

# **Polyamine Cell Signaling**

# Polyamine Cell Signaling

*Physiology, Pharmacology, and Cancer Research*

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# Preface

Polyamines are organic cations found in all eukaryotic cells and intimately involved in, and required for, distinct biological functions. An increasing body of evidence indicates that the regulation of cellular polyamines is a central convergence point for the multiple signaling pathways driving various cellular functions. Over the last decade, considerable progress has been made in understanding the molecular functions of cellular polyamines. These significant findings provide a fundamental basis to not only define the exact role of polyamines in physiology, but also to develop new therapeutic approaches for cancers and other diseases.

The major objective of this book is to provide a timely and long lasting guide for investigators in the fields of polyamines, physiology, pharmacology, and cancer research. It will provide a foundation based on research and address the potential for subsequent applications in clinical practice. *Polyamine Cell Signaling: Physiology, Pharmacology, and Cancer Research* is divided into four main parts:

- Part I: Polyamines in Signal Transduction of Cell Proliferation
- Part II: Polyamines in Cellular Signaling of Apoptosis, Carcinogenesis, and Cancer Therapy
- Part III: Polyamines in Cell Motility and Cell–Cell Interactions
- Part IV: Polyamine Homeostasis and Transport

This book not only covers the current state-of-the-art findings relevant to cellular and molecular functions of polyamines, but also provides the underlying conceptual basis and knowledge regarding potential therapeutic targeting of polyamines and polyamine metabolism. These points are addressed by internationally recognized experts in their contributions to this book.

We would like to take this opportunity to thank Humana Press, especially Mr. Harvey Kane and Ms. Erika Wasenda, who have made a great effort to make this book possible. We are indebted to all the contributors who have shared and contributed their invaluable research experiences and knowledge with us and to the medical community at large. And last but not least, we express our sincere thanks to our families for their generous support throughout the years.

**Jian-Ying Wang**  
**Robert A. Casero, Jr.**

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**POLYAMINES IN SIGNAL TRANSDUCTION OF CELL  
PROLIFERATION**

## Polyamine Structure and Synthetic Analogs

Patrick M. Woster

### 1. Introduction

The polyamines putrescine (1,4-diaminobutane), spermidine (1,8-diamino-4-aza-octane, 2), and spermine (1,12-diamino-4,9-diazadodecane, 3) (Fig. 1) are ubiquitous polycationic compounds that are found in significant amounts in nearly every prokaryotic and eukaryotic cell type. Spermidine and spermine primarily exist in aqueous solution at pH 7.4 as fully protonated polycations and possess the pKa values indicated in Fig. 1 (1). This high degree of positive charge is an important factor in the biological functions of these molecules, and, as will be discussed later in this chapter, alterations in the pKa of polyamine nitrogens can affect and disrupt their cellular function. Polyamines are widely distributed in nature and are known to be required in micromolar to millimolar concentrations to support a wide variety of cellular functions. However, data that establish the precise role of the polyamines and their analogs in cellular processes are incomplete. The ongoing identification of new functions for the polyamines ensures that new avenues for research are arising continuously in an extremely diverse set of disciplines. The human and mammalian pathways for polyamine metabolism have been extensively studied, and analogous pathways have been elucidated for a relatively small number of organisms. There are important interspecies differences in polyamine metabolism, especially among eukaryotic cells, plants, and some bacteria and protozoa. In some prokaryotes, only putrescine and spermidine are synthesized, whereas in other cases, such as certain thermophilic bacteria, polyamines with chains longer than spermine are found. In some parasitic organisms, there are additional enzymes that are not present in the host cell, and, as such, provide a target for the design of specific antiparasitic agents. 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 (2). Keeping in mind the diverse nature of polyamine distribution and function, it is reasonable

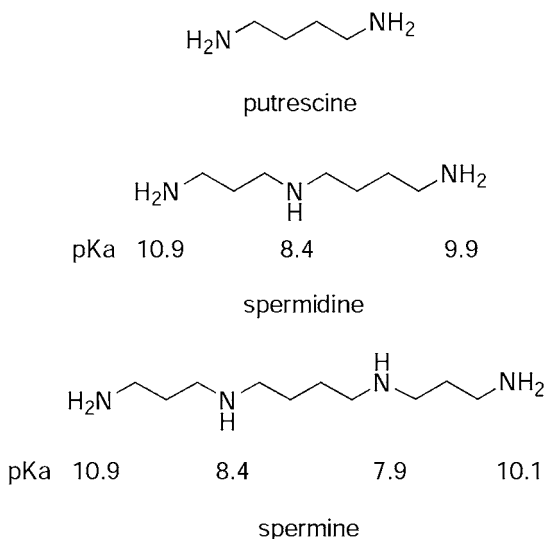


Fig. 1. Structures of putrescine, spermidine, and spermine, and pKa values for spermidine and spermine.

to assume that carefully designed polyamine analogs could have the potential to disrupt polyamine metabolism, and thus such agents have been investigated as potential therapeutic agents *in vitro* and *in vivo*. The polyamine pathway represents an important target for chemotherapeutic intervention because depletion of polyamines results in the disruption of a variety of cellular functions and may, in specific cases, result in cytotoxicity (3,4). This chapter will summarize the development of synthetic derivatives of the polyamines, and describe their use as potential chemotherapeutic agents. A comprehensive review of polyamine biosynthesis inhibitors (4) and a review of the role of polyamines in normal and tumor cell metabolism (5) have recently been published.

## 2. Polyamine Biochemistry

The biosynthesis and catabolism of the polyamines putrescine, spermidine, and spermine are carefully controlled processes in all eukaryotic cell types. The mammalian polyamine biosynthetic pathway is shown in Fig. 2. Although definitive mechanisms for the various functions of the polyamines have not been fully elucidated, it is known that they are absolutely required for normal cell homeostasis. Inhibition of the polyamine pathway, therefore, is viewed as a valid target for the design of antitumor or antiparasitic agents. Available compounds that specifically inhibit individual enzymes in the pathway are extremely useful as research tools to elucidate the cellular functions of the naturally occurring polyamines. Specific inhibitors have now been developed for the enzymes in the forward polyamine biosynthetic pathway, ornithine decarboxylase (ODC), *S*-adenosylmethionine decarboxylase (AdoMet-DC), and for the aminopropyl-

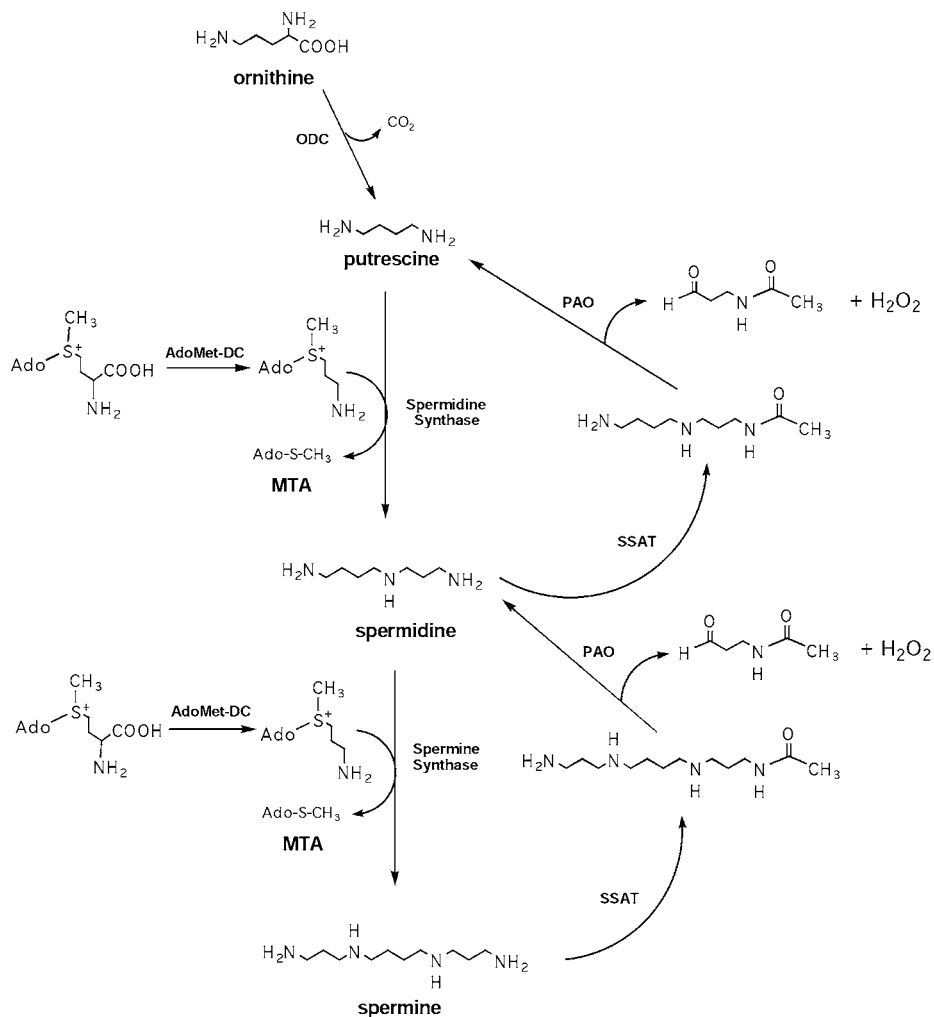


Fig. 2. The mammalian polyamine metabolic pathway.

transferases spermidine synthase and spermine synthase. These inhibitors produce a variety of responses ranging from cessation of cell growth to overt cytotoxicity (6,7). The range of these activities appears to be both agent- and cell type-specific.

Polyamine metabolism can be viewed as having forward and reverse component pathways, although careful cellular control of these enzymes and the polyamine transporter act in concert to maintain appropriate levels of the individual polyamines. Ornithine is converted to putrescine by ODC, a typical pyridoxal phosphate-requiring amino acid decarboxylase. ODC is one of the control points in the pathway, producing a product that is committed to polyamine biosynthesis. ODC levels are modulated by

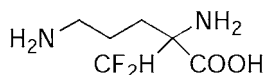


Fig. 3. The structure of  $\alpha$ -difluoromethylornithine (DFMO).

synthesis and degradation of ODC protein, with a half-life of about 10 min. In mammalian cells, the degradation of ODC is facilitated by a specific ODC-antizyme (8), a protein that also appears to downregulate polyamine transport. Competitive inhibitors of ODC have proven to be of limited value, and most useful inhibitor of ODC to date,  $\alpha$ -difluoromethylornithine (DFMO; Fig. 3) (9), is an irreversible inactivator of the enzyme. The discovery of DFMO provided an enormous stimulus to the field of mammalian polyamine biology, and led to marketing of the drug as a treatment for *Pneumocystis carinii* secondary infections. DFMO is also quite effective for the treatment of late-stage West African trypanosomiasis.

Putrescine is next converted to spermidine by the aminopropyltransferase spermidine synthase. A second closely related but distinct aminopropyltransferase, spermine synthase, then adds an additional aminopropyl group to spermidine to yield spermine, the longest mammalian polyamine. The byproduct for the spermidine and spermine synthase reactions is 5'-methylthioadenosine, generated from the cosubstrate, decarboxylated *S*-adenosylmethionine (dc-AdoMet). 5'-Methylthioadenosine is a potent product inhibitor for the aminopropyl transfer process, and must be rapidly hydrolyzed by 5'-methylthioadenosine-phosphorylase to maintain the forward pathway. Selective inhibition of the individual aminopropyltransferases has proven to be a significant problem because of the similarity of the reactions catalyzed by the two enzymes. The transition state analogs *S*-adenosyl-1,8-diamino-3-thiooctane (10) and *S*-adenosyl-1,12-diamino-3-thio-9-azadodecane (11) remain the only known specific inhibitors of the individual aminopropyltransferases.

The aminopropyl donor for both aminopropyltransferases is dc-AdoMet, produced from AdoMet by the action of AdoMet-DC. AdoMet-DC, like ODC, is a highly regulated enzyme in mammalian cells and belongs to a small class of proteins known as pyruvoyl enzymes. All of the known forms of AdoMet-DC contain a covalently bound pyruvate prosthetic group that is required for activity (12), and formation of a Schiff's base between AdoMet and this pyruvate is a prerequisite for the reaction to occur. The antileukemic agent methylglyoxal bis(guanylhydrazone) (Fig. 4) is a potent competitive inhibitor of the putrescine-activated mammalian enzyme, with a  $K_i$  value of less than 1  $\mu M$  (6), but is of limited use as a chemotherapeutic agent because of excessive toxicity. The AdoMet analog 5'-{[(*Z*)-4-amino-2-butenyl]methylamino}-5'-deoxyadenosine (AbeAdo; Fig. 4), is a potent enzyme-activated inhibitor of AdoMet-DC from *Escherichia coli*, and produces a long-lasting, dose-dependent decrease in AdoMet-DC activity in vivo (13). Several additional inactivators of AdoMet-DC have been described (4).

The so-called reverse polyamine metabolic pathway provides further control of cellular polyamine levels through acetylation and subsequent oxidative deamination processes. In the cell nucleus, spermidine is acetylated on the four carbon end by sper-



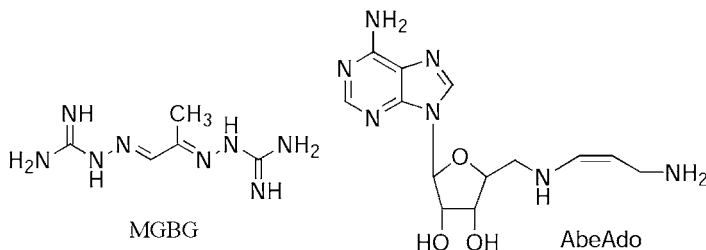


Fig. 4. Structures of the AdoMet-DC inhibitors of MGBG and AbeAdo.

midine- $N^8$ -acetyltransferase, possibly altering the compound's binding affinity for DNA. A specific deacetylase can then reverse this enzymatic acetylation (14). Neither of these enzymes affects the level of histone acetylation. Cytoplasmic spermidine and spermine are acetylated on the three carbon end by spermidine/spermine- $N^1$ -acetyltransferase (SSAT; Fig. 2) (4,15). This enzyme is the first and rate-limiting step in the catabolic interconversion of putrescine, spermidine, and spermine; its kinetics and substrate specificity have been described elsewhere (4). Acetylated spermidine or spermine can be exported from the cell or oxidized by acetyl polyamine oxidase (PAO) to form 3-acetamidopropionaldehyde and either putrescine or spermidine, respectively (Fig. 2). SSAT and PAO together serve to reverse polyamine biosynthesis, facilitating the interconversion 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, allow the cell considerable control of intracellular polyamine concentrations.

A final method of controlling intracellular polyamine levels is afforded by one or more specific polyamine transport mechanisms (16,17). 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 (18). Specific polyamine transporters have been detected in yeast, *Trypanosoma cruzi* epimastigotes, *Crithidia fasciculata*, and *Leishmania donovani*. The process of polyamine transport in mammalian cells is poorly understood, and, to date, none of the proteins involved has 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. Several factors have been shown to alter the polyamine transport system, and, as a result, cellular homeostasis. It is worth noting that the polyamine system is significantly upregulated in a variety of tumor cells; thus, this system is regarded as a potential target for cancer chemotherapy (19). Efforts to synthesize specific polyamine transport inhibitors have recently begun in several laboratories; a complete discussion of these efforts is beyond the scope of this chapter and has been previously reviewed (17,19).

### 2.1. Symmetrical, Terminally Alkylated Polyamine Analogs

The development of analogs of spermidine and spermine as potential antitumor agents was initiated in the mid-1980s. Initially, these analogs were structurally similar

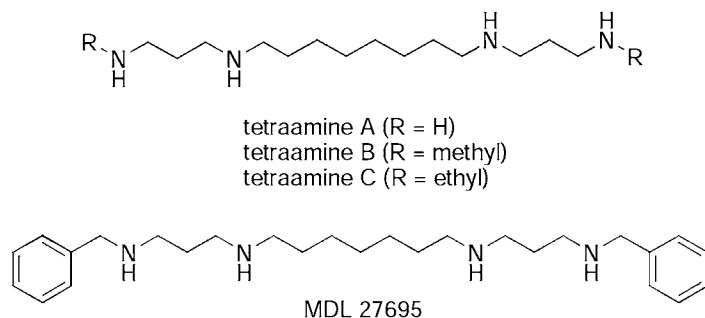


Fig. 5. The earliest known polyamine analogs with antitumor effects.

to the natural polyamines in that they had terminal primary amine groups, with variations in the length of the intermediate carbon chains. Edwards and coworkers synthesized a series of diamines and triamines related to spermidine and a series of tetraamines derived from 1,8-diaminooctane; these analogs were evaluated for antitumor activity in cultured L1210 cells (20). In the series of diamines and triamines, substitution of alkyl groups at the terminal nitrogens, or replacing the central nitrogens with other heteroatoms, failed to produce spermidine analogs with antitumor effects superior to norspermidine. However, compounds with eight carbons between the central nitrogens, such as tetraamine A (Fig. 5), generally showed significant antitumor activity. Tetraamine A increased survival time in male mice inoculated with L1210 leukemia from 7.7 to 16.2 d. Coadministration of spermidine was shown to reverse the antitumor activity of this compound, presumably because of a competition for the polyamine transport system, whereas coadministration of a polyamine oxidase inhibitor potentiated the observed antitumor activity, suggesting that these analogs may be metabolized by polyamine oxidase. Tetraamines B and C (R = CH<sub>3</sub> or R = CH<sub>2</sub>CH<sub>3</sub>, respectively) were also active in the L1210 model, but substitution of larger alkyl groups resulted in a reduction of activity. In a related study, a series of *bis*benzyl analogs related to MDL 27695 and an additional series of substituted tetraamines were evaluated for the ability to inhibit proliferation of HeLa cells (21). The *bis*(benzyl)polyamine analog MDL 27695 (Fig. 5) and tetraamine A were active antiproliferative compounds, exhibiting IC<sub>50</sub> values of 5 and 50 μM, respectively. Interestingly, no correlation between the DNA binding properties and antitumor activity of these analogs was detected.

Subsequent attempts to develop polyamine analogs as potential modulators of polyamine function focused on the synthesis of symmetrical, terminally substituted *bis*(alkyl)polyamines. These analogs were designed in response to the finding that natural polyamines use several feedback mechanisms that autoregulate their synthesis (22) and that they can be taken into cells by the energy-dependent transport systems described previously. Several symmetrically substituted polyamine analogs have been synthesized that enter the cell using the polyamine transport system. These analogs specifically slow the synthesis of polyamines by downregulation of the biosynthetic

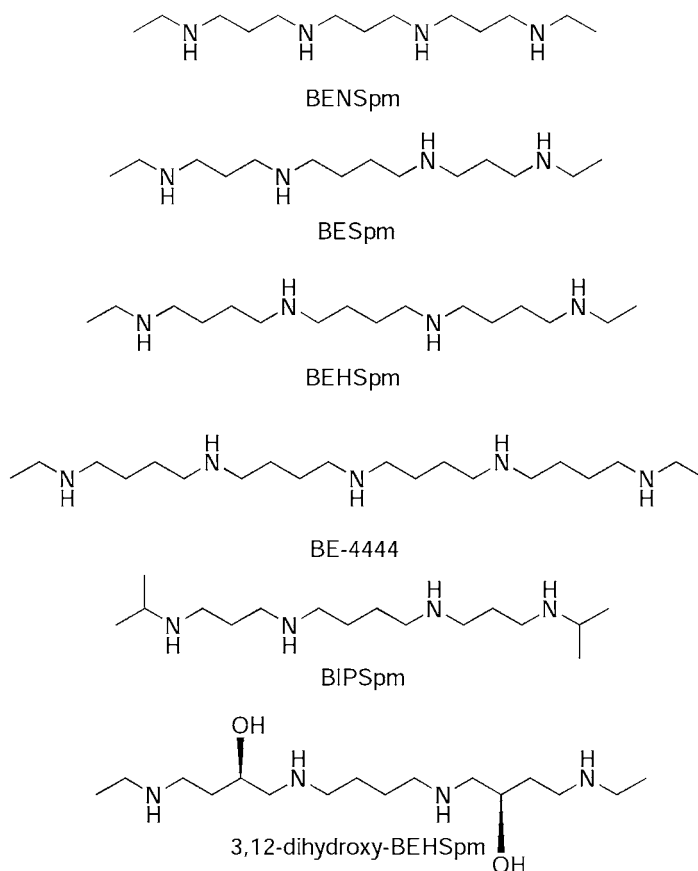


Fig. 6. Structures of *bis*(ethyl)polyamine analogs BENSpm, BESpm, BEHSpm, BIPSpm, BE-4444, and 3,12-dihydroxy-BEHSpm.

enzymes ODC and AdoMet-DC, but cannot substitute for the natural polyamines in terms of their cell growth and survival functions (4,23). As will be discussed, some but not all alkylpolyamine analogs are potent inducers of SSAT in cultured tumor cells, an effect that leads to the induction of apoptosis. The most successful of the symmetrically substituted polyamine analogs to date are the *N,N'*-*bis*(ethyl)polyamines shown in Fig. 6: *bis*(ethyl)norspermine (BENSpm), *bis*(ethyl)spermine (BESpm), *bis*(ethyl)homospermine (BEHSpm), and 1,20-(ethylamino)-5,10,15-triazanonadecane (BE-4444). These compounds have been shown to possess a wide variety of therapeutic effects and illustrate that small structural changes in alkylpolyamine analogs can result in surprisingly significant changes in biological activity.

A major advantage of the *bis*(ethyl)polyamines lies in the fact that their synthesis is extremely straightforward and depends only on the availability of the appropriate parent polyamine backbone. Functionalization of the terminal nitrogens can be readily

accomplished by protecting all of the nitrogens in the parent chain with a tosyl or mesityl protecting group, producing an intermediate that possesses acidic hydrogens at the terminal nitrogen moieties, and unreactive central nitrogens. Alkylation of the terminal nitrogens is then accomplished by a sodium hydride-catalyzed reaction with ethyl bromide followed by deprotection of the nitrogens and recrystallization from ethanol/water (23–25). The *N,N'*-bis(ethyl)polyamines are readily transported into mammalian cells by the same transport mechanism as the natural polyamines (26). Treatment of mammalian cells with these analogs leads to a reduction of putrescine, spermidine, and spermine, downregulation of ODC and AdoMetDC, and, depending on the cell lines used, cytostasis or cytotoxicity (23,26). These effects are accompanied by a tremendous induction of SSAT activity, in some cases as much as 1000-fold. Preliminary structure/activity correlations, based only on data from the symmetrically alkylated polyamine analogs, 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 (23). It was further determined that the greatest induction of SSAT was dependent on the presence of “protected” aminopropyl or aminobutyl moieties (27–29). Adding terminal nitrogen *bis*(alkyl) substituents larger than ethyl resulted in a dramatic reduction in antitumor activity (23,30,31). Compounds with a 3-3-3 carbon skeleton were more effective than the corresponding 3-4-3 analogs, and spermine-like compounds (3-3-3 or 3-4-3) are more effective than spermidine-like analogs (3-3 or 3-4). However, these data were collected using only symmetrically substituted *bis*-alkylpolyamines, and as a result, only *bis*(ethyl)-substituted analogs were advanced to clinical trials. Among these analogs, the most promising were  $N^1,N^{11}$ -*bis*(ethyl)norspermine (BENSpm; Fig. 6), which has a 3-3-3 backbone;  $N^1,N^{14}$ -*bis*(ethyl)norspermine (BEHSpm; Fig. 6), which has a 4-4-4 carbon skeleton; and 1,20-*bis*(ethylamino)-5,10,15-triazanonadecane (BE-4444; Fig. 6), which has a 4-4-4-4 architecture. Interestingly, BEHSpm proved to be useful as an antidiarrheal agent (32) and was advanced to clinical trials for this indication.

BENSpm 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 and xenografts, human MALME-3 melanoma xenografts, melanocytes, human bladder cancer cells, and ovarian carcinoma tumor cells (4). In CaCO<sub>2</sub> colon cancer cells, the analog causes induction of SSAT, downregulation of ODC, and depletion of cellular polyamines, resulting in cytotoxicity (33). In addition, SSAT induction appears to be the common event leading to cytotoxicity in non-small-cell lung (SCLC) tumor explants (34). Presumably because of the proprietary nature of data concerning BENSpm, no human clinical trials involving the compound have been published, although it is known anecdotally that these trials were initiated. The closely related analog BEHSpm (Fig. 6) does not show similar promise as an antitumor agent (35), but is being developed as an effective treatment for AIDS-related diarrhea (32,35,36). The potent antidiarrheal activity of BEHSpm has been demonstrated in several animal models, and in human clinical trials involving patients with AIDS-related diarrhea. A limited structure/activity study was conducted, and the closely related analog  $N^1,N^{12}$ -*bis*(isopropyl)spermine (BIPSp; Fig. 6) proved

to be the most active antidiarrheal in the series (32). The pharmacokinetics of BENSpm (37) and BEHSpm (38) for in vivo metabolism have been described. BENSpm is metabolized by *N*-de-ethylation and stepwise removal of aminopropyl equivalents by SSAT and PAO, with a half-life of 73 min. BEHSpm was metabolized almost exclusively to homospermine, which cannot serve as a substrate for SSAT and thus persists in tissues for a period of weeks (liver  $T_{1/2} = 15.4$  d). Chronic administration of BEHSpm results in tissue accumulation of the analog and homospermine, resulting in disruption of normal polyamine metabolism. The metabolically programmed alkylpolyamine 3,12-dihydroxyBEHSpm (Fig. 6) retained the antidiarrheal activity of the parent BEHSpm, and exhibited a significantly diminished tissue half-life, presumably from the metabolic “handles” provided by the hydroxyl groups (39). The alkylpolyamine BE-4444 (Fig. 6) was originally designed based on the hypothesis that analogs with chain lengths different from spermine could exhibit enhanced binding to DNA and thus exert antiproliferative effects (40). BE-4444 has been shown to be effective in cultured U-251, MG, SF-126, and SF-188 brain tumor cells at a concentration of 5  $\mu\text{M}$  (40,41) and against DU-145, LNCaP, and PC-3 prostate cancer cells in vitro and in vivo (42).

Specific alterations to the polyamine backbone structure of BESpm, BEHSpm, and BE-4444 has resulted in a series of “second-generation” *bis*(ethyl)polyamines with impressive antitumor and antiparasitic activity. Restriction of rotation in the central region of the polyamine chain in BESpm by including a *cis*- and *trans*-cyclopropyl or cyclobutyl ring, a *cis*- and *trans*-double bond, a triple bond, and a 1,2-disubstituted aromatic ring produced analogs (Fig. 7) with varying antitumor activity in a panel of human tumor cell lines (A549, HT-29, U251MG, DU145, PC-3, and MCF7) (43). There was little difference between the *cis* and *trans* isomers in the cyclopropyl, cyclobutyl, and double bond-containing analogs, and the triple bond and aromatic substituents rendered the resulting analogs inactive. All of the analogs were imported by the polyamine transport system, suggesting that the lack of activity was because of diminished DNA binding affinity. In like fashion, insertion of a central dimethylsilane group resulted in a significant decrease in growth inhibition when compared with the *bis*(ethyl)polyamine analogs (44). It was later found that the *bis*(ethyl)spermine analog  $N^1, N^{12}$ -*bis*(ethyl)-*cis*-6,7-dehydrospermine (SL-11047; Fig. 8) was an effective treatment for *Cryptosporidium parvum* infections, producing cures in a murine model (45). By contrast, the 4-4-4 (homospermine) analog SL-11093 (Fig. 8), which contains a *trans* cyclopropyl moiety in the central region, was an effective antitumor agent in vitro, and in vivo against DU-145 nude mouse xenografts (46). Compounds with a 4-4-4 or 4-4-4-4-4 backbone that featured *trans*-cyclopropyl or a *trans*-cyclobutyl moieties in noncentral regions of the chain were more active in vitro against prostate tumor cell lines (LnCap, DU145, DUPRO, and PC-3), and inclusion of a *cis* unsaturation in one of the terminal aminobutyl groups also enhanced activity, presumably by enhancing DNA binding (47). The *trans*-*bis*-cyclopropyl analogs *bis*(cyclopropane)tetramine A and *bis*(cyclopropyl)hexamine B (Fig. 8) were effective antitumor agents against Du-Pro and DU-145 prostate tumor cells in vitro (48). In general, structural modifications to homospermine-like backbones that are analogous to those made to the BESpm backbone (i.e., *cis*- and *trans*-cyclopropyl, cyclobutyl, and double-bond moieties) afforded

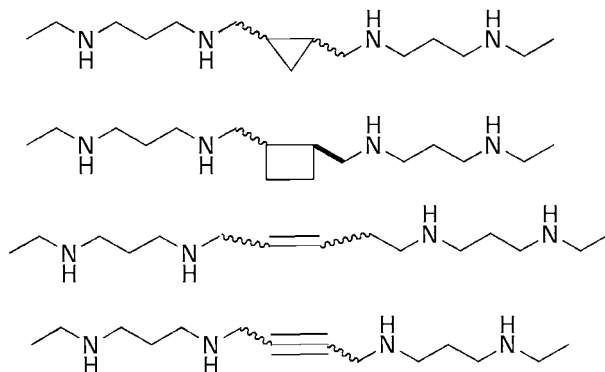


Fig. 7. Conformationally restricted analogs of BESpm.

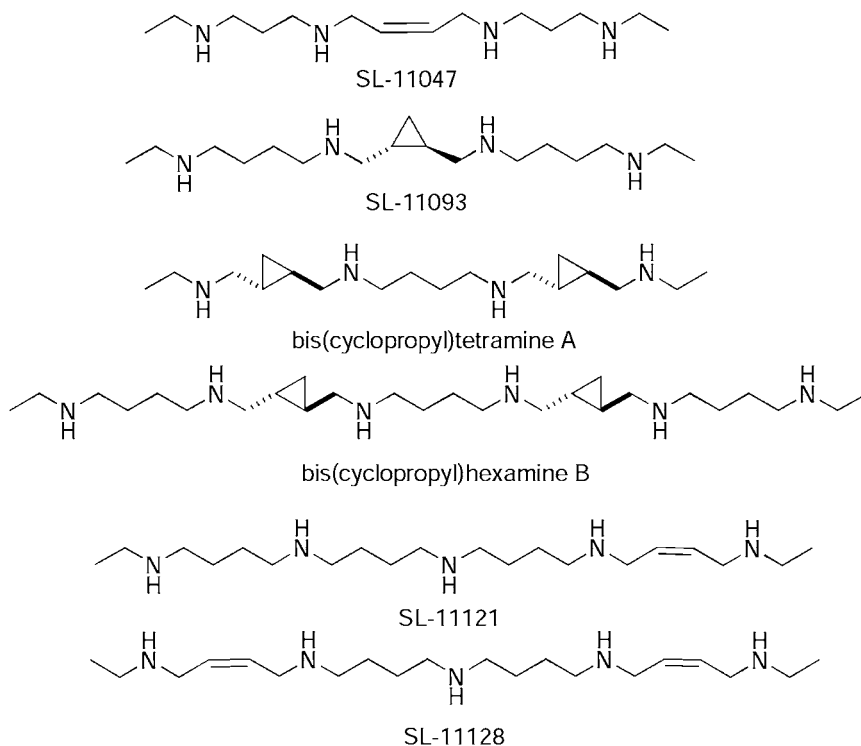


Fig. 8. Conformationally restricted *bis*(ethyl)polyamine analogs with enhanced antitumor activity against prostate tumor cells in culture.

analogues with enhanced antitumor activity and diminished systemic toxicity. In addition, insertion of a *cis* double bond into the terminal aminobutyl moieties of BE-4444

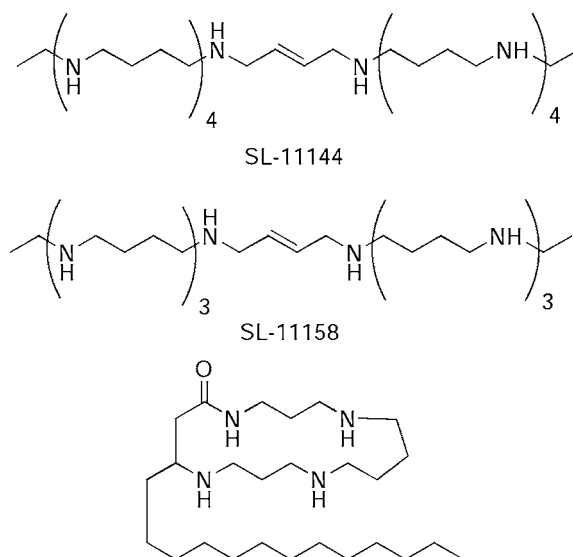


Fig. 9. Structures of oligamines and macrocyclic polyamines with antitumor activity.

(i.e., SL-11121 and SL-11128, Fig. 8) also affords analogs that are equipotent to BE-4444 with respect to  $ID_{50}$  values, but that are an order of magnitude more cytotoxic in a dose-response study (49).

Recently, a series of *bis*(ethyl)oligamine analogs have been described that show promise as potential chemotherapeutic agents. In the limited series of oligamines that were evaluated, the decamine SL-11144 and the octamine SL-11158 (Fig. 9) proved to be most growth inhibitory against a panel of prostate tumor cells in vitro (LnCap, DU-145, DuPro, and PC-3). Not surprisingly, their activity roughly correlated with their ability to aggregate DNA (50). It has been shown that macrocyclic polyamines known as budmunchiamines act as potent antitumor agents by virtue of their ability to selectively deplete adenosine triphosphate. Based on this observation, a series of five macrocyclic polyamines with the representative structure shown in Fig. 9 were synthesized and evaluated as antitumor agents in the DuPro and PC-3 prostate cell lines (51). All five of these analogs were readily imported by cells and caused a dramatic depletion of cellular polyamines. These compounds also proved to be cytotoxic in the tumor lines tested, and the degree of cytotoxicity roughly correlated to their ability to deplete adenosine triphosphate.

One unusual characteristic of the *bis*(ethyl)polyamines is their ability to produce cell type-specific cytotoxicity in two representative lung cancer cell types, NCI H157 non-SCLC and H82 SCLC. Soon after the first alkylpolyamines were described, it was shown that the *bis*(ethyl)polyamines were cytotoxic to DFMO-resistant H157 cells (29) that are clinically characterized as being refractory to all treatment modalities. By contrast, the *bis*(ethyl)polyamines are relatively ineffective against DFMO-sensitive SCLC lines. The mechanisms underlying the observed differential sensitivities are still being elucidated, but it was noted that unusually high induction of SSAT (in some cases

>1000 fold) in cell types that respond to *bis*(ethyl)polyamine analogs, but not in the refractory cell lines, and a lack of SSAT induction in the refractory SCLC line H82 (15,29). In H157 cells in culture, the induction of SSAT correlated with a time- and dose-dependent increase in SSAT steady-state messenger RNA levels, suggesting a transcriptional level of control over SSAT synthesis. The correlation between high induction of the SSAT activity and cytotoxicity was subsequently demonstrated for other examples of human malignancies, including human melanomas.

## 2.2. Unsymmetrically Substituted Alkylpolyamine Analogs

Structure activity studies involving the *bis*(ethyl)polyamines revealed much about the role of charge and flexibility in the polyamine backbone structure. However, the most useful compounds were symmetrically substituted with ethyl groups at the terminal nitrogens, and it was concluded that substituents of greater size than ethyl would render a molecule inactive. It was clear that unsymmetrically substituted alkylpolyamines needed to be synthesized to determine the optimal substituent pattern for the terminal nitrogens, and to explore the chemical space surrounding the terminal alkyl groups. Compounds that possessed unsymmetrically substituted terminal nitrogens were first described in 1993 (52), the first of which being *N*<sup>1</sup>-propargyl-*N*<sup>11</sup>-ethyl norspermine (PENSp<sub>m</sub>) and *N*<sup>1</sup>-cyclopropylmethyl-*N*<sup>11</sup>-ethyl norspermine (CPENSp<sub>m</sub>), which are shown in Fig. 10. In general, the synthesis of these and other unsymmetrically substituted analogs is more difficult because it requires selective protection and deprotection of the internal and external nitrogens. Preliminary results indicated that both PENSp<sub>m</sub> (IC<sub>50</sub> = 1.1 μM) and CPENSp<sub>m</sub> (IC<sub>50</sub> = 1.1 μM) were as active or more active than BESp<sub>m</sub>, both with respect to SSAT induction and cytotoxicity in H157 cells in culture. These analogs also retained the cell type-specific cytotoxic activity observed after treatment with BESp<sub>m</sub>, and their activity was directly correlated to their ability to induce SSAT. This increase in SSAT activity was accompanied by a cell-specific increase in steady-state SSAT messenger RNA that was similar in magnitude to that observed after treatment of the H157 cells with BESp<sub>m</sub> or BENS<sub>m</sub>. These data suggest a similar mechanism of induction of SSAT for these compounds, and 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*<sup>1</sup>-cycloheptylmethyl-*N*<sup>11</sup>-ethyl norspermine (CHENSp<sub>m</sub>, Fig. 10), was subsequently synthesized and evaluated (4) and was found to retain antitumor activity (IC<sub>50</sub> = 0.25 μM against non-SCLC cells) while producing a less-pronounced cell type-specificity. However, when the induction of SSAT and polyamine levels in the H157 cell line were measured, the analog showed striking differences from the parent analog BESp<sub>m</sub>. Treatment of H157 cells with 10 μM BESp<sub>m</sub> 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 analog itself, a response that is typical among SSAT-inducing alkylpolyamines. Surprisingly, treatment with 10 μM CHENSp<sub>m</sub> had almost no effect on the levels of putrescine, spermidine, and spermine, and caused only a 15-fold induction of SSAT activity (4). These data suggested that the cytotoxic effects produced by BENS<sub>m</sub>,



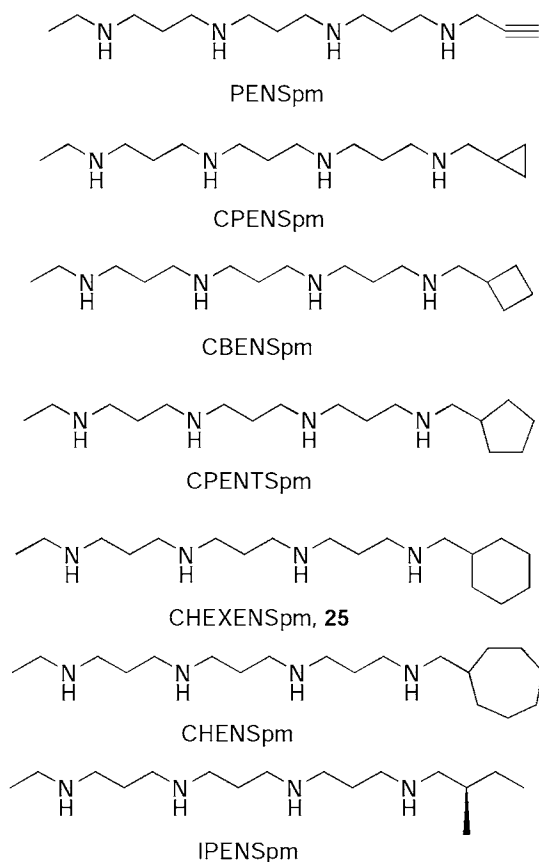


Fig. 10. The unsymmetrically substituted alkylpolyamines PENSpm, CPENSpm, CBENSpm, CPENTSpM, CHEXENSpm, CHENSpm, and IPENSpm.

PENSpm, and CPENSpm in H157 cells could be mediated by different cellular mechanisms than the effects produced by CHENSpm. In response to these findings, three additional compounds were synthesized that possessed substituents containing the intervening ring sizes (CBENSpm, CPENTSpM, and CHEXENSpm), as shown in Fig. 10. All three analogs were generally cytotoxic in both the H157 and H82 cell lines, but there was no correlation between the induction of SSAT and the  $IC_{50}$  value in the H157 line, as shown in Fig. 11. In this cycloalkyl series, the induction of SSAT decreased dramatically as a function of ring size, whereas the  $IC_{50}$  values were remarkably constant (0.4–0.7  $\mu M$ ). 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.

Based on data obtained for unsymmetrically substituted alkylpolyamine analogs, it is evident that the structure/activity relationships (SAR) of these polyamine analogs is

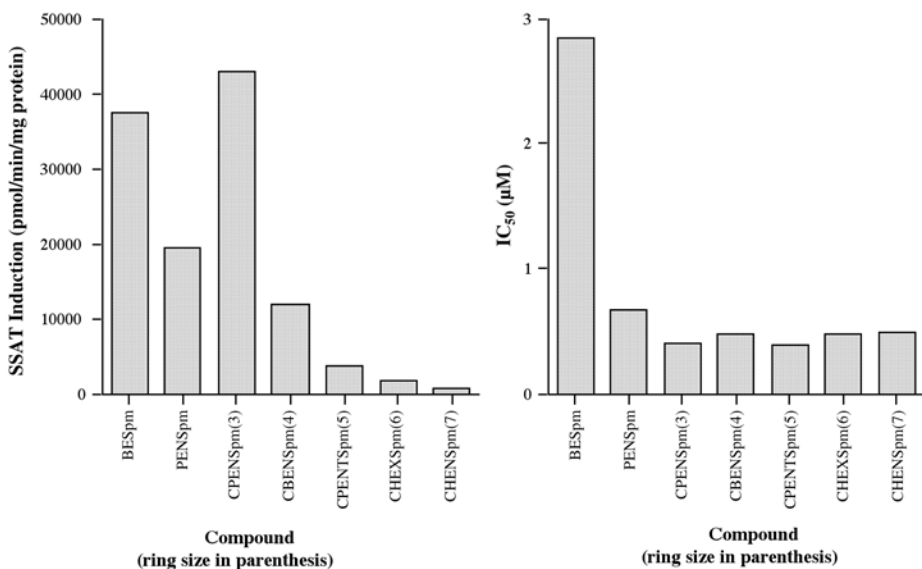


Fig. 11. 96 h SSAT induction and IC<sub>50</sub> values in the NCI H157 cell line for selected alkylpolyamine analogs.

more complex than originally postulated. Optimal activity is obtained from molecules that possess secondary terminal nitrogens, but only one of the two terminal nitrogens of the backbone need be substituted with a small alkyl group. Structurally similar compounds, like CPENSpm and CHENSpm, which differ only in the number of carbons in the cycloalkyl substituents, appear to inhibit cell growth by completely different mechanisms. It has also been noted that compounds that significantly induce SSAT tend to be more cell type-specific in their activity than those that do not (4).

In addition to the lung cancer model, unsymmetrically substituted polyamine analogs have been evaluated in prostate cancer model systems (53). CPENSpm and CHENSpm were cytotoxic to the DU145 cell line at concentrations  $\geq 1 \mu\text{M}$  with significant accumulation of each analog, and CHENSpm was found to be cytotoxic to the DU145, PC-3, and LnCap cell lines at  $30 \mu\text{M}$ . Although the effects of these analogs on the polyamine metabolic pathway appear to be modest in the prostate lines, the cytotoxicity produced in these cells at low concentrations encourages further study.

The data outlined here suggest that unsymmetrically substituted alkylpolyamines can exhibit varying degrees of SSAT induction based on the size of their terminal alkyl substituents. The SAR model for unsymmetrically substituted alkylpolyamines that superinduce SSAT is shown in Fig. 12A (4). Analogs in this subclass generally possess a 3-3-3 or 3-4-3 carbon skeleton and a *bis*(alkyl) substitution pattern on the terminal nitrogens. Unsymmetrically substituted analogs induce as well or better than the parent analogs BESpm and BENSpm. When R<sub>1</sub> is ethyl, R<sub>2</sub> can vary in size from small (e.g., ethyl, cyclopropylmethyl) to medium size; however, the size of only one of the substituents can be increased beyond ethyl. The central nitrogens are separated by 5.0–5.8 Å,

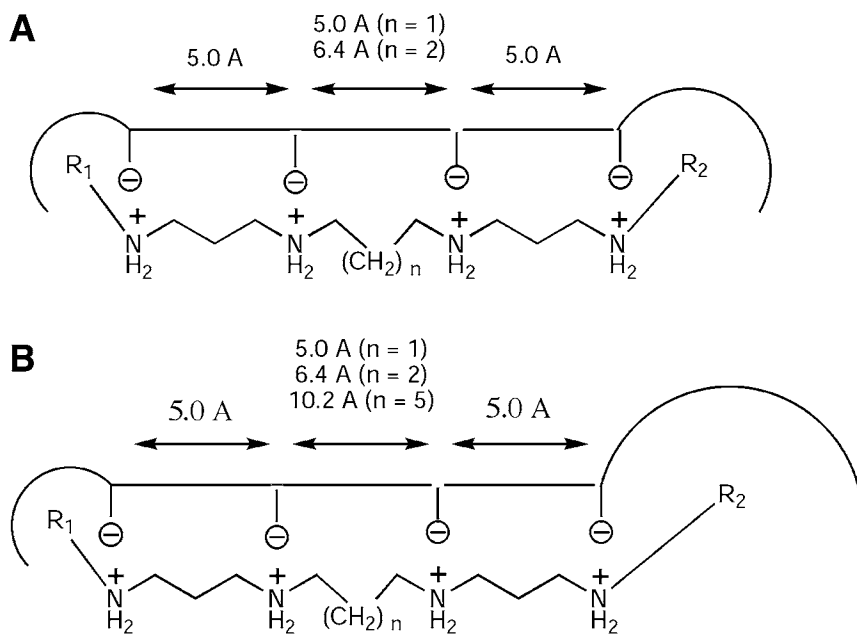


Fig. 12. SAR models for alkylpolyamine analogs. (A) SSAT induction-dependent antitumor agents. (B) SSAT induction-dependent alkylpolyamine antitumor agents and antiparasitic agents.

and each terminal nitrogen is 5.0 Å from the adjacent central nitrogen. The binding pocket for R<sub>1</sub> will accept only a small alkyl group, whereas the R<sub>2</sub> binding pocket can accommodate medium sized groups up to the size of cyclopentylmethyl. Both spermine and spermidine analogs can bind to this site, because both types of analogs are capable of superinducing SSAT. The cytotoxicity of agents that fit these criteria can be directly related to the superinduction of SSAT.

The model for the design of SSAT induction-independent alkylpolyamine analogs is shown in Fig. 12B (4). The requirement for *bis*(alkyl) substitution remains, but the binding pockets for R<sub>1</sub> and R<sub>2</sub> seem to be less restrictive. The most active analogs have a small (ethyl) or medium sized R<sub>1</sub> and a large R<sub>2</sub> (e.g., cycloheptylmethyl or cyclohexylmethyl). It is required that one of the alkyl groups must be small (e.g., ethyl) whereas the other must be larger than cyclopentylmethyl. Agents with two large terminal alkyl substituents may possess activity, but are not likely to superinduce SSAT. Active analogs with a 3-3-3 and with a 3-7-3 carbon skeleton have been identified, indicating that active 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. 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 data suggest that agents that fit these criteria produce cytotoxicity through an as yet undetermined pathway. It has also been noted that analogs

with a 3-7-3 architecture can act as antiparasitic agents, a topic that is beyond the scope of this chapter.

### 3. Mechanisms for Alkylpolyamine-Induced Cytotoxicity

It is clear that alkylpolyamines are capable of producing rapid cytotoxicity in lung and prostate tumor cell lines, but the mechanistic aspects of these effects must still be fully elucidated. However, the induction of programmed cell death (PCD) appears to be a common result after treatment with alkylpolyamines from both structural classes mentioned previously. This effect was first observed in the MCF-7 and MDA-MB-468 breast cancer lines, and later in the H-157 non-SCLC human lung tumor cell line after treatment with CPENSpm (54). In the case of the breast cancer lines, greater than 90% growth inhibition was observed after prolonged treatment with CPENSpm in each of six cell lines tested. The  $IC_{50}$  values for inhibition by CPENSpm in these six breast tumor lines ranged from 0.2 to 1.3  $\mu M$ . 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  $\mu M$  concentration and after 96 h with as little as 1  $\mu M$ . Similar results were observed in other breast cancer lines including: T47D, Zr-75-1, MDA 231, and Hs578t. 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 (53). High molecular weight ( $\geq 50$  kbp) DNA fragmentation was observed after 24 h exposure to 10  $\mu M$  CPENSpm. Similar results were observed with 10  $\mu M$  BENSpm, but only after 48 h, although the initiation of PCD in Chinese hamster ovary (CHO) cells is quite rapid at high concentrations of the analog (4). Although these results clearly indicate that the unsymmetrically substituted analogs induce PCD, the underlying cellular mechanism(s) had not been elucidated.

Acetylation and subsequent oxidation of polyamines by the SSAT/ PAO pathway is known to produce  $H_2O_2$  as a byproduct. During superinduction of SSAT, PCD produced by CPENSpm in H157 cells may result from oxidative stress resulting from  $H_2O_2$  overproduction. When catalase is added in combination with CPENSpm, high molecular weight DNA fragmentation and early fragmentation of the nuclei are greatly reduced (55). Inhibition of PAO by the specific inhibitor *N,N'*-bis(2,3-butadienyl)-1,4-butane-diamine (MDL 72527) resulted in a significant reduction in the formation of high molecular weight DNA, and similarly reduced the number of apoptotic nuclei formed after CPENSpm treatment. These results strongly suggest that  $H_2O_2$  production by PAO has a role in compound CPENSpm-induced cytotoxicity in H157 cells. Catalase or MDL 72527 had no effect on the formation of high molecular weight DNA fragments or apoptotic bodies when coadministered with CHENSpm, supporting the contention that CPENSpm and CHENSpm produce apoptosis by different mechanisms. Treatment of wild-type H157 cells with both CPENSpm and CHENSpm leads to the activation of caspase-3 and cleavage of poly (adenosine 5'-diphosphate-ribose) polymerase (4). In H157 cells that overexpress Bcl-2, many of the known steps of the cell death program, including caspase-3 activation, poly (adenosine 5'-diphosphate-ribose) polymerase cleavage, and the release of cytochrome c from the mitochondria, were blocked in analog-treated H157 cells. However, the overexpression of Bcl-2 was

only able to alter the kinetics of PCD, not completely block it. Thus, both CPENSpm and CHENSpm are capable of inducing PCD in a caspase-3-independent manner.

Several groups have demonstrated that some polyamine analogs (e.g., BE-4444, CHENSpm) that do not superinduce SSAT can still produce PCD. Consistent with this hypothesis is the observation that CPENSpm and CHENSpm have dramatically different effects on the cell cycle (56). After 24 h treatment of H157 non-SCLC with 10  $\mu\text{M}$  CPENSpm, no significant effects on cell cycle are observed by flow cytometric analysis. However, under the same conditions, 10  $\mu\text{M}$  CHENSpm produces a dramatic G<sub>2</sub>/M cell cycle block in normal and Bcl-2-overexpressing H157 cells (4). The analog *S*-1- $\{N-[(2\text{-methyl-}1\text{-butyl)]\text{amino}\}-11-[N-(\text{ethyl})\text{amino}]-4,8\text{-diazaundecane}$  (IPENSpm; Fig. 10) was subsequently found to produce a similar G<sub>2</sub>/M cell-cycle arrest (57). All three analogs demonstrated similar cytotoxic effects in the human non-SCLC line, NCI H157, where they were found to be cytotoxic at concentrations greater than 0.1  $\mu\text{M}$ , but significant induction of SSAT activity was only observed in cells treated with CPENSpm. The effects of all three compounds on the cell cycle progress were analyzed by flow cytometry after a 24-h exposure to 10  $\mu\text{M}$  of each compound. As previously observed, CPENSpm treatment had no significant effect on the cell cycle. However, both CPENSpm and IPENSpm produced a significant G<sub>2</sub>/M cell-cycle arrest and a concurrent decrease in the G<sub>1</sub> fraction. All three analogs, as well as the natural tetraamine, spermine, stimulate tubulin polymerization in the absence of microtubule-associated proteins and other polymerization stimulants, and the rate of polymerization was greatest in the case of CHENSpm (4.9 times faster than spermine). In the presence of microtubule-associated protein-rich tubulin, CHENSpm remained the most effective promoter of tubulin polymerization, whereas CPENSpm and spermine showed significant decreases in their ability to effect tubulin polymerization. These data suggest that CPENSpm, but not CHENSpm, are possibly competing for binding at the site normally occupied by microtubule-associated proteins.

The symmetrically and unsymmetrically substituted alkylpolyamines described have been of great value in determining the mechanisms of analog-induced cytotoxicity. However, the alkyl substituents in these molecules are representative of only a minute portion of the available chemical diversity for the terminal alkyl substituents. Recently, more than 200 alkylpolyamines have been synthesized and evaluated as antitumor agents in an effort to refine the SAR model described in Fig. 12. Preliminary biological evaluation of these analogs was conducted using a high-throughput screen based on 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT cell) viability determination in NCI H157 lung tumor cells. The structures of several of these analogs that demonstrate new structural directions to exploit are shown in Fig. 13, along with their IC<sub>50</sub> values in the MTT high-throughput screen. The compounds designated 39-TDW-47C, 39-TDW-12C, and 46-TDW-34C were selected for in vivo studies in an A549 lung tumor xenograft model. Preliminary studies indicate that all three of these analogs are effective in limiting tumor growth in the xenograft model. It is important to note that the compounds shown in Fig. 13 contain structural features that have not previously been included in polyamine analogs described. The data indicate that it is possible to synthesize active alkylpolyamines that contain aralkyl substituents, heteroatoms,

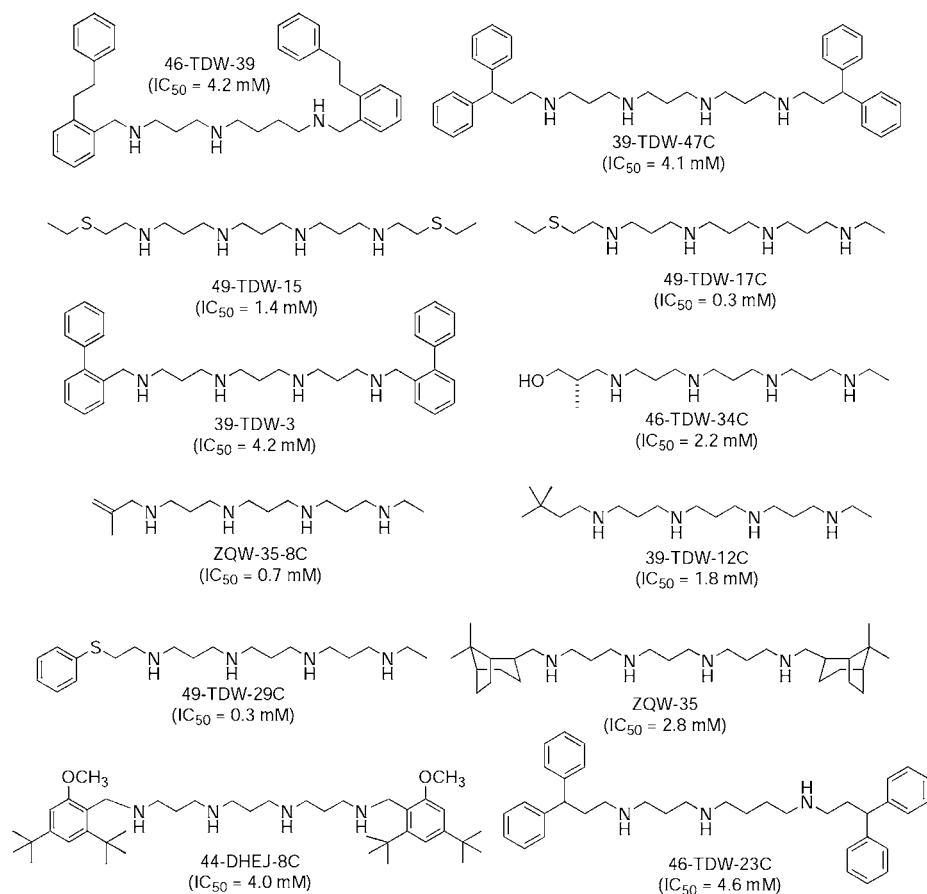


Fig. 13. Novel, structurally diverse alkylpolyamine analogs.

and unsaturations in the terminal alkyl substituents. Additional analogs in this series are being synthesized and used to determine the structural requirements for binding at the various alkylpolyamine effector sites.

#### 4. Future Directions for Polyamine Drug Discovery

As recently as 20 yr 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 known (58). These research advances have resulted in one marketed agent (i.e., DFMO), two agents that were not developed because of the economic status of the target population