in the series (R = H) increased survival time in male mice inoculated with L1210 leukemia from 7.7 to 16.2 days. Coadministration of spermidine was shown to reverse the antitumor activity of this compound, presumably due to a competition for the polyamine transport system. In addition, coadministration of a PAO inhibitor potentiated the observed antitumor activity, suggesting that these analogues may be metabolized by PAO. Analogues of general structure **10** in which R = CH_3 or R = CH_2-CH_3 also showed activity in the L1210 model, but substitution of larger alkyl groups resulted in a reduction of activity. Polyamine levels did not appear to be dramatically reduced following treatment with these tetraamine analogues, and the ability of the compounds to interact with SSAT was never established. In a related study, these tetraamines, a series of bis(benzyl) analogues related to MDL 27695 (**11**), and an additional series of substituted tetraamines were evaluated for the ability to inhibit proliferation of HeLa cells.[95] The ability of each compound to displace ethidium bromide from calf thymus DNA was also determined, although no correlation between the DNA binding properties and antitumor activity was detected. Not surprisingly, the bis(benzyl)polyamine analogue MDL 27695 (**11**) and the tetraamine **10** (R = H) were active antiproliferative compounds, exhibiting IC_{50} values of 5 and 50 μM, respectively.

Subsequent attempts to develop polyamine analogues as potential modulators of polyamine function focused on the synthesis of symmetrical, terminally substituted bis(alkyl)polyamines. Design of these analogues was based on findings demonstrating that natural polyamines utilize several feed-back mechanisms which autoregulate their synthesis[96−98] and that they can be taken into cells by the energy-dependent transport systems described above.[96] To date, a number of symmetrically substituted polyamine analogues have been synthesized which enter the cell using the polyamine transport system. Many of these analogues specifically down-regulate the synthesis of polyamines but cannot substitute for the natural polyamines in their cell growth

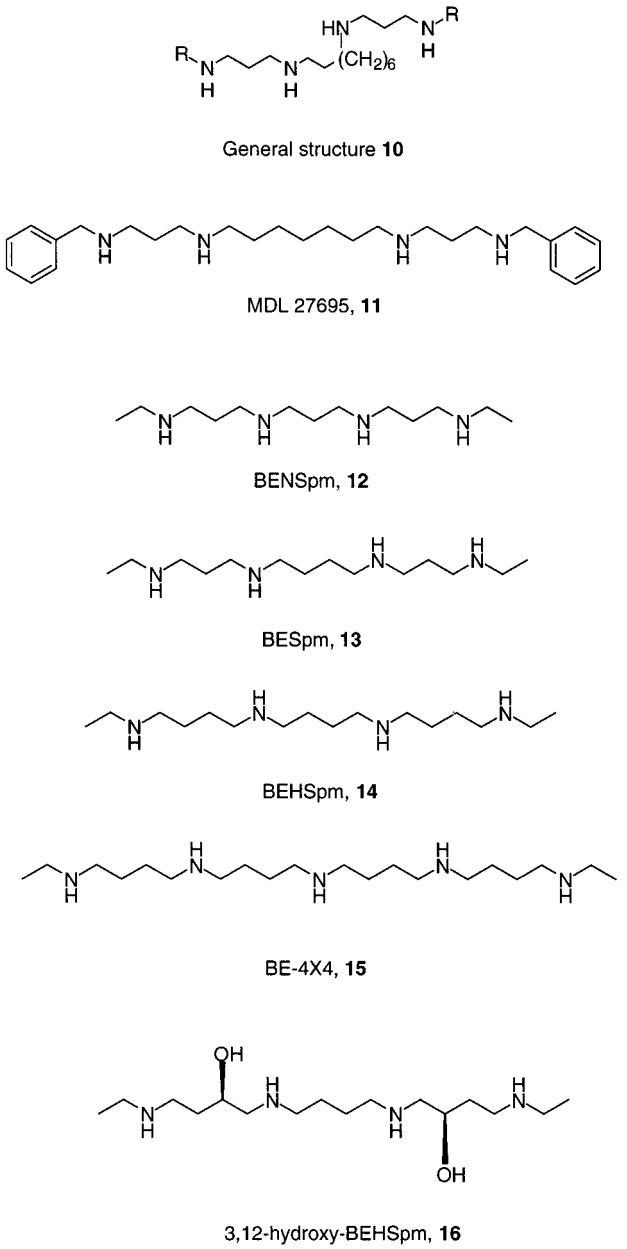


Figure 5. Structures of biologically active bis(alkyl)- and bis(aralkyl)polyamine analogues.

and survival functions.[96,99−101] The most successful of the symmetrically substituted polyamines analogues to date are the *N*,*N*′-bis(ethyl)polyamines (Figure 5): bis(ethyl)norspermine (BENSpm, **12**), bis(ethyl)spermine (BESpm, **13**), bis(ethyl)homospermine (BEHSpm, **14**), and 1,20-(ethylamino)-5,10,15-triazanonadecane (BE-4X4, **15**). These compounds have been shown to possess a wide variety of therapeutic effects and illustrate the fact that small structural changes in alkylpolyamine analogues can result in surprisingly significant changes in biological activity. It has recently been shown that conformational restriction of the central carbon chain of alkylpolyamines[102] or insertion of a central dimethylsilane group[103] results in a significant decrease in growth inhibition when compared to the bis(ethyl)-polyamine analogues.

The synthesis of symmetrically substituted bis(alkyl)-polyamines is straightforward and depends on the availability of the appropriate parent polyamine[101,104,105] to afford the desired target compounds with structures similar to general structure **10** (R = alkyl), following recrystallization from ethanol/water, as the corresponding tetrahydrobromide salts.

The *N*,*N*′-bis(ethyl)polyamines are readily transported into mammalian cells, apparently by the same transport mechanism as the natural polyamines.[98] Treatment of most mammalian cell types with these analogues leads to a reduction of all three natural polyamines (Spd, Spm, Put), decreases in ODC activity and AdoMetDC activity, and ultimately cytostasis or cytotoxicity, depending on the cell lines utilized.[98,101,106] In addition to these effects, the observed cytotoxic response is accompanied by a tremendous induction of SSAT activity, in some cases as much as 1000-fold. Preliminary SAR correlations, based only on data from the symmetrically alkylated polyamine analogues, suggested that monoalkylation at both terminal nitrogens of spermidine or spermine was important for optimal antiproliferative activity and that alkylation at an internal nitrogen reduced in vitro activity.[101] It was further determined that the greatest induction of SSAT was dependent on the presence of "protected" aminopropyl or aminobutyl moieties.[17,107−109] Terminal nitrogen bis(alkyl) substituents larger than ethyl led to analogues exhibiting a dramatic reduction in antitumor activity.[101,106,110] These studies also suggested that compounds with a 3−3−3 carbon skeleton were more effective that the corresponding 3−4−3 analogues and that spermine-like compounds are more effective that spermidine-like analogues. Because these data were collected using only symmetrically substituted bis(alkyl)polyamines, the alkylpolyamines currently undergoing clinical trials are all bis(ethyl)-substituted analogues. Among the most effective compounds to date are BENSpm (**12**), which has three aminopropyl moieties, and BE-4X4 (**15**), which has four aminobutyl moieties. The SAR data described above were used as the rationale for the synthesis of additional series of compounds, as described below.

Perhaps the most successful alkylpolyamine to date is the analogue BENSpm, **12**. This analogue has shown exceptional promise as an antitumor agent, in both in vitro and in vivo studies. Early studies indicated that BENSpm was an effective antitumor agent in cultured human pancreatic adenocarcinoma cells[111] and xenografts,[112] human MALME-3 melanoma xenografts,[113,114] melanocytes,[115] human bladder cancer cells,[116] and ovarian carcinoma tumor cells.[117] Growth inhibition in $CaCO_2$ colon cancer cells is associated with a reduction in the protooncogene *c-MYC*, but not with a reduction of *c-MYC* mRNA.[118] In these cells, the analogue causes induction of SSAT, downregulation of ODC, and depletion of cellular polyamines, resulting in cytotoxicity. In the NCI H209 SCLC lung tumor line, which expresses the *v-Ha-ras* oncogene and overexpresses *c-MYC*, sensitivity to BENSpm is retained.[119] SSAT induction appears to be the common event leading to cytotoxicity in non-SCLC tumor explants.[120] SSAT was detected by immunochemical staining in the tumor tissue, but not in the surrounding normal lung tissue. Thus, immunochemical staining of SSAT in treated tissue may prove to be a prognostic indicator of drug response. Presumably because of the proprietary nature of data concerning BENSpm, no studies concerning its

therapeutic use have been published. BENSpm is currently undergoing phase II clinical trials for use against several human solid tumors.

The closely related analogue BEHSpm (**14**) does not show promise as an antitumor agent but is being developed as an effective treatment for AIDS-related diarrhea (ARD).[121,122] The effects of BEHSpm on ARD were examined because polyamines are known to reduce gastrointestinal motility.[123] The potent antidiarrheal activity of BEHSpm has been demonstrated in several animal models and in human clinical trials involving patients with ARD. Interestingly, the drug is also effective at lowering blood pressure without a concomitant increase in heart rate.[124] This effect can be abrogated by arginine and NG-nitro-L-arginine methyl ester (L-NAME), suggesting the involvement of the nitric oxide pathway.

The pharmacokinetics of BENSpm (**12**)[125] and BEHSpm (**14**)[126] in vivo metabolism have been described. BENSpm is metabolized by N-deethylation and stepwise removal of aminopropyl equivalents, with a half-life of 73 min. By contrast, BEHSpm was metabolized almost exclusively to homospermine, which was found to persist in tissues for a period of weeks (liver $t_{1/2} = 15.4$ days). Because chronic administration of BEHSpm could result in tissue accumulation of the analogue, resulting in disruption of normal polyamine metabolism, a strategy was devised to enhance the clearance of the analogue in vivo.[122] Molecular modeling studies suggested that the 3,12-dihydroxy derivative of BEHSpm would have similar effects as the parent analogue, and this compound (3,12-dihydroxy-BEHSpm, **16**) was synthesized as the 3*R*,12*R* isomer. This so-called "metabolically programmed" alkylpolyamine retained the antidiarrheal activity of the parent BEHSpm but exhibited a significantly diminished tissue half-life, presumably due to the metabolic "handles" provided by the hydroxyl groups.

The alkylpolyamine BE-4X4 (**15**)[127] was originally designed based on the hypothesis that analogues with chain lengths different from spermine could exhibit enhanced binding to DNA and thus exert antiproliferative effects. This hypothesis was formed using molecular modeling techniques, as well as cell culture experiments.[128] As was the case with BENSpm, the proprietary nature of the research surrounding the compound has limited the publication of data. To date, BE-4X4 has been shown to be effective in cultured U-251, MG, SF-126, and SF-188 brain tumor cells at a concentration of 5 μM.[127,128] Recent studies have demonstrated that the analogue is effective against DU-145, LNCaP, and PC-3 prostate cancer cells in vitro and reduced tumor cell growth in a DU-145 nude mouse xenograft model.[129]

One unusual characteristic of the bis(ethyl)polyamines is their ability to produce cell-type-specific cytotoxicity in two representative lung cancer cell types: the NCI H157 non-SCLC and the H82 SCLC lines. Soon after the first alkylpolkyamines were described, it was shown that DFMO-resistant non-SCLC responded in a rapid cytotoxic manner to treatment with the bis(ethyl)-polyamines.[17,109,130] These results are particularly interesting because the H157 tumor type is clinically characterized as being refractory to all treatment modalities. As will be detailed below, although the analogues are cytotoxic to the large cell phenotype and quite active against other non-SCLC lung tumors, they are relatively ineffective against DFMO-sensitive SCLC lines.[17,109] This effect was first observed following treatment of H157 and H82 cells in culture with 10 μM BESpm (**13**) (Table 1). The mechanisms underlying the observed differential sensitivities are still being elucidated; however, one consistent finding was the unusually high induction of SSAT (in some cases >1000-fold) in cell types which respond to bis(ethyl)polyamine analogues and a lack of SSAT induction in the refractory SCLC line H82.[17,108−109,131] In non-SCLC, the induction of SSAT correlated with a time- and dose-dependent increase in SSAT steady-state mRNA levels, suggesting a transcriptional level of control over SSAT synthesis.[109] The correlation between high induction of the SSAT activity and cytotoxicity was subsequently demonstrated for other examples of human malignancies including human melanomas.[111−113] However, definitive proof of a direct functional correlation was lacking.

Table 1. Effect of 10 μM BESpm (**13**) on NCI H157 (non-SCLC) and NCI H82 (SCLC) Cell Lines[131]

	polyamines (nmol/mg protein)				
treatment	PUT	SPD	SPM	SSAT[a]	cytotoxicity
H157	0.82	4.45	8.60	40 ± 3.4	
H157 + 10 μM **13**	ND[b]	ND	ND	6511 ± 644	yes
H82	3.82	13.74	17.51	24 ± 0.7	
H82 + 10 mM **13**	ND	3.07	10.06	60 ± 1.8	no

[a] SSAT is expressed as nmol product formed/mg protein in 24 h. [b] ND, not detectable (below 0.05 nmol/mg protein).

Unsymmetrically Substituted Alkylpolyamine Analogues

Although the symmetrical bis(alkyl)polyamines typified by BENSpm (**12**) and BESpm (**13**) had been synthesized and evaluated for antitumor activity, no efficient synthesis leading to unsymmetrically substituted polyamines was described prior to 1993. In general, the synthesis of these analogues is more difficult, since it requires selective protection and deprotection of the internal and external nitrogens. This has been accomplished by assembling the compounds in aminopropyl or aminobutyl subunits. Saab et al.[132] were the first to describe a synthesis for unsymmetrically substituted alkylpolyamines. It is noteworthy that a nearly identical synthetic route to unsymmetrically substituted alkylpolyamines was published independently by another group in 1994.[110] The reader is directed to the references for these synthetic routes and associated nitrogen-protecting strategies, which can be used to produce selectively functionalized mono- and bis(alkyl)-substituted alkylpolyamines.[94,95,99−101,103−106,110,122,132−137]

The first unsymmetrically substituted polyamine analogues to be evaluated were N^1-propargyl-N^{11}-ethylnorspermine (PENSpm, **17**) and N^1-cyclopropylmethyl-N^{11}-ethylnorspermine (CPENSpm, **18**), shown in Figure 6.[132] Preliminary results indicated that both **17** and **18** were as active or more active than BESpm (**13**), with respect to both SSAT induction and cytotoxicity, in H157 non-SCLC cells in culture. The IC_{50} values for **17** and **18** were and 1.1 and 0.8 μM, as determined from their 96 h dose response curves.[132] These analogues also retained the cell-type specific cytotoxic activity typical of **12** and **13**, wherein the non-SCLC cell line

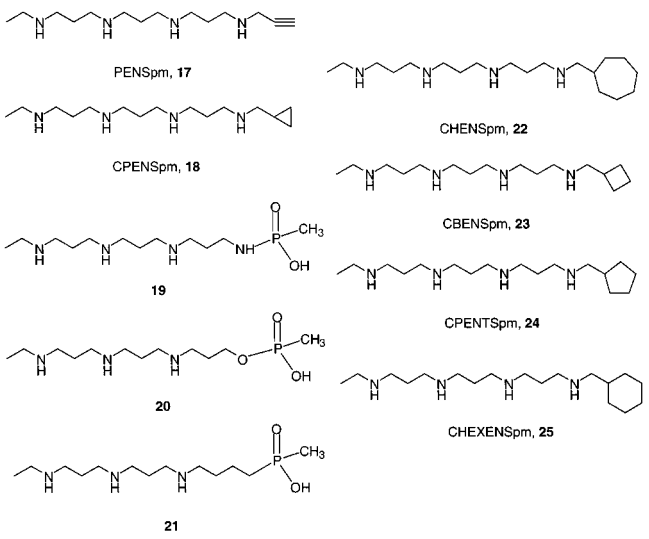


Figure 6. Structures for unsymmetrically substituted alkylpolyamine analogues **10**−**16**.

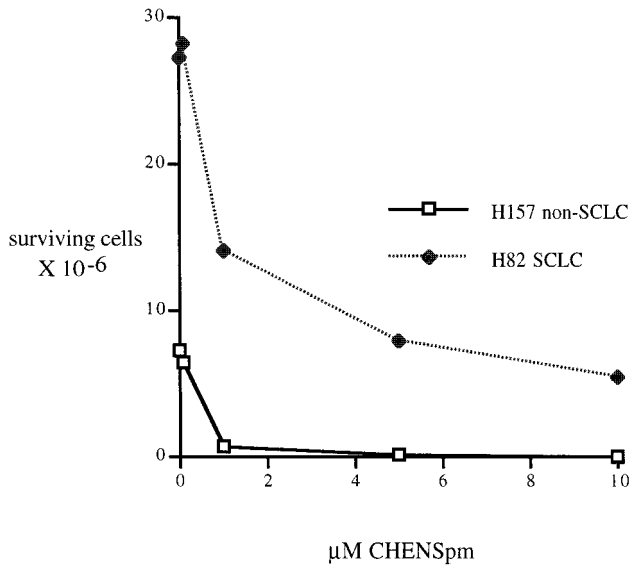


Figure 7. Effect of CHENSpm (**22**) on H157 non-SCLC and H82 SCLC lung tumor cells following 96-h treatment.

was rapidly killed, while the SCLC cell line responded in a cytostatic manner. As was observed in the case of **13**, analogue cytotoxicity was directly correlated to its ability to induce SSAT. This increase in SSAT activity was accompanied by a cell-specific increase in steady-state SSAT mRNA which was similar in magnitude to that observed after treatment of the H157 cells with BESpm or BENSpm. These data suggest a similar mechanism of induction of SSAT for these compounds.

The SSAT inhibitory activity of **17** and **18** was also examined, using crude extracts of SSAT from superinduced H157 cells. At saturating concentrations of spermidine, **17** and **18** were capable of inhibiting SSAT by 50% at concentrations of ∼40 and ∼100 μM, respectively. These concentrations are similar to the concentration of **13** required to achieve the same level of inhibition. However, the inhibition of SSAT in intact, sensitive non-SCLC cells is of no biochemical significance, since SSAT is superinduced to levels where the intracellular concentration of the analogue is low with respect to the concentration of enzyme. Thus, although alkylpolyamine analogues are effective SSAT inhibitors in cell lysates, they cannot be used to examine the effects of specific inhibition of SSAT in responsive, intact cells.

To produce a specific, noninducing inhibitor for SSAT, the synthesis and evaluation of the phosphonamidate and phosphinate SSAT transition-state analogue inhibitors **19** and **20** were undertaken.[137−139] The phosphonamidate analogue **19** proved to be too labile under the conditions of the SSAT assay to act as an inhibitor and prompted the design and synthesis of the more stable phosphinate **20**. Phosphinate **20** proved to be an effective inhibitor of purified human SSAT, exhibiting a K_i value of 250 μM. However, this analogue did not appear to be transported into mammalian cells and in fact acted as an inhibitor of polyamine uptake. The phosphinate **20** competitively reduced the uptake of bis(ethyl)-spermine into NCI H157 cells by 45% in vitro, as determined by HPLC analysis. Recently, the synthesis of a related analogue, the polyamine phosphonate **21**, has been completed. This analogue acts as a more potent inhibitor of human SSAT, exhibiting a K_i of 50 μM. The studies involving the activity of **37** are not complete, but preliminary data suggests that the analogue is also poorly transported into NCI H157 cells in culture. This poor transport may be due to the fact that **37** is negatively charged at physiological pH and is therefore a poor substrate for the polyamine transport system. Studies aimed at producing transition-state analogue inhibitors of SSAT that are transported into mammalian cells are ongoing.

Data gathered from the unsymmetrically substituted alkylpolyamines **17** and **18** supported the hypothesis that there may be a functional relationship between cytotoxicity and SSAT induction in the non-SCLC and SCLC cell lines. A third compound in this series, N^1-cycloheptylmethyl-N^{11}-ethylnorspermine (CHENSpm, **22**) (Figure 6), was subsequently synthesized and evaluated and was found to retain the activity (IC_{50} = 0.25 μM against non-SCLC cells) seen with **13** and **18** while producing a less-pronounced cell type specificity, as shown in Figure 7. However, when the induction of SSAT and polyamine levels in the sensitive non-SCLC line were measured, the analogue showed striking differences from the parent analogue **12** (Table 2). Treatment of H152 cells with 10 μM **12** depleted the natural polyamines to undetectable levels and produced a 2849-fold increase in SSAT activity. Under these conditions, the only polyamine that was present in the cell in significant amounts was the analogue itself. Surprisingly, treatment with 10 μM **22** had almost no effect on the levels of putrescine, spermidine, and spermine and caused only a 15-fold induction of SSAT activity. These data suggested that the cytotoxic effects produced by **12**, **13**, and **18** in H157 cells could be mediated by different cellular mechanisms than the effects produced by **22**.

Because the structures of **18** and **22** differ only in the size of the cycloalkylmethyl substituent, three additional compounds were synthesized which possessed substituents containing the intervening ring sizes, as shown in Figure 6. The analogues, N^1-cyclobutylmethyl-N^{11}-ethylnorspermine (CBENSpm, **23**), N^1-cyclopentylmethyl-N^{11}-ethylnorspermine (CPENTSpm, **24**), and N^1-

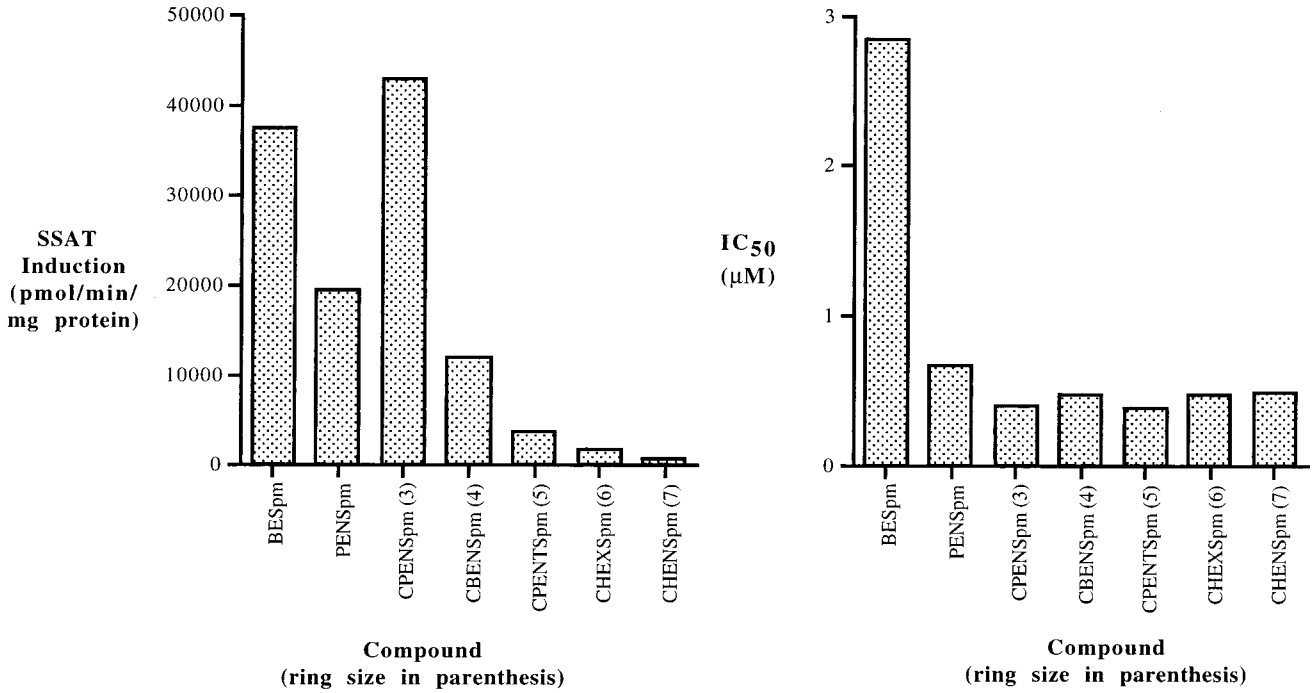


Figure 8. SSAT induction and IC_{50} data for selected alkylpolyamine analogues.

Table 2. Polyamine Levels and SSAT Induction in NCI H157 non-SCLC Tumor Cells following 24-h Treatment with BENSpm (**12**) and CHENSpm (**22**)

treatment	PUT	SPD	SPM	**12**	**22**	SSAT activity[a]	-fold increase
control	2.00	9.83	14.92	0.00	0.00	52	
10 μM **12**	0.00	0.00	0.51	36.07	0.00	148141	2849
10 μM **22**	4.28	6.44	15.97	0.00	14.66	781	15.01

[a] SSAT activity is expressed as pmol product/mg protein/min.

cyclohexylmethyl-N^{11}-ethylnorspermine (CHEXENSpm, **25**) were synthesized and evaluated for antitumor activity and for their ability to induce SSAT in the H157 and H82 cells. The cell type specificities of **23**−**25** were not as pronounced as that seen with **18** and **22**, in that all three analogues were generally cytotoxic in both cell lines. However, there was no correlation between the induction of SSAT and the IC_{50} value, as shown in Figure 8. Compound **17** produced about 50% of the SSAT induction caused by **13**, while **18** produced significantly more induction than **13**. In the cycloalkyl series, the induction of SSAT decreased dramatically as a function of ring size, while the IC_{50} values were remarkably constant (0.4−0.7 μM). These data clearly support the contention that there are at least two mechanisms by which unsymmetrically substituted alkylpolyamines produce cytotoxicity in H157 non-SCLC cells. To date, more than 50 related alkylpolyamine analogues have been produced. The structures for some of these analogues (**26**−**36**) appear in Figure 9, and a summary of their cellular effects in SCLC and non-SCLC cells appears in Table 3.

As was mentioned above, SAR correlations for symmetrically substituted alkylpolyamine analogues suggested that terminal nitrogen substituents larger than ethyl led to analogues exhibiting a dramatic reduction in antitumor activity.[101,106,110] These studies also suggested that compounds with a 3−3−3 carbon skeleton were more effective that the corresponding 3−4−3

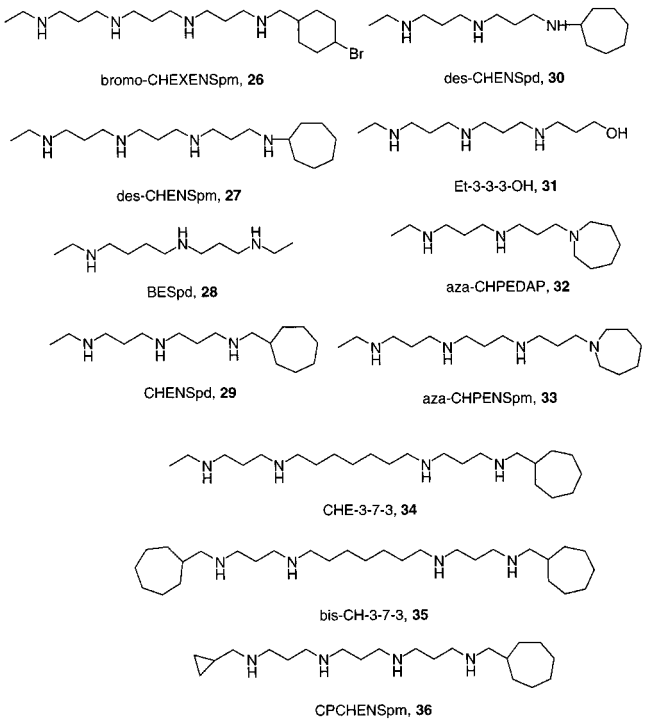


Figure 9. Structures for unsymmetrically substituted alkylpolyamine analogues **26**−**36**.

analogues and that spermine-like compounds are more effective that spermidine-like analogues. On the basis of data from the unsymmetrically substituted analogues, this does not appear to be entirely the case. These data indicate that only one of the two terminal nitrogens of the norspermine backbone needs be substituted with a small alkyl group for significant antitumor activity. It is likely that compounds such as CPENSpm (**18**) and CHENSpm (**22**), which differ only in the size of their ring substituents, inhibit cell growth by different mechanisms or that the induction of SSAT is not the primary

Table 3. Effects of Polyamine Analogues on NCI H157 and NCI H182 Tumor Cells in Vitro

	cytokinetic response[a]		effects on enzymes[b]				
			induct	inhib		effect on	
compd	H157	H82	SSAT	ODC	AdoMet-DC	polyamine pools[c]	analogue[d]
12	T	S	H	Y	Y	↓	H
13	T	S	H	Y	Y	↓	H
17	T	S	H	N		↓	ND
18	T	S	H	Y		↓	H
22	T	S/T	N	N	N	↓	H
23	T	T	H	Y		↓	H
24	T	T	H	Y	Y	↓	H
25	T	T	L	N	N	↓	H
26	T	S/T	L	N	N	↓	H
27	T	S/T	H			↓	H
28	T	S	M	Y	N	↓	H
29	T	S	H			↓	H
30	S	N	M	N		↓	H
31	S	N	H	N	N	↓	H
32	S	N	L			NC	ND
33	T	S/T	H			↓	ND
34	S	S	N	N	N	NC	L
35	T	T	N			NC	ND

[a] Cytokinetic effects on H157 non-SCLC and H82 SCLC after 96-h treatment: T, cytotoxic at concentrations <10 μM; S, cytostatic at ≤10 μM; S/T, cytostatic ≤5 μM and cytotoxic >5 μM; N, no effect. [b] Effect of 10 μM treatment of H157 non-SCLC cells with indicated analogue for 24 h on the various rate-limiting steps in polyamine metabolism induction of SSAT: H, >500-fold induction; M, >50-fold induction; L, <50-fold induction; N, no induction. Inhibition of ODC and AdoMet-DC: Y, >50% by 24 h; N, <50% inhibition by 24 h. [c] Effects of exposure of H157 non-SCLC to indicated analogue on natural polyamines and accumulation of analogue. For polyamines: ↓, decrease or NC, no significant change in all three polyamines by 96-h treatment with <10 μM analogue. [d] For indicated analogue: H, >20 nmol/mg protein within 24 h treatment; L, <20 nmol/mg protein with 24 h; ND, unable to detect by HPLC.

cellular mechanism involved in the cytotoxicity of these compounds. However, current trends continue to suggest that compounds that significantly induce SSAT tend to be more cell-type-specific in their activity than those which do not. These data further suggest that spermidine analogues such as CHENSpd (**29**) and desCHENSpd (**30**) were not as potent as the corresponding spermine analogues, consistent with previously published reports, although the potency of these analogues was comparable to that of the parent compound **13**. Nearly all of the compounds examined elicited a significant cell-type-specific response with regard to the H157 and H82 lung tumor lines, and none were found which induced SSAT in the H82 line. The best inducers of SSAT in the H157 cell line were those analogues that were most similar sterically to **12** and **13**. Interestingly, the analogues CHE-3-7-3 (**34**) and bis-CH-3-7-3 (**35**), both of which possess a seven-carbon central region, did not cause a typical cell-type-specific response. However, these agents possess significant antiparasitic activity, as will be discussed below.

It is noteworthy that small structural changes can have significant effects on both SSAT induction and the cytokinetic response. This point is best illustrated by the cycloheptyl-substituted norspermidine and norspermine analogue pairs, CHENSpd (**29**)/des-CHENSpd (**30**) and CHENSpm (**22**)/des-CHENSpm (**27**), shown in Figure 9. The des-compounds differ from their counterparts by a single methylene spacer group between the terminal nitrogen and the cycloheptyl ring. Both of the spermine analogues produce a dose- and time-dependent response with respect to growth and survival in the non-SCLC line H157 (unpublished observations). At concentrations greater than 0.1 μM both **22** and **27** were found to be cytotoxic after 96-h treatment times. At concentrations of 10 μM (24-h exposure), each of the analogues accumulated to a significant extent in NCI H157 cells and significant cell death was observed. Although the spermidine analogues **29** and **30** demonstrated dose-dependent growth inhibition, there was no evidence of net cell loss, suggesting that these compounds were cytostatic rather than cytotoxic. Interestingly, **29** (a spermidine-like analogue) and **27** (a spermine-like analogue) produced the largest induction of SSAT observed among the cycloheptyl-substituted analogues. Each induced SSAT from ~20 to >14 000 pmol/mg protein/min. Consistent with their high induction of SSAT, they also produced the greatest depletion of the natural polyamines. Only **29** (5-fold) and **27** (2-fold) induced SSAT mRNA to a measurable extent. These results suggest that posttranscriptional regulation plays a significant role in the expression of SSAT in response to these agents.

In addition to the lung cancer model, unsymmetrically substituted polyamine analogues have been evaluated in prostate cancer model systems.[142] The effects of **18**, **22**, and the parent analogue **12** were evaluated in three representative human prostate cancer cell lines. Two lines, PC-3 and DU145, are androgen receptor-negative lines, and LNCAP is an androgen receptor-positive line. Dose−response studies were performed within a concentration range from 0.1 to 30 μM for 120 h. All three lines were significantly growth-inhibited by the three analogues, with Du145 being the most sensitive of the three lines (Figure 10). Compounds **18** and **22** were cytotoxic to the DU145 cell line at concentrations ≥1 μM, and **22** was found to be cytotoxic to all three lines

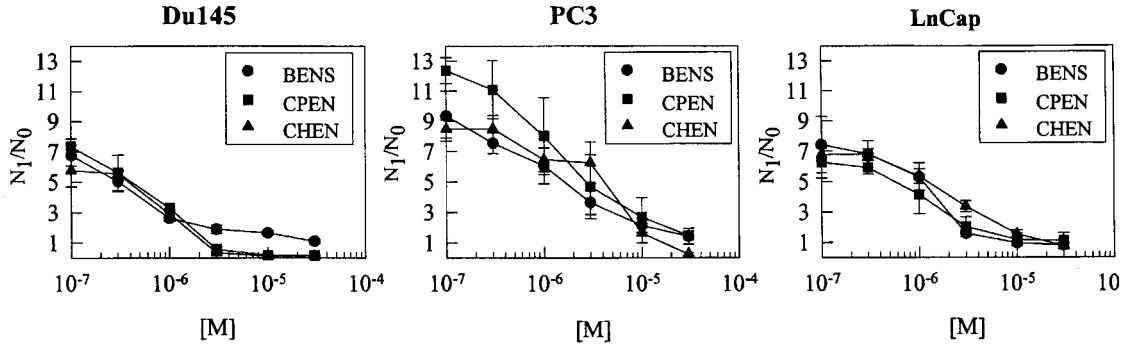
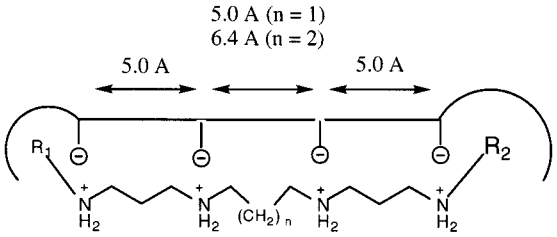
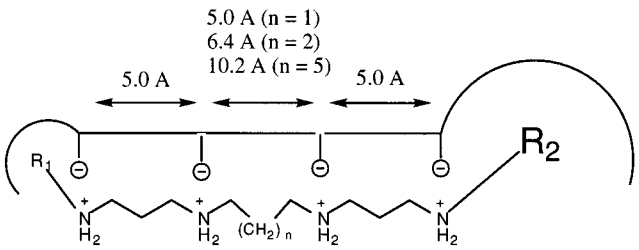


Figure 10. Effects of BENSpm (**12**), CPENSpm (**18**), and CHENSpm (**22**) on three prostate tumor cell lines in vitro.



a. Model for design of SSAT induction-dependent antitumor agents

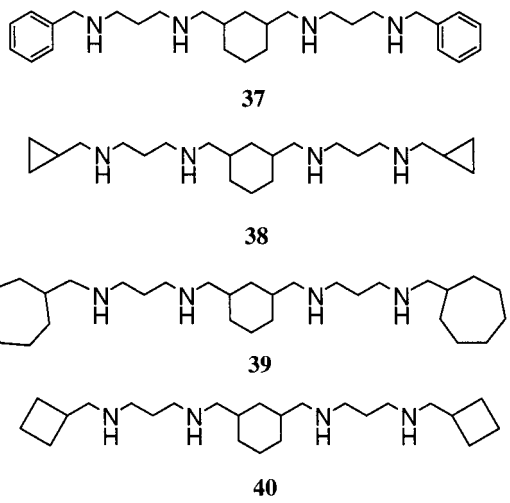


b. Model for design of SSAT induction-independent antitumor agents

Figure 11. SAR models developed for SSAT induction-dependent and SSAT induction-independent alkylpolyamine antitumor agents.

at 30 μM. All three prostate lines accumulated significant concentrations of each analogue. However, the effects on the polyamine metabolic enzymes were relatively small compared to the effects observed in the lung cancer systems. ODC and AdoMetDC were largely unaffected by treatment with the analogues. The maximal induction of SSAT was observed in PC3 cells where **18** and **12** induced SSAT >70- and 180-fold, respectively, after 24-h treatment. The maximal induction of SSAT in the remaining two lines was less than 50-fold with any of the compounds. Similar to results in the H157 non-SCLC line, compound **22** did not induce SSAT in the prostate lines. Although the effects on the polyamine metabolic pathway appear to be modest in the prostate lines, their responsiveness to low concentrations and the cytotoxic activity in these lines with the newly synthesized analogues continue to encourage further study.

The SAR model for the design of SSAT induction-associated analogues is shown in Figure 11a. To date, the analogues that have produced the highest levels of SSAT induction have been those with a 3−3−3 or 3−4−3 carbon skeleton. There appears to be a requirement for bis(alkyl) substitution on the terminal nitrogens, and unsymmetrically substituted analogues induce as well or better than the parent analogue **12**. When R_1 is ethyl, R_2 can vary in size from small (e.g. ethyl, cyclopropylmethyl) to medium (e.g. cyclopentylmethyl). However, the size of only one of the substituents can be increased beyond ethyl (e.g. CPCHENSpm (**36**) (Figure 6) is inactive and does not induce SSAT, since both R_1 and R_2 are larger than ethyl). These data suggest the SAR model shown in Figure 11a for analogues which act at the SSAT induction effector site. The central nitrogens bind to putative negative charges that are separated by 5.0−5.8 Å, and each terminal nitrogen interacts with a putative anionic site that is 5.0 Å from each central anionic site. Two adjacent binding areas for alkyl substituents are suggested: the pocket for R_1 will accept only a small alkyl group, while the R_2 binding pocket can accommodate medium-sized groups



37

38

39

40

Figure 12. Structures of alkylpolyamine analogues **37**−**40**.

up to the size of cyclopentylmethyl. Both spermine and spermidine analogues can bind to this site, since both types of analogues are capable of superinducing SSAT. The current hypothesis is that agents that fit these criteria produce cytotoxicity following superinduction of SSAT (see below).

The model for the design of SSAT induction-independent analogues is shown in Figure 11b. In this model, there again seems to be a preference for bis-substituted analogues. The pockets for R_1 and R_2 seem to be less restrictive, although the most active analogues have a small R_1 (ethyl) and large R_2 (e.g. cycloheptylmethyl, cyclohexylmethyl). To fit this catagory, at least one of the alkyl groups must be larger than a cyclopentylmethyl (e.g. **24** (Figure 6) is an inducer of SSAT, while **25** is not). Active analogues with a 3−3−3 and 3−7−3 carbon skeleton have been identified, indicating that compounds may be synthesized with *n* varying between 1 and 5. Thus, the requirement for the intermediate chain seems to be less restrictive than in the SSAT inducer series. However, steric bulk on the intermediate chain is not well-tolerated (e.g. compounds **37**−**40**, shown in Figure 12, have very low activity). These data suggest an effector site which has internal anionic sites between 5 and 10 Å apart and terminal anionic sites that are roughly 5 Å away from the respective internal anionic sites. The pocket for R_1 binding prefers a small- or medium-sized alkyl group, while the R_2 pocket prefers a large group with considerable steric bulk and hydrophobic character. The current hypothesis is that agents that fit these criteria produce cytotoxicity through an as yet unknown pathway (see below).

Initial in vivo testing of the bis(ethyl)polyamines was conducted using a t.i.d. dosing schedule and ip administration.[113] However, during the preliminary evaluation of **18** and **22**, reports of better responses with diffusion pumps were reported in a non-SCLC model with the symmetrically substituted analogues.[143] Initial attempts to repeat these results were initiated in a similar study using the A549 non-SCLC cell line[109] and the same reported dosing schedule for compound **12**, 240 and 120 mg/kg/day × 5 days. However, in the A549 model, the highest concentration was 100% lethal. All animals survived the lower dose, and a 30-day growth delay was observed compared to controls. However, by 50 days posttreatment all tumors resumed near control growth

Table 4. Sensitivity of Representative Breast Cancer Cell Lines to CPENSpm (**18**)

cell line	estrogen receptor status	IC_{50} (μM)
MCF-7	+	1.1
T47D	+	0.4
ZR-75-1	+	1.1
MDA-MDB-468	−	0.6
MDA-MB-231	−	1.3
Hs578T	−	0.2

rates. Therefore, trials in the prostate DU145 model involving **12** were performed with a high dose rate of 200 mg/kg/day × 5 days. Both BENSpm (**12**) treatment regimens produced significant reductions in tumor volume during a 40- and/or 80-day time period. On the basis of these encouraging results, LD_{50} determinations for **18** and **22** were completed using diffusion pumps. The maximal practical dosages for **18** and **22** were determined to be 100 mg/kg/day × 5 days and 20 mg/kg/day, respectively, and these in vivo trials are currently in progress.

Programmed Cell Death − A Common Mechanism for Analogue-Induced Cytoxicity

Although the rapid cell death produced by alkylpolyamine analogues has been well-characterized, the pathways involved in this cytotoxicity have not been adequately defined. The first biochemical evidence which suggested a common mechanism was the finding that polyamine analogues could produce cytotoxicity through programmed cell death (PCD). This effect was first observed following treatment with the unsymmetrically substituted analogue CPENSpm (**18**)[144,145] in the MCF-7 and MDA-MB-468 breast cancer lines, as well as in H-157 non-SCLC human lung tumor cell line. In the case of the breast cancer lines, greater than 90% growth inhibition was observed after prolonged treatment with compound **18** in each of six cell lines tested. The IC_{50} values for inhibition by **18** in these six breast tumor lines are shown in Table 4. In the breast cancer lines MCF-7 and MDA-468, high-molecular-weight DNA fragmentation and formation of oligonucleosomal-sized fragments were observed as early as 72 h at a 10 μM concentration and after 96 h with as little as 1 μM. Similar results in other breast cancer lines including T47D, Zr-75-1, MDA-231, and Hs578t were also observed.

In the case of the NCI H157 lung cancer model, PCD was found to occur at earlier exposure times than observed in the breast tumor lines.[146] High-molecular-weight (≥50 kbp) DNA fragmentation was observed after a 24-h exposure to 10 μM **18**. Similar results were observed with 10 μM **13**, but only after 48 h, although the initiation of PCD in CHO cells is quite rapid at high concentrations of the analogue.[147] Interestingly, nucleosomal ladder formation was observed after a 36-h exposure to **18** but was never observed in cells treated with **13**, even at exposure times in excess of 140 h. In the case of the lung cancer line, it was also possible to visualize the nuclear fragmentation and formation of apoptotic bodies commonly associated with PCD/apoptosis after Hoechst staining (data not shown). After 48-h exposure, essentially all of the visualized nuclei displayed apoptotic bodies. Although the above results clearly indicate that the unsymmetrically substituted analogues induce PCD, the underlying cellular mechanism(s) had not been elucidated.

As has been mentioned, both **18** and **13** cause dramatic induction of SSAT.[97,148] However, no direct evidence existed to demonstrate a role for SSAT/PAO catabolic pathway in cell death. The catalysis of polyamines by the SSAT/PAO pathway is known to produce H_2O_2 as a byproduct, suggesting that PCD produced by treatment with **18** in H157 may be, in part, due to oxidative stress resulting from H_2O_2 production. To determine whether H_2O_2 was involved in the observed toxicity, catalase was added in combination with **18**.[149] This co-addition significantly reduced the high-molecular-weight DNA fragmentation observed and reduced the early fragmentation of the nuclei as observed by Hoechst staining. These results demonstrated that H_2O_2 was, in fact, the insulting ROS leading to PCD. Additionally, the combination of **18** with inhibitors of endonuclease activity reduced the amount of DNA damage produced, demonstrating that the DNA damage was mediated by endonuclease activity, rather than a direct drug effect. The inhibition of PAO by the specific inhibitor *N*,*N*-bis(2,3-butadienyl)-1,4-butanediamine (MDL 72,527) resulted in a significant reduction in the formation of high-molecular-weight DNA and similarly reduced the number of apoptotic nuclei formed. These results strongly suggest that H_2O_2 production by PAO has a role in compound **18**-induced cytotoxicity in H157 cells. Interestingly, co-addition of catalase or MDL 72527 had no effect on the formation of high-molecular-weight DNA fragments or apoptotic bodies following treatment with 10 μM **22**, supporting the contention that the two agents produce apoptosis by different mechanisms.

Since Bcl-2 protein family members have been implicated in protecting cells from oxidative stress-induced apoptosis,[150] the levels of Bcl-2 family members thought to modulate cell death were determined. H157 cells were treated for increasing times up to 30 h with 10 μM **18**, and Western blot analyses were conducted using antibodies against Bcl-2, β-actin, Bcl-x_L/Bcl-x_S, and Bax. There was no change in the level of any of the Bcl-2 family members, suggesting that the induction of PCD was not a result of changes in the expression of Bcl-2 family proteins. However, it does not exclude the possibility that increased expression of Bcl-2 may protect cells from polyamine analogue-induced PCD. To address this issue, NCI H157 transfected with a cDNA expression vector for the antiapoptotic protein Bcl-2 was developed. The H157 cell line normally does not express Bcl-2 at detectable levels, and therefore background levels were not a concern. In the transfected H157 line, exogenous Bcl-2 was highly expressed, an event which has been demonstrated in several systems to block or delay PCD induced by various stimuli. It has been suggested that Bcl-2 activity is associated with its ability to modulate the permeability of the outer mitochondrial membrane to various components including cytochrome *c* and through its antioxidant activity.[150−153] In the case of **22** treatment, the mitochondrial membrane potential was clearly effected.[154] Release of cytochrome *c* with subsequent activation of caspase-3 was also observed as early events. However, in the case of treatment with compound **18**,[155] cytochrome *c* release

was not observed until most cells were determined to be dead, suggesting that cytochrome *c* release was not an important early determinant of PCD in cells treated with **18**. The treatment of wild-type H157 cells with both **18** and **22** leads to the activation of caspase-3 and cleavage of poly(ADP-ribose) polymerase (PARP).[155] The overexpression of Bcl-2 was found to block many of the known steps of the cell death program, including caspase-3 activation, PARP cleavage, and release of cytochrome *c* from the mitochondria in analogue-treated H157 cells. However, the overexpression of Bcl-2 was only able to alter the kinetics of PCD, not completely block it. The results with **18** and **22** are particularly interesting with respect to the demonstration that both compounds are capable of inducing PCD in a caspase-3-independent manner. Although it appears that the drug-induced activation of the caspase cascade plays a role in the analogue-induced cytotoxicity, it is clearly not required for the activity of either analogue. The results of these experiments demonstrate that polyamine analogues activate a classical PCD response, which can include the release of cytochrome *c* and the activation of the caspase cascade, leading to the morphological and biochemical steps in the PCD pathway. However, the results also clearly demonstrate that multiple parallel pathways exist which allow inhibition of the caspase cascade without affecting the ultimate extent of PCD. These findings are particularly significant because they indicate the ability of the analogues to function in cells with a fully competent PCD pathway and in those in which the PCD pathway has been compromised.

Polyamines, Polyamine Analogues, and Reactive Oxygen Species

As outlined above, the metabolism of polyamines can produce reactive oxygen species (ROS), specifically through the PAO-mediated generation of H_2O_2. It has also been postulated that the natural polyamines may act as antioxidants under certain situations, although no formal proof has been provided.[156] The natural polyamines are found in millimolar concentrations in the cell and are generally thought to be associated with DNA, RNA, and negatively charged proteins. Therefore, their location places them in the most effective areas for protection against free radical attack. One possibility for the observed toxicity of polyamine analogues is that polyamine depletion by the combined activity of increases in catabolism, decreases in biosynthesis, and replacement by the analogues produces a cellular state that made them more susceptible to DNA damage. To test this hypothesis, the ability of polyamines and their analogues to protect DNA from ROS-mediated strand breakage was determined using an in vitro Cu(II)/H_2O_2 oxygen-radical generating system. Strand breakage of ΦX-174 plasmid DNA was observed indirectly by the conversion of supercoiled double-stranded DNA to open circle and linear forms.[157] In the actual assays, 0.2 μg of DNA was incubated in the presence of 30 μM H_2O_2, 10 μM Cu(II), and various concentration of the natural polyamine or analogue at 37 °C. Spermine was found to be nearly as effective at protecting DNA in this system as *N*-acetyl-L-cysteine, reduced glutathione, and the H_2O_2-metabolizing enzyme, catalase. Interestingly, the polyamine analogue **18** was nearly as effective as spermine in protecting DNA from strand breakage.

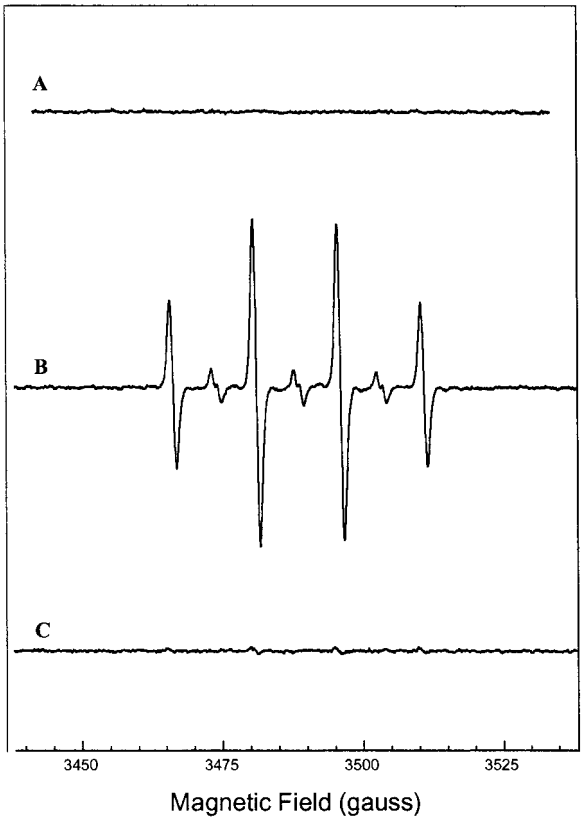


Figure 13. Effects of spermine on formation of the DMPO-OH adduct from H_2O_2/Cu(II) in the presence of 50 mM DMPO: (A) 30 μM H_2O_2 + DMPO; (B) 30 μM H_2O_2 + 10 μM Cu(II) + DMPO; (C) 30 μM H_2O_2 + 10 μM Cu(II) + 1 mM spermine + DMPO.

However, N^1-acetylspermine was not capable of the same protection. The inorganic cation Mg^{2+} was also not capable of protecting the DNA from strand breaks. Among the analogues tested, the order of efficacy in their ability to protect DNA was observed to be **18** > **24** > **22**, suggesting that ring size may limit the ability of the analogue in its protective capacity. Interestingly, the symmetrically substituted analogues **12** and **13** did not provide similar protection at the same concentration (1 μM).

Although the above data are consistent with the hypothesis that the polyamines are acting directly as free radial scavengers, they do not eliminate at least two other possibilities: the proposal that the polyamines and their analogues can alter chromatin structure, and thus alter its susceptibility to attack by other agents,[158] and the contention that the polyamines could act as metal ion chelators[159] and inhibit the formation of the hydroxyl radical by Cu(II)/Cu(I) cycling. To rule out the first possibility, electron spin resonance (ESR) spectroscopy studies were conducted in the absence of DNA, using 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) as a spin-trap label.[160] The results of these experiments, shown in Figure 13, clearly demonstrate a decrease in measurable hydroxyl radical in the presence of physiological concentrations of spermine. These studies suggest that changes in chromatin structure may not be necessary for the protective effect of the polyamines, since they were performed in the absence of DNA. However, they do not exclude the possibility that the polyamines are chelating the copper ion. To address this

question directly, bis-α-[^{13}C]spermine (**41**) was synthesized and subjected to the oxygen radical-generating system described above in the absence of DNA. The products of this reaction were then analyzed by ^{13}C NMR (Figure 14). The enriched spermine spectrum showed a single resonance at 39 ppm; in each experiment involving the free radical-generating system, the ^{13}C NMR peak corresponding to enriched spermine disappeared and two new peaks appeared, one downfield at 60.3 ppm and one upfield at 19.6 ppm. This phenomenon was observed in each experiment at a variety of concentrations of copper chloride and peroxide and at two concentrations of spermine. These data clearly show that spermine is converted to at least two adducts in the presence of hydroxyl radical and support the contention that spermine is capable of acting as a free radical scavenger.

High-resolution mass spectrometry was next used to determine the structures of the adducts formed and to help to elucidate the chemical pathways involved in the reaction between spermine and hydroxyl radicals and other ROS. The reaction mixtures generated as described above were analyzed by chemical ionization (CI) mass spectroscopy. Unlabeled spermine produced the expected molecular ion peak at *m/z* 203 (data not shown), corresponding to the protonated parent molecule, as well as major peaks at *m/z* 146 (loss of one aminopropyl) and 89 (loss of a second aminopropyl). The CI mass spectrum generated from bis-α-[^{13}C]spermine (**41**) (20 nM sample) exhibited the expected molecular ion peak at *m/z* 205 and a second peak appearing at *m/z* 147, corresponding to the loss of a single α-[^{13}C]-aminopropyl group. The structures of these fragments, as well as their empirical formulas and expected mass spectral peaks, are shown in Table 5. Using the same technique described above, the reaction mixtures from the free radical scavenging experiments were pooled, lyophilized, and subjected to CI mass spectral analysis. Major peaks appeared at *m/z* 203, 148, 130, and 90. Potential identities of the peaks at *m/z* 203, 130, and 190 are listed in Table 6, while the identity of the peak at *m/z* 148 has yet to be determined.

Table 5. CI MS Fragments from Bis-α-[^{13}C]spermine

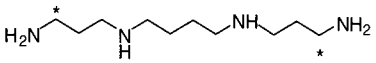
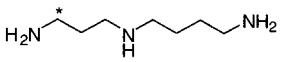
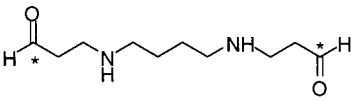
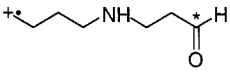
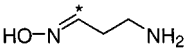
structure of fragment	formula	MW	*m/z*
	$^{13}C_2{}^{12}C_8H_{26}N_4$	204	205
	$^{13}C^{12}C_6H_{19}N_3$	146	147

Table 6. CI MS Fragments from Free Radical Scavenging Experiments

structure of fragment	formula	MW	*m/z*
	$^{13}C_2{}^{12}C_8H_{20}N_2O_2$	202	203
	$^{13}C^{12}C_6H_{14}NO$	129	130
	$^{13}C^{12}C_2H_8N_2O$	89	90

On the basis of the observed fragments from the CI mass spectral studies outlined above, it is postulated that spermine can indeed act as a free radical scavenger in the cell. These results suggest that formation and metabolism of the adduct formed from spermine and hydroxyl radical is similar to that observed following

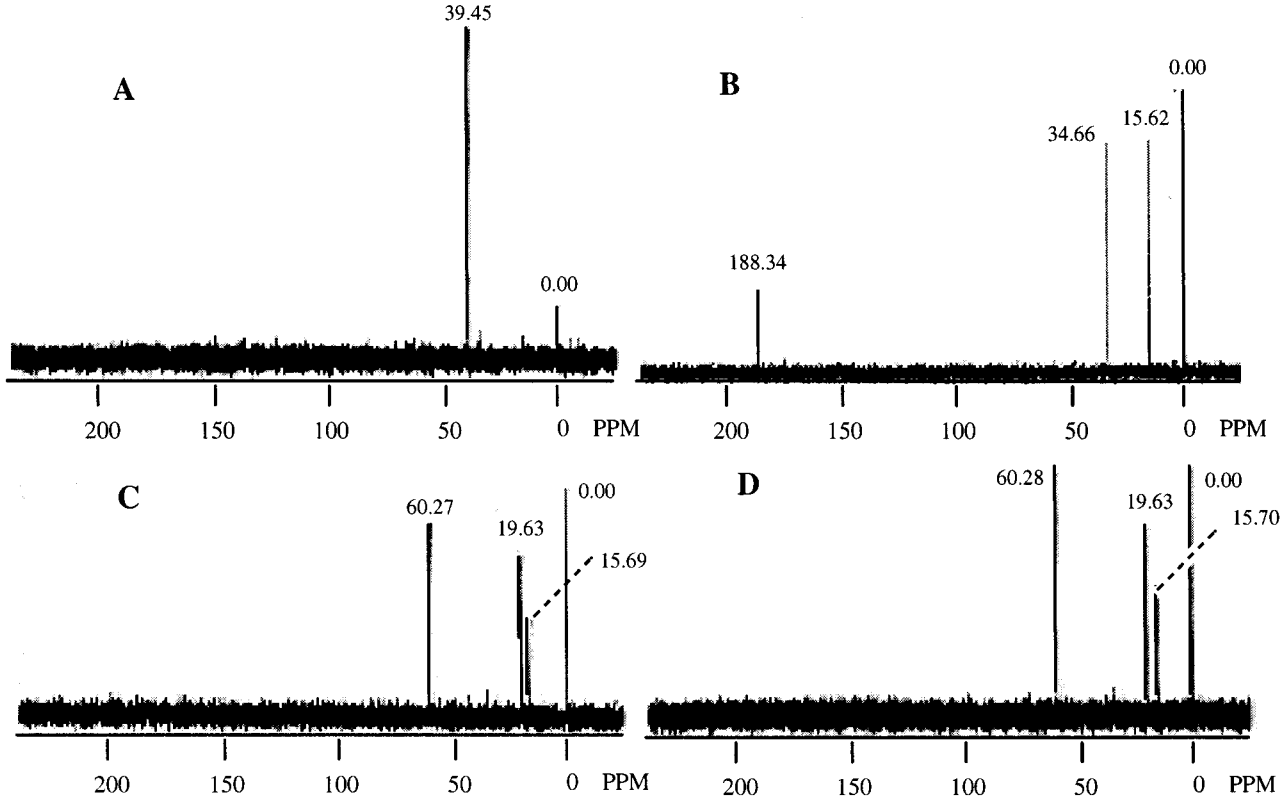


Figure 14. NMR data for [^{13}C]spermine free radical scavaging experiments: (A) bis-α-^{13}C-enriched spermine; (B) 1% TSP in D_2O; (C) 1 mM bis-α-^{13}C-enriched spermine + 0.1 M H_2O_2 + 33.3 mM $CuCl_2$; (D) 0.5 mM bis-α-^{13}C-enriched spermine + 0.5 M H_2O_2 + 167 mM $CuCl_2$.

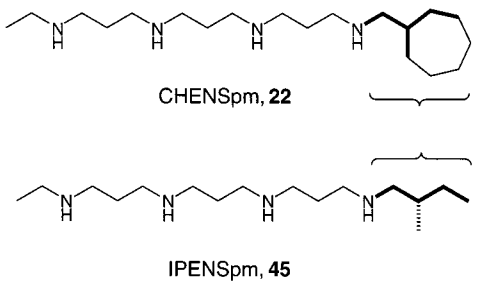


Figure 15. Structural comparison of the alkylpolyamine analogues CHENSpm (**22**) and IPENSpm (**45**).

flavin-containing monooxygenase (FMO)-mediated N-oxygenation of tyramine[161] and other aliphatic primary amines.[162] In the presence of hydroxyl radical, bis-α-[^{13}C]spermine (**41**) could form the corresponding unstable bis-*N*,*N*-dihydroxyspermine (**42**) (Scheme 1). Spontaneous dehydration of this intermediate could then afford the corresponding bisoxime **43**, which would then be readily hydrolyzed to form the dialdehyde **44**. While the NMR and mass spectral data support the formation of adducts **43** and **44**, it is likely that there are additional steps in the pathway or additional adducts formed, since there are peaks in each spectra which clearly do not arise from any of the intermediates in Scheme 1. It should be pointed out that this proposed pathway is speculative, and thus the identification of additional adducts is an ongoing concern. The results of these studies are the first to definitively demonstrate the role of polyamines as free radical scavengers and open up an entirely new area in polyamine research which may have significant potential in terms of therapeutic applications.

Polyamine Analogues That Alter Tubulin Polymerization

Several groups have demonstrated that some polyamine analogues which do not highly induce SSAT can still produce PCD.[128,149,154,155,163−165] Although both **18** and **22** induce PCD, it is likely that they do so through a combination of potentially dissimilar mechanisms. Consistent with this hypothesis is the observation that they have dramatically different effects on the cell cycle.[149,154] Following 24-h treatment of H157 non-SCLC with 10 μM **18**, no significant effects on cell cycle are observed by flow cytometric analysis. However, under the same conditions, 10 μM **22** produces a dramatic G_2/M cell cycle block in normal and Bcl-2-overexpressing H157 cells.[154] The analogue (*S*)-1-[*N*-(2-methyl-1-butyl)amino]-11-(*N*-ethylamino)-4,8-diazaundecane (IPENSpm, **45**) (Figure 15) was subsequently synthesized and found to produce a similar G2/M cell cycle arrest. These data are not surprising, in light of the structural similarity of the two analogues, as shown in Figure 15. Using molecular mechanics techniques, it has been shown that the two analogues, **22** and **45**, are virtually superimposable, indicating that the geometry of the larger alkyl group determines the activity of the compound. On the basis of these observations, the potential mechanisms leading to the observed G_2/M block initiated by **22** and **45** were investigated.[166] All three analogues demonstrated similar cytotoxic effects in the human non-SCLC line NCI H157. In 96-h dose−response studies each compound was found to be cytotoxic at concentrations greater than 0.1 μM. However, it should be noted that significant induction of SSAT activity was only observed in cells treated with **18** and not in cells treated with **22** or **45** (Figure 16). The effects of all three compounds on the cell cycle progress were analyzed by flow cytometry after a 24-h exposure to 10 μM of each compound. As previously observed, CPENSpm treatment had no significant effect on the cell cycle.

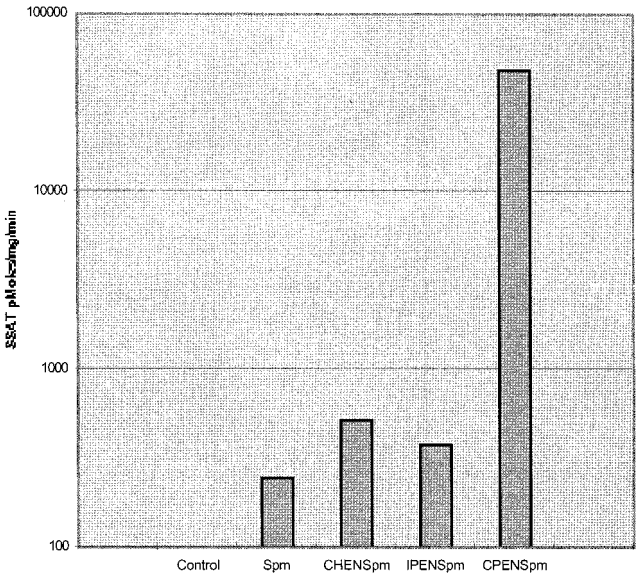
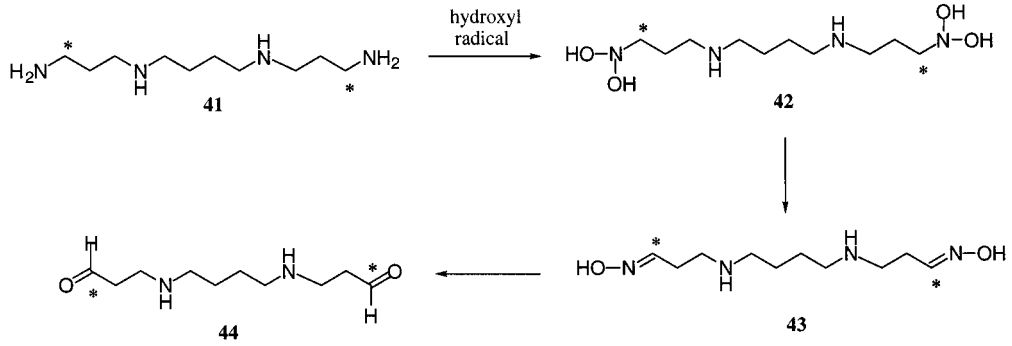


Figure 16. SSAT induction by polyamine analogues **18**, **22**, and **45**. NCI H157 cells were treated with 10 μM of the indicated compounds for 24 h followed by analysis of their SSAT activity. Note the ordinate is a log scale. Only CPENSpm (**18**) treatment was found to superinduce SSAT. Results are from a representative experiment performed in triplicate, within which the standard deviation was uniformly less than 10%.

Scheme 1



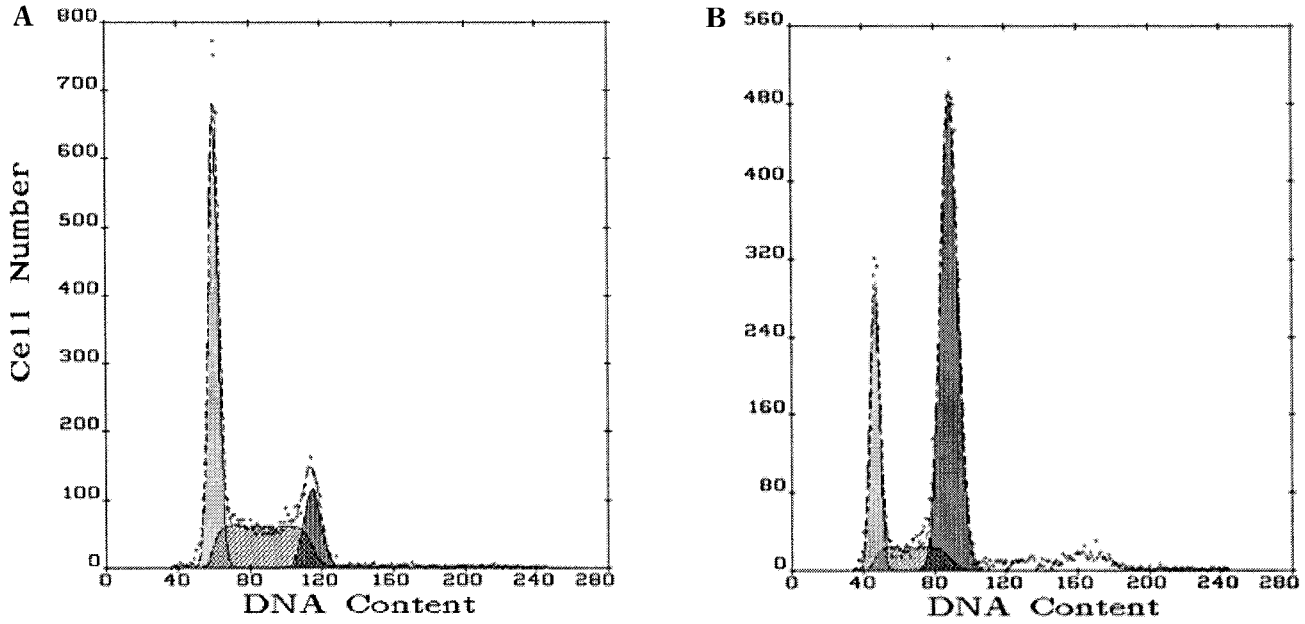


Figure 17. Effects of treatment with **22** on the cell cycle in NCI H157 cells. Subconfluent H157 cells were exposed to: (A) no treatment; (B) 10 μM CHENSpm (**22**) for 24 h. Results are from a minimum of three trials, each with nearly identical results.

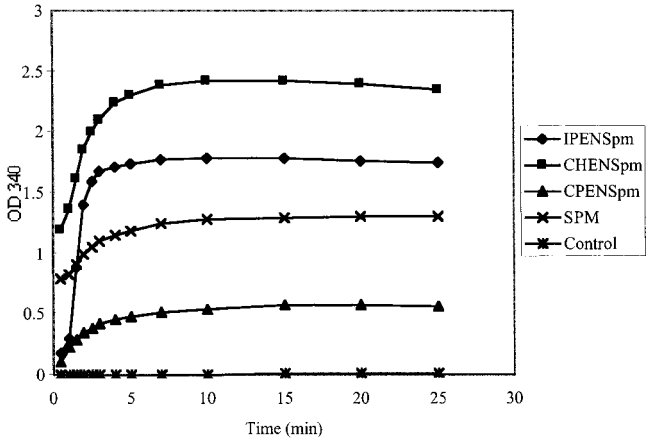


Figure 18. Effects of CPENSpm (**18**), CHENSpm (**22**), IPENSpm (**45**), and spermine on tubulin polymerization. Buffer containing the polyamine was equilibrated at 37 °C. The polymerization was initiated by the 10-fold dilution of 10 mg/mL tubulin which had been thawed immediately before the reaction. The first OD_{340} reading was taken at 30 s. Each point represents the mean of 2−3 polymerization reactions; values generally varied <10%.

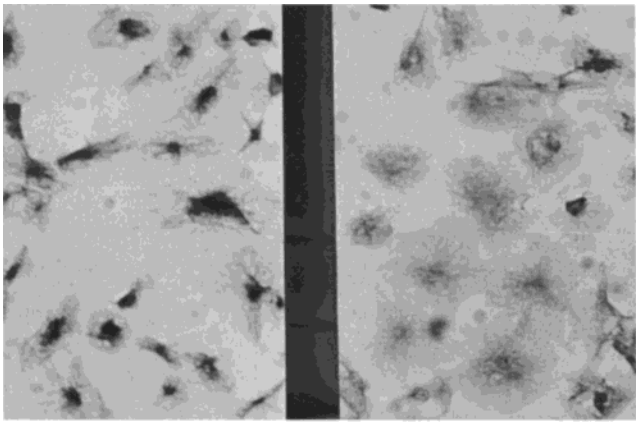


Figure 19. Immunohistochemistry of NCI H157 cells probed using antitubulin monoclonal antibody. The nucleus was stained with methyl green. Representative pictures of three separate experiments. The DAB staining was performed simultaneously to ensure comparable results: (left) control; (right) 24-h treatment with 10 μM CHENSpm (**22**).

However, both **22** and **45** had a profound effect, wherein each treatment resulted in a significant G_2/M block and a concurrent decrease in the G_1 fraction (Figure 17). All three analogues, as well as the natural tetraamine, spermine, stimulate tubulin polymerization in the absence of microtubule-associated proteins (MAPs) and other polymerization stimulants (Figure 18). In the absence of the analogues or spermine, there was no detectable polymerization, as determined by either increase in absorbance or observable microtubule formation. The rate of polymerization was greatest in the case of **22**, and proceeded 4.9 times faster than spermine. The analogues altered the equilibrium OD_{340} from control in the following order: **22** > **45** > spermine > **18**.

To further investigate elements that could compete for interaction with tubulin in the intact cell, the polymerization assay was repeated with MAP-rich tubulin. MAPs are believed to interact with the tubulin at least in part through their polylysine cationic regions. Compound **22** remained the most effective agent in terms of increasing the rate and equilibrium value for tubulin polymerization; however, in the presence of MAPs, **18** and spermine showed significant decreases in their ability to effect tubulin polymerization. These data suggest that the analogues are possibly competing for binding at the site normally occupied by MAPs.

CHENSpm (**22**) altered the microtubule density in the putative centrosome adjacent to the nucleus when analyzed by immunohistochemical staining of tubulin (Figure 19). The cytoplasmic microtubules do not appear to be effected by treatment; however, the strong staining of the microtubules emanating from the centrosomal area is absent or significantly reduced. Higher concentrations of **18** caused rapid apoptosis and thus were not amenable to immunohistochemistry. Interestingly, **45**

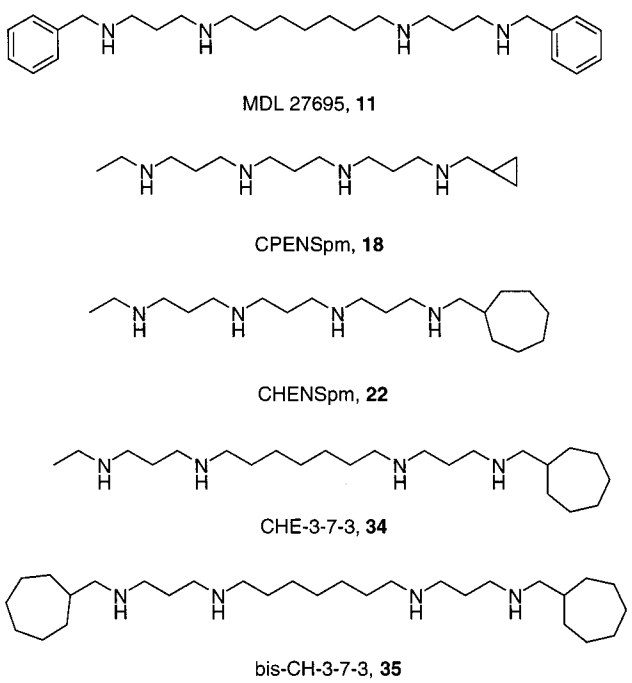


Figure 20. Structures of alkylpolyamine analogues evaluated as antitrypanosomal agents.

was also tested for its ability to alter cellular microtubules. There were no observable immunohistochemical changes in the density or distribution of the microtubules after treatment with **45**, suggesting that the effects on tubulin polymerization initiated by **22** and **45** proceed by different overall mechanisms.

IPENSpm (**45**) has emerged as an extremely interesting lead compound, due to its antitumor activity, its alteration of in vitro tubulin polymerization, its spindle effects, and the production of a profound G2/M cell cycle block. Preliminary in vivo toxicity studies have been performed using a 5-day Alzet diffusion pump protocol, as described above. From these preliminary studies, 45 mg/kg/day × 5 appears to be the maximally tolerated dose where the animals exhibited minimal weight loss (<8%) and all treated animals survived. On the basis of these data, in vivo efficacy studies with **45** in the lung and prostate models have been initiated.

Polyamine Analogues with Activity Against Parasitic and Opportunistic Infection

Following an initial series of papers describing the antiparasitic effects of polyamine analogues in 1989, interest in this avenue of research waned until the mid-1990s. Initially, a series of bis(benzyl)polyamine analogues were described[167] which exhibited potent effects on cell growth and intracellular polyamine levels in cultured rat hepatoma (HTC) cells.[168] In HTC cells, treatment with a 1 μM concentration of MDL 27695 (**11**) (Figure 20) led to a rapid loss of ODC and AdoMet-DC activity and a complete cessation of cell growth, although cellular polyamine levels did not change dramatically. Interestingly, and unlike alkyl-substituted 3−3−3 and 3−4−3 polyamine analogues, these aromatic-substituted polyamines were originally developed for their marked antimalarial activity.[167,169] These analogues, and in particular MDL 27695 (**11**), appear to act by regulation of the polyamine biosynthetic pathway. It has been suggested that the antiproliferative and antiparasitic activities of **11** are due to the ability of the analogue to be rapidly metabolized by PAO.[168] The metabolism of the alkylpolyamine analogue bis(ethyl)-norspermine, which involves N-dealkylation followed by SSAT acetylation and oxidation by PAO, has also been described.[125] MDL 27695 has emerged as a promising lead in the search for novel antiparasitic agents, since it was the most potent antiparasitic in a limited series of polyamine analogues, exhibiting an IC_{50} value of 1 μM against cultured *Plasmodium falciparum*.[169] Compound **11** was growth inhibitory against chloroquine-resistant *P. falciparum* in vitro and in a murine model was found to cure *P. berghei* infections in combination with DFMO.[167]

The success of MDL 27695 (**11**) as an antimalarial agent prompted the evaluation of the compound for activity against *Leishmania donovani*. This analogue shows excellent activity against both antimony-susceptible and antimony-resistant strains of the organism and eliminated 77−100% of *L. donovani* amastigotes from mouse peritoneal macrophages in vitro at a concentration of 1 μM.[170] The compound also showed good in vivo activity, exhibiting ED_{50} values of 2.5 and 1 mg/kg in mice and hamsters, respectively. The mechanism of action of **11** in *L. donovani* is different from that of antimonials, since the drug is effective against susceptible *and* resistant strains. There is evidence that **11** acts by disrupting DNA and RNA synthesis in this organism. MDL 27695 has also been shown to be effective orally and by intramuscular administration in mice infected with *L. donovani*, at doses of 100 mg/kg twice a day (14 days) and 15 mg/kg three times a day (5 days), respectively.[171] In this regard, the analogue is significantly superior to the antimonial drug sodium stibogluconate.

A logical extension of the work described above would be to synthesize and evaluate a series of polyamine analogues related to MDL 27695. A preliminary series of analogues based on MDL 27695 have been synthesized and evaluated for antiparasitic activity in vitro against one strain of *T. brucei brucei* (LAB EATRO 110) and three strains of *T. brucei rhodesiense* (KETRI 243, KETRI 243 As-10-3, and KETRI 269).[133] These organisms were selected as targets because the trypanosomal polyamine metabolic pathway has been fully described.[25] The same analogues were evaluated for antitumor activity against the H157 non-SCLC and H82 SCLC lines in culture. In particular, MDL 27695 (**11**), CHE-3-7-3 (**34**), and bis-CH-3-7-3 (**35**), shown in Figure 20, were compared against the known antitumor analogues CPENSpm (**18**) and CHENSpm (**22**). These preliminary biological studies suggested new SAR trends within the broad class of bis-alkylated polyamine analogues. Interestingly, analogues with a 3−3−3 carbon skeleton (e.g. **18** and **22**), as previously reported, were potent antitumor agents in vitro (IC_{50} against NCI H157 0.4 and 0.5 μM, respectively) but had little antitrypanosomal activity in in vitro screens, as shown in Table 7. By contrast, agents with a 3−7−3 carbon skeleton (e.g. **34** and **35**) showed potent antiparasitic activity (Table 8) but only weak antitumor activity (IC_{50} against NCI H157 2.1 and >10.0 μM, respectively). The analogues **34** and **35** showed potency against all organisms tested

Table 7. In Vitro Activity for Alkylpolyamine Analogues Against H157 non-SCLC and H82 SCLC

compd	IC_{50} (μM) H157	H82	compd	IC_{50} (μM) H157	H82
BESpm, **13**	2.9		MDL 27695, **11**	0.5	1.7
CPENSpm, **18**	0.7	5.2	CHE-3-7-3, **34**	>10[a]	9.0
CHENSpm, **22**	0.4	0.7	bis-CH-3-7-3, **35**	2.1	1.5

[a] No significant effects were seen at any concentration tested up to 10 μM. Each data point is the average of two determinations which in each case differed by less than 5%.

Table 8. In Vitro Growth Inhibition Values of Four Strains of *T. brucei* by Bis-Substituted Polyamine Analogues[a]

compd	IC_{50} (μM) LAB 110	K 243	K 269	K 243 As-10-3
MDL 27695, **11**	14.5	15.1	12.3	13.0
CHE-3-7-3, **34**	18.0	18.4	21.0	26.2
bis-CH-3-7-3, **35**	0.125	0.98	0.69	0.78
PENSpm, **17**	>100	>100	>100	>100
CPENSpm, **18**	>100	>100	>100	>100
CHENSpm, **22**	>100	>100	>100	>100
melarsen oxide	0.001	0.04	0.5	

[a] Each data point represents the average of two determinations which differed by less than 5% in all cases. >100 refers to the fact that there was no appreciable inhibitory activity up to the highest concentration tested.

Table 9. In Vitro Growth Inhibition Values of Four Strains of *T. brucei* by Alkylpolyamine Analogues[a]

compd	IC_{50} (μM) LAB 110	K 243	K 269	K 243 As-10-3
46	4.05	4.15	5.1	56
47	73.0	81.5	>100	>100
48	3.25	14.0	1.71	2.15
51	>100	>100		
53	>100	>100		
54	>100	>100		
57	0.24	0.19	0.75	0.20
58	22	18.5	16.5	5.5

[a] Each data point represents the average of two determinations which differed by less than 5% in all cases. >100 refers to the fact that there was no appreciable inhibitory activity up to the highest concentration tested.

which was superior to that of MDL 27695 and which approached the potency of the known trypanocide melarsen oxide. Bis-CH-3-7-3 (**35**) is especially noteworthy, since it had potent activity against the aresenical-resistant strain K 243 As-10-3, against which melarsen oxide is inactive. These data strongly suggest that it is possible to synthesize alkylpolyamine analogues of high potency which can be specifically targeted to parasitic cells.

On the basis of the encouraging results with the analogues described above, a second generation of potential trypanocidal agents was synthesized (Figure 21, unpublished results). The antitrypanosomal activity of some of these agents is summarized in Table 9. In this case, the most active analogue, BW-1 (**57**), was more potent than the previously described agent **35** and also showed excellent activity against arsenical-resistant trypanosomes. Studies aimed at the synthesis of additional analogues, as well as experiments designed to determine the site of action of these analogues, are underway.

The microsporidia are obligate intracellular parasites which primarily infect the CNS, and microsporidial infections have been found in a variety of animals. In recent years, increasing attention has been given to microsporidia, since opportunistic infections due to the organism are a significant problem in immunocompromised patients. At the present time, there are no suitable agents available to treat this type of infection. The second-generation alkylpolyamines listed above were thus evaluated against *Enterocytozoon cuniculi* in vitro to determine whether they had inhibitory activity against microsporidia. The results of these studies are summarized in Table 10. All of the analogues tested had at least modest activity, but the analogue BW-1 (**57**) was again strikingly more potent than the other analogues tested (IC_{50} = 0.47 μM). Since microsporidia is an intracellular parasite, the activity against the organism as well as toxicity to the host cell needs to be considered. Only two of the analogues tested had low host cell toxicity; however, the toxicity observed with **57** is variable and may not be as severe in vivo as in cell culture. As a result, **57** is currently undergoing in vivo efficacy trials. As is the case for antitrypanosomal activity, studies aimed at the synthesis of additional analogues, elucidation of structures that minimize host cell toxicity, and experiments designed to determine the mechanism of action of these analogues are underway.

Table 10. In Vitro Growth Inhibition Activity Against *E. cuniculi* Produced by Alkylpolyamine Analogues[a]

compd	activity	toxicity to monolayer
72	86% inhibition (100 μM)	Y
73	100% inhibition (100 μM)	Y
74	100% inhibition (100 μM)	Y
75	100% inhibition (250 μM)	Y
76	100% inhibition (100 μM)	Y
77	IC_{50} = 232 μM	N
78	76% inhibition (100 μM)	Y
79	IC_{50} = 36 μM	N
80	IC_{50} = 204 μM	Y
81	47% inhibition (50 μM)	Y
82	87% inhibition (500 μM)	Y
57	IC_{50} = 0.47 μM	Y
84	100% inhibition (50 μM)	Y

[a] *E. cuniculi* was grown on RK-13 cells infected as a monolayer (average 30% infected cells). The percentage of infected cells was determined after 7 days in the presence of the test compound and compared to the percentage of infected control cells. All values listed are derived from 3−5 growth curves.

An interesting series of polyamine analogues were developed by following structure−function leads in the design of inhibitors for the enzyme trypanothione reductase (TR). Trypanosome polyamine metabolism progresses as far as spermidine, but the organisms do not make spermine. Instead, spermidine is used to form trypanothione, a peptide conjugate which has activity similar to glutathione and protects the organism from oxidative stress.[25] A molecular modeling study meant to detect pharmacophore-like patterns in the active site of TR indicated that diphenyl sulfide derivatives with prolonged or branched polyamino side chains had the correct spatial and electronic characteristics to act as putative TR inhibitors. The inhibition results within the synthesized series confirmed the main working hypothesis of the molecular modeling study. These compounds showed activity in vitro using purified TR and on *T. cruzi* and *T. brucei* trypomastigotes and were also evaluated in vivo in infected mice.[172] Subsequently, a series of spermine and spermidine derivatives contain-

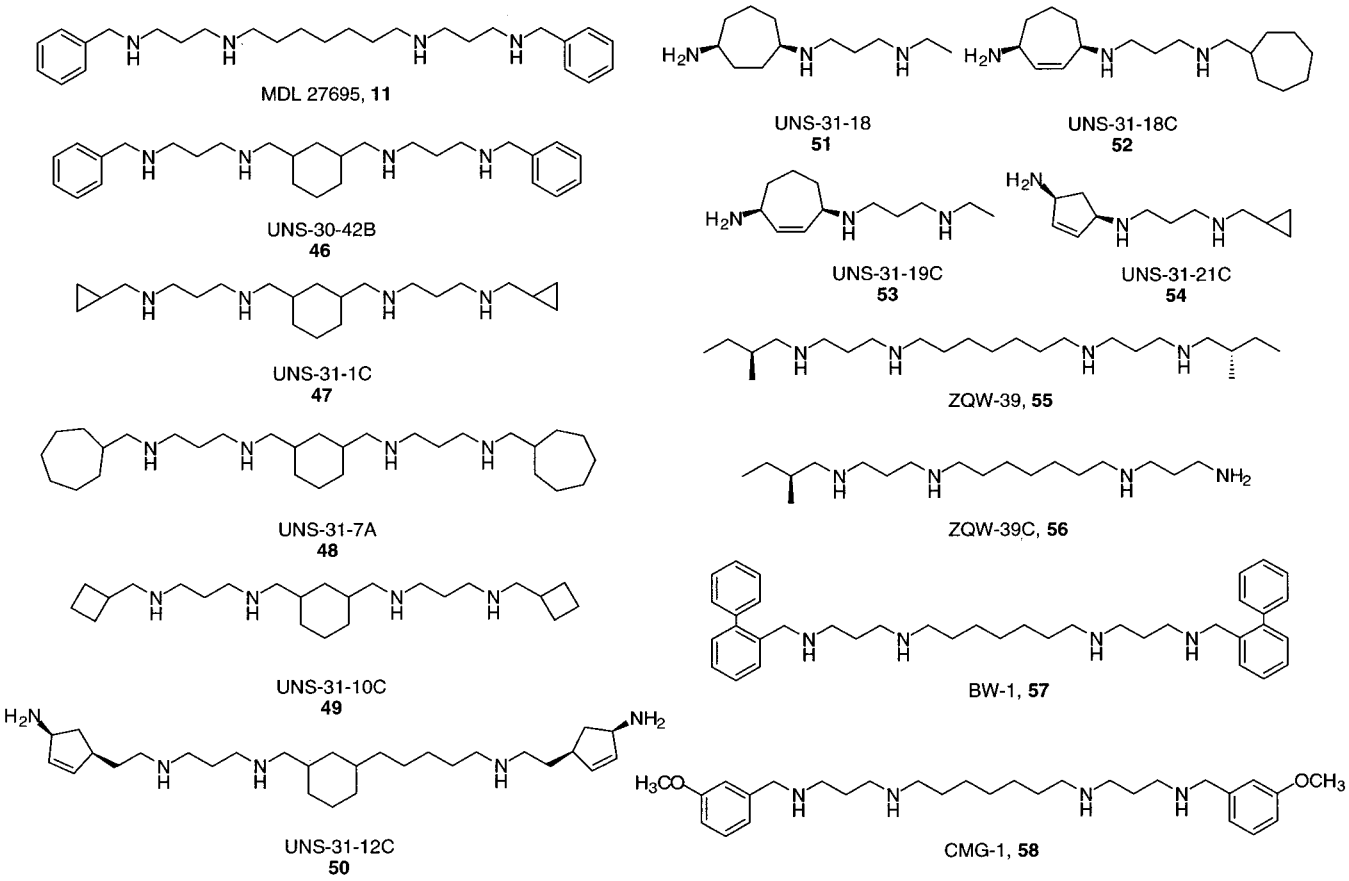


Figure 21. Structures of alkylpolyamine analogues evaluated as antitrypanosomal and antimicrosporidial agents.

ing 2-aminodiphenyl sulfide substituents were prepared and tested for inhibitory effects against *T. cruzi* TR. The compounds exhibited IC_{50} values between 0.3 and 3 μM, making them the most potent TR inhibitors described to date.[173] As an extension of this work, a new series of symmetrically substituted 1,4-bis(3-aminopropyl)piperazines was synthesized and evaluated against TR and for in vitro trypanocidal activity. The most trypanocidal analogue in this series was found to be totally inactive toward TR and thus constitutes a lead structure for the identification of potential *T. cruzi* targets.[174] It is possible that the 1,4-bis(3-aminopropyl)piperazines could have a mechanism similar to that of the alkylpolyamines shown in Figure 21.

Future Directions for Polyamine Drug Discovery

This Perspective began with the statement that polyamines occur naturally in nearly every prokaryotic and eukaryotic cell type, placing them among the most ubiquitous of all biochemical entities. Clearly these polycationic compounds have many functions within cells, and research has only begun to discover and characterize these functions. As recently as 20 years ago, the polyamine biosynthetic pathway was still being elucidated and the enzymes were being characterized. Drug discovery efforts were focused on finding specific inhibitors for these enzymes and at determining the cellular consequences of selective depletion of individual polyamines. The polyamine metabolic pathway is now well-defined; the enzymes have been characterized, cloned, and expressed in bacterial vectors; and in the case of AdoMet-DC, the crystal structure of the enzyme is now known.[34] Polyamines have been found to be a factor in a host of biochemical processes in a variety of plants, animals, bacteria, and humans. These research advances have resulted in one marketed agent (DFMO), two agents that should be marketed but will not due to the size and status of the target population (AbeAdo, MDL 27695), and 2−3 agents which will likely be marketed soon (BENSpm, bis(ethyl)-4X4, BEHSpm). Because of the progress made in the study of the polyamine pathway, there is now evidence that alkylpolyamine analogues can act at a variety of cellular targets that are independent of the biosynthetic and catabolic enzymes.[175] In terms of drug discovery and development, research efforts will likely be concentrated in three general areas:

1. Polyamine Transport. As was outlined above, a number of research groups are involved in elucidating the structure and functions of polyamine cellular binding and transport proteins.[53−93] The polyamine transport assemblies have been characterized for a number of microorganisms, but much remains to be learned about the structure of the mammalian forms of the polyamine transporter. The polyamine transporter in tumor cells appears to accept a fairly wide variety of substrates, and as such it is a factor in the success of any potential drug which is designed to alter the function of the polyamine pathway or to regulate known and/or putative polyamine effector sites. Although the known inhibitors of enzymes in the polyamine pathway are effective, intact cells have numerous routes to compensate for depletion of individual polyamines and

can often overcome the effects of these drugs. Thus, inhibition of the transport system, which mediates both influx and efflux of polyamines from the cell, may prove to be a more reliable way to disrupt polyamine metabolism. Because polyamine transport is elevated in rapidly dividing cells, drugs which rely on this process for entry into intact cells can show target specificity by virtue of preferential uptake. Essentially all of the alkylpolyamines described above fit into this category. Conversely, because of elevated transport levels in rapidly dividing cells, specific inhibitors of polyamine transport would be expected to have dramatic effects on cell viability in target cells while showing minimal effects on normal cells. As was described above, the design and synthesis of such inhibitors is already underway,[87−91] and this process will certainly accelerate as soon as the crystal structures of the mammalian transport proteins have been elucidated.

2. New Target Validation. The polyamines putrescine, spermidine, and spermine are among the most ubiquitous organic compounds found in nature, and they exist as polycations at physiological pH. Thus, it is not surprising that these simple molecules can interact with a wide variety of cellular constituents, such as RNA, DNA, nucleotides, proteins, and other acidic substances.[176] Polyamines have been shown to interact with the cellular lipid bilayer[177] and have also been shown to promote membrane fusion.[178] Spermine and closely related synthetic analogues have been shown to modulate the activity of various ion-gated channels,[179−181] and there is a well-documented polyamine binding site on the NMDA receptor complex.[182,183] Polyamines have also been shown to produce a neuroprotective effect in rat brain slices.[184] Agents are now being developed which are designed to specifically interact with the NMDA−polyamine binding site.

There are numerous recent reports of interactions between polyamines and various cellular transcription factors which are important in human cancers. Polyamines appear to activate the nuclear factor κB in breast tumor cells, presumably by promoting binding of the transcription factor to its response elements.[175] Inhibition of polyamine synthesis, and subsequent depletion of cellular polyamines, has been shown to produce p53 gene expression, but not apoptosis, in small intestinal epithelial cells.[185] Other studies have shown a functional relationship between polyamine levels and expression of the p53 oncogene pathway.[185−188] Polyamines have also been suggested to play a role the c-myc pathway[189] and in the expression of caspase-3.[190] Thus, there are a variety of new polyamine−protein binding interactions which could be validated as targets for novel antitumor agents. In addition, as mentioned above, polyamines and their analogues can protect DNA from damage by ROS,[156,157,160] and there is evidence that the natural polyamines can protect DNA bases from radiation-induced degradation.[191] These data suggest the potential for the development of novel polyamine analogues as chemopreventative agents. Clearly, there are a number of emerging targets for intervention using rationally designed analogues of the natural polyamines. As these targets are refined and validated, drug discovery efforts will take advantage of emerging structural biology data and focus on the design of target-specific analogues.

3. Refinement of SARs. Although much has been learned about the polyamine biosynthetic and metabolic pathways, there are now numerous drugs that exert their effects at sites which are independent of the biosynthetic and catabolic enzymes. Thus, the second-generation agents which target the functions of the natural polyamines will likely be designed to bind to nonenzymatic polyamine binding sites. As was mentioned above, there are SAR models available for some of the effects of alkylpolyamine analogues, but these models are crude and limited in number. In addition, the effector sites for some of the effects of synthetic alkylpolyamines remains obscure. During the next few years, many research groups will be working toward defining these targets and developing agents which specifically bind to these sites and modulate their function. The ubiquitous nature of the polyamines, and the wide variety of effects they produce, virtually guarantees that new polyamine effector sites will be discovered and that these sites will provide new avenues for drug design and development. In the opinion of the authors of this Perspective, polyamine research and analogue development have matured to the point where they are considered mainstream drug discovery endeavors, with the potential to result in the discovery of important therapeutic agents with novel mechanisms of action.

Biography

Patrick M. Woster is Associate Professor of Medicinal Chemistry in the College of Pharmacy and Allied Health Professions at Wayne State University. He received a B.S. in pharmacy from the University of Nebraska Medical Center in 1978 and a Ph.D. in medicinal chemistry from the University of Nebraska in 1986. Following postdoctoral work in the Department of Chemistry at Rensselaer Polytechnic Institute and in the Department of Medicinal Chemistry at the University of Michigan, he joined the WSU Faculty in 1988. Ongoing research projects in his laboratory include synthesis of alkylpolyamines as antitumor or antiparasitic agents, synthesis of *S*-adenosylmethionine analogues as mechanism-based enzyme inhibitors, and synthesis of furanocoumarins as inhibitors of cytochrome P450.

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