Published in final edited form as:
J Med Chem. 2009 August 13; 52(15): 4551–4573. doi:10.1021/jm900187v.

Recent Advances in the Development of Polyamine Analogues as Antitumor Agents§

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Introduction

Since the publication of our previous *Perspective* dealing with polyamine drug discovery,[1] the field has undergone numerous changes. Most prominent among these changes, from a medicinal chemistry point of view, is that polyamine drug discovery efforts have partially shifted away from an emphasis on inhibitors of polyamine biosynthetic and catabolic enzymes. This shift is in part due to the fact that multiple compensatory mechanisms are available that are able to maintain homeostasis in cellular polyamine pools,[2] and thus the utility of specific enzyme inhibitors as drugs is limited. Specific inhibitors are available for every enzyme in the polyamine biosynthetic pathway,[1, 3-6] and for the polyamine transport system.[7-9] Although these inhibitors have significant effects on their respective target enzymes, only one inhibitor, α-difluoromethylornithine (DFMO) has reached the market. DFMO was originally designed as an antitumor agent, but the drug was not effective enough to warrant continued Phase II trials. However, it has been shown to be an effective cure for infection caused by *Trypanosoma brucei gambiense* (West African Sleeping Sickness),[10, 11] and has recently shown considerable potential as a cancer chemopreventive agent (see below).[12-14] Although no other polyamine biosynthesis inhibitor has been advanced to the market, the ubiquitous nature of the natural polyamines would lead one to conclude that these molecules have numerous cellular effector sites that are frequently dysregulated in cancer, and as such should provide a target rich environment for therapeutic intervention. Recent medicinal chemistry efforts in the polyamine field have focused on the discovery of compounds that produce cellular effects that are either independent of, or in addition to the polyamine metabolic enzymes. In addition, polyamine chains have been used to make hybrid drug molecules in order to improve cellular import, increase affinity for chromatin or to serve as carriers. This Perspective will focus on developments in polyamine drug discovery since our previous article.[1]

Polyamine Metabolism as a Drug Target

The role of natural polyamines in cellular homeostasis

The polyamines putrescine (1,4-diaminobutane, **1**), spermidine (1,8-diamino-4-azaoctane, **2**) and spermine (1,12-diamino-4,9-diazadodecane, **3**) (Figure 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 Figure 1.[15] This high degree of positive charge is an important factor in the biological functions of these molecules, and as

§This manuscript is dedicated to a mentor and friend, Professor Edward B. Roche, in honor of his 70th birthday.

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will be discussed below, 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 establishes the precise role of the polyamines and their analogues in cellular processes remains 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 many organisms. There are important interspecies differences in polyamine metabolism, especially within eukaryotic cells (plant versus animal) and between higher eukaryotes, 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 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 other 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. It is reasonable to assume that carefully designed polyamine analogues could have the potential to selectively disrupt polyamine metabolism, and thus such agents have been investigated as potential therapeutic agents in vitro and in vivo. Depletion of polyamines results in the disruption of a variety of cellular functions, and may in specific cases result in cytotoxicity.[1, 16, 17] Comprehensive reviews of polyamine biochemistry, polyamine biosynthesis inhibitors and the role of polyamines in normal and tumor cell metabolism have recently been published.[1, 16-22]

DFMO in cancer chemoprevention

DFMO was originally evaluated as an antitumor agent in the early 1980's, with limited success. Phase I studies suggested a dose of 2.25 g/m^2 every 6 hours for patients with advanced solid tumors or lymphomas.[23] Phase II studies were conducted in patients with melanoma,[24] small cell lung carcinoma,[25] colon cancer[25] and prostate cancer,[26] among others. The drug was generally well tolerated, although significant but infrequent adverse effects including thrombocytopenia, transient hearing loss and osmotic diarrhea were noted. The results of these studies did not warrant continued evaluation of the drug as an antitumor agent.[25] DFMO was eventually approved for use in *Pneumocystis carinii* infection in AIDS patients.[27] In 1985, it was discovered that DFMO was curative for infections with *Trypanosoma brucei gambiense* (West African Sleeping Sickness).[28] It was also curative in a mouse model for *Trypanosoma brucei rhodesiense*,[29] but the same effect was not observed in humans. Reports outlining the use of DFMO as a chemopreventative agent began to appear after 1985, and the first report of chemoprevention by DFMO in combination with a non-steroidal anti-inflammatory agent (piroxicam) appeared shortly thereafter.[30] DFMO has been shown to suppress skin carcinogenesis in patients with moderate to severe actinic keratosis with no systemic toxicities.[31, 32] More recently, DFMO dosed at 500 mg per day in a one-year randomized trial reduced prostate putrescine content and slowed prostate growth in men with a family history of prostate cancer.[33] In this study, no grade 3 or 4 toxicities, including ototoxicity, were observed. In a landmark study, Meyskens et al. demonstrated that 500 mg of DFMO in combination with 150 mg of sulindac daily over a 3 year period led to a dramatic reduction in the recurrance of colon adenomas in patients with prior disease, with remarkably few toxicities.[34] Subsequent studies analyzing the potential toxicities associated with long-term DFMO/non-steroidal anti-inflammatory agent combination treatment suggest minimal risk for the development of ototoxicity[35] and adverse cardiovascular events.[36] It is clear from these data that DFMO has considerable potential for use as a cancer chemopreventative agent.

Analogues of the Natural Polyamines Spermidine and Spermine

Symmetrical Terminally Alkylated Polyamine Analogues

The development of analogues of spermidine and spermine as potential antitumor agents was first attempted in the late 1980's. 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.[37] Compounds with eight carbons between the central nitrogens, such as tetramines A-C (**4a, b** and **c**, Figure 2), generally showed significant antitumor activity. In a related study, a series of (bis)benzyl analogues related to MDL 27695 (**5**, Figure 2), and an additional series of substituted tetraamines were evaluated for the ability to inhibit proliferation of HeLa cells.[38] Compounds **4a** and **5** were active antiproliferative compounds, exhibiting IC_{50} values of 5 and 50 μM, respectively. Interestingly, no correlation between the DNA binding properties and antitumor activity of these analogues was detected.

Subsequent attempts to develop polyamine analogues as potential modulators of polyamine function focused on the synthesis of symmetrical, terminally-substituted bis(alkyl)polyamines. These analogues were designed in response to the finding that natural polyamines utilize several feed-back mechanisms which autoregulate their synthesis,[2, 39, 40] and that they can be taken into cells by the energy dependent polyamine transport system. A variety of symmetrically substituted polyamine analogues have been synthesized which enter the cell using the polyamine transport system, including analogues **6-11** (Figure 3). These analogues specifically slow the synthesis of polyamines through down regulation of the biosynthetic enzymes ornithine decarboxylase (ODC) and S-adenosylmethionine decarboxylase (AdoMet-DC), but cannot substitute for the natural polyamines in terms of their cell growth and survival functions.[1, 41] As will be discussed below, some but not all alkylpolyamine analogues 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 polyamines analogues to date are the N,N'-*bis* (ethyl)polyamines shown in Figure 3: bis(ethyl)norspermine (BENSpm, **6**), bis(ethyl)spermine (BESpm, **7**),[41-43] bis(ethyl)homospermine (BEHSpm, **8**)[41-43] and 1,20-(ethylamino)-5,10,15-triazanonadecane (BE-4444, **9**).[44, 45] 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.

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.[41, 46,47] These syntheses can also be readily scaled up to pilot-plant quantities. The N,N'-*bis*(ethyl)polyamines are readily transported into mammalian cells by the same transport mechanism as the natural polyamines.[40] Treatment of mammalian cells with these analogues leads to a reduction of putrescine, spermidine and spermine, downregulation of ODC and AdoMetDC, and depending on the cell lines utilized, cytostasis or cytotoxicity.[39, 41] These effects are often accompanied by a tremendous induction of SSAT activity, in some cases as much as 1000-fold. However, these data were collected using only symmetrically substituted (bis)alkylpolyamines, and as a result, only bis(ethyl)-substituted analogues were advanced to clinical trials. Among these analogues, the most promising were **6**, which has a 3-3-3 backbone, **8**, which has a 4-4-4 carbon skeleton and **9**, which has a 4-4-4-4 architecture. Interestingly, BEHSpm proved to be useful as an antidiarrheal agent,[48-50] and was advanced to clinical trials for this indication. Phase I and II clinical studies involving **6** revealed that it was safe for administration, and had minimal toxicity, but the compound did not show any significant clinical effects in patients with breast or lung malignancies.[51-53] However, compelling pre-clinical data suggested that the study of additional polyamine analogues was warranted. It should be noted that the possibility of combination trials using **6** with standard chemotherapeutic agents is being considered to demonstrate synergy in in vitro models.[54]

Phase I clinical studies involving **8** were discontinued due to neuro- and hepatotoxicity issues. However, the toxicities observed with **8** could be reduced by the introduction of hydroxyl groups into the intermediate chain, such as in **11**, probably due to the fact that these hydroxylated analogues are more rapidly cleared through phase 2 metabolism.[48, 55]

Conformationally Restricted Symmetrically Alkylated Polyamine Analogues

Specific alterations to the polyamine backbone structure of **6-11** has resulted in a series of "second generation" bis(ethyl)polyamines (Figure 4) with impressive antitumor and antiparasitic activity. Restriction of rotation in the central region of the polyamine chain in **8** by including a *cis*- and *trans*- cyclopropyl (**12**) or -cyclobutyl (**13**) ring, a *cis*- and *trans* double bond (**14**), a triple bond (**15**), and a 1,2-disubstituted aromatic ring (**16**) produced analogues with varying antitumor activity in a panel of human tumor cell lines (A549, HT-29, U251MG, DU145, PC-3 and MCF7).[56] By contrast, insertion of a central dimethylsilane group resulted in a significant decrease in growth inhibition when compared to the bis(ethyl)polyamine analogues.[57] It was later found that the *cis*-**14** (CGC-11047, Figure 5) was an effective treatment for *Cryptosporidum parvum* infections, producing cures in a murine model.[58] By contrast, the 4-4-4 (homospermine) analogue CGC-11093 (**17**, Figure 5), 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.[59] Compound **17** was recently found to be effective in combination with the proteosome inhibitor bortezomib for the treatment of myeloma. Compounds with a 4-4-4 or 4-4-4-4-4 backbone that featured *trans*-cyclopropyl or a *trans*-cyclobutyl moieties in non-central 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,[60] presumably by enhancing DNA binding.[61] The *trans*-bis-cyclopropyl analogues **18** and **19** (Figure 5) were effective antitumor agents against Du-Pro and DU-145 prostate tumor cells in vitro.[62] In general, structural modifications to homospermine-like backbones that are analogous to those made to the backbone of 7 (i.e. *cis*-and *trans*-cyclopropyl, -cyclobutyl and double bond moieties) afforded analogues with enhanced antitumor activity and diminished systemic toxicity. In addition, insertion of a *cis* double bond into the terminal aminobutyl moieties of **9** (i.e. as in CGC-11121, **20** and CGC-11128, **21**, Figure 5) also affords analogues that are equipotent to **9** with respect to ID_{50} values, but that are an order of magnitude more cytotoxic in a dose-response study.[63] Recently, *cis*-**14** was shown to effectively inhibit the growth of both small cell (NCI H82 and H69) and non-small cell (NCI A549 and H157) lung cancer cells in vitro.[64] In the non-small cell lung cancer cells, a profound down regulation of ODC activity was observed, along with a significant increase in polyamine catabolism, polyamine pool depletion and an accumulation of *cis*-**14**. These effects were significantly less prominent in the small cell lung tumor lines. Importantly, *cis*-**14** was found to significantly delay the progression of established tumors in an in vivo A549 xenograft model. Finally, **14** and **17** were found to cause significant suppression and regression of laser-induced choroidal neovascularization following periocular injection, most likely by initiating apoptosis in proliferating vascular cells.[65] These data suggest a potential role for these analogues in the treatment of macular degeneration. Thus, *cis*-**14** represents a promising new polyamine analogue that warrants further clinical evaluation.

Symmetrically Substituted Oligamines and Macrocyclic Polyamine Analogues

Recently, a series of bis(ethyl)oligamine analogues have been described that show promise as potential chemotherapeutic agents. In the limited series of oligamines that were evaluated, the decamine CGC-11144 (**22**) and the octamine CG-11158 (**23**) (Figure 6) proved to be most growth inhibitory against a panel of prostate tumor cells in vitro (LnCap, DU-145, DuPro and PC-3). These analogues exhibited IC_{50} values that were an order of magnitude lower than previously studies polyamine analogues. Not surprisingly, their activity roughly correlated

with their ability to aggregate DNA.[66] Compound **22** was also found to have effective antineoplastic action against human breast cancer cells *in vitro* and *in vivo*, and it was shown that multiple apoptotic mechanisms are associated with its cytotoxic effect in specific human breast cancer cell lines.[67] These oligoamines significantly inhibit cell growth and activate AP-1 members c-Jun and c-Fos in MDA-MB-435cells,[68] suggesting that c-Jun plays a protective role in oligoamine-induced cell death. In addition, **22** and two related oligamines specifically suppressed the mRNA and protein expression of estrogen receptor α and estrogen receptor target genes in the estrogen receptor-positive MCF-7 and T47D human breast cancer cell lines,whereas neither estrogen receptor β nor other steroid hormonal receptors were affected. These results suggest a novel antiestrogenic mechanism for specific polyamine analogues in human breast cancer cells.[69] In addition to antitumor effects, compound **23** and related analogues have been shown to have significant inhibitory activity against *Enterocytozoon bieneusi*, the most common cause of chronic diarrhea in HIV/AIDS, and for which there is no effective therapy.[70]

It has been shown that macrocyclic polyamines known as budmunchiamines[71] act as potent antitumor agents by virtue of their ability to selectively deplete ATP. Based on this observation, a series of 5 macrocyclic polyamines with the representative structure **24** (Figure 6) were synthesized and evaluated as antitumor agents in the DuPro and PC-3 prostate cell lines.[72] All 5 of these analogues 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 ATP.

A relatively large number of symmetrically substituted alkylpolyamines have been synthesized, and a number of these were advanced to pre-clinical trials, and in some cases human clinical trials. A subset of these agents are continuing to be developed, and are now being evaluated in Phase I clinical trials. In these clinical trials, some adverse effects have been noted. Patients with advanced malignancies who were treated with 6 at a dose of 83 mg/kg/m^2 developed a complex of unusual neurological symptoms that included unilateral weakness, dysphagia, dysarthria, numbness, paresthesias, and ataxia after treatment had been completed.[73] In a similar study, doses of **6** that exceeded 94 mg/kg/m2 produced CNS dose-limiting toxicities characterized by aphasia, ataxia, dizziness, vertigo and slurred speech.[53] A later Phase I study in patients with non-small cell lung cancer showed that **6** at 25-231 mg/m^2 could be administered safely, and dose-limiting toxicities were mainly gastrointestinal.[52] Clinical trials involving **8** were discontinued due to grade 3 or 4 adverse effects included nausea, vomiting, constipation, ileus, elevations of aspartate aminotransferase (AST) and alkaline phosphatase, hyperbilirubinemia, and ventricular bigeminy.[74] Despite these findings, human clinical evaluation of second generation bis(ethyl) polyamine analogues such as **17** and **21-23** is warranted. A summary of the antitumor effects of symmetrically substituted polyamine analogues **4-24** appears in Table 1.

Unsymmetrical, Terminally Alkylated Polyamine Analogues

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 erroneously 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. The first examples in this series, N^1-propargyl-N^{11}-ethylnorspermine (PENSpm, **25**) and N^1-cyclopropylmethyl-N^{11}-ethylnorspermine (CPENSpm, **26**), which possess unsymmetrically substituted terminal nitrogens, were described in 1993 (Figure 7).[75] In general, the synthesis of these and other

unsymmetrically substituted analogues is more difficult, since it requires selective protection and deprotection of the internal and external nitrogens. Studies involving unsymmetrically substituted alkylpolyamine analogues such as **25-31** (Figure 7) were extensively discussed in earlier publications,[76-86] and in our previous Perspective.[1] The data described in these manuscripts clearly support the contention that there are at least two mechanisms by which unsymmetrically substituted alkylpolyamines produce cytotoxicity in NCI H157 non-small cell lung carcinoma cells, and that each of these mechanisms appears to lead to apoptosis as a common endpoint.[81, 85] Similar results were obtained in the DU145 and PC-3 androgen receptor-negative prostate cell lines, and the LnCap androgen receptor-positive prostate cell lines,[84] as well as in several breast tumor cell lines.[79, 83] The selective cytotoxicity of alkylpolyamines in these cell lines may be due to the fact that the analogues are concentrated in tumor cells, wherein the polyamine transport system is significantly up regulated.[82]

In addition to production of apoptosis by at least 2 mechanisms, unsymmetrically substituted alkylpolyamines can have dramatically different effects on the cell cycle.[80] Symmetrically substituted alkylpolyamines such as **6-11**, and unsymmetricl alkylpolyamines with small substituents such as **25** and **26**, either produce no effect on cell cycle, or induce a G_1 cell cycle arrest, while analogues with one large substituent (e.g. **30** and **31**) produce a G_2/M cell cycle arrest.[86]

Chemical Diversity in Unsymmetrical Alkylpolyamine Analogues

The symmetrically and unsymmetrically substituted alkylpolyamines described above have been of great value in determining the mechanisms of analogue-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 120 alkylpolyamines were synthesized and evaluated as antitumor agents in an effort to develop an SAR model for alkylpolyamine analogues with antitumor activity. Preliminary biological evaluation of these analogues was conducted using a high throughput screen based on MTT cell viability determination in NCI H157 lung tumor cells. These analogues were designed with varying polyamine backbone structure (3-4, 3-3-3, 3-4-4 and 3-7-3), and variation of the terminal alkyl substituents has been attempted to determine the optimal overall structure for antitumor effects. As previously described,[75, 79, 83-88] alkylpolyamine analogues have a variety of cellular effects, including cytotoxicity, induction of apoptosis, disruption of tubulin polymerization, induction of spermidine/spermine-N^1-acetyltransferase (SSAT) and spermine oxidase (SMO), and inhibition of trypanothione reductase. To date, 27 alkylpolyamine analogues have been identified that exhibit IC_{50} values of 5.0 μM or less against the NCI H157 non-small cell lung tumor line in vitro, as shown in Figures 8-10. Many of these analogues share the properties of **6**, in that they are taken up into tumor cells by the polyamine transporter, where they deplete polyamines and down regulate polyamine biosynthetic enzymes, but do not substitute for the natural polyamines in terms of their cellular function. Analogues of spermidine exemplified by compounds **32-35** (3-4 architecture, Figure 8) are capable of producing potent antitumor effects, although in this series symmetrically substituted compounds with terminal aralkyl substituents were much more potent than symmetrically substituted alkylpolyamines or unsymmetrically substituted alkyl- or aralkylpolyamines. Six compounds based on the natural polyamine spermine with a 3-4-3, 3-5-3 or 3-4-4 polyamine backbone were also shown to possess IC_{50} values less than 5 μM, as seen in Figure 9 (**36-41**).

A primary synthetic goal in this area has been to produce a large number of norspermine (3-3-3) analogues related to the parent compound **6**. Numerous symmetrically- and unsymmetrically substituted derivatives have been produced that incorporate chemical diversity into the terminal alkyl substituent as a means to elucidate the structure-activity relationships for alkylpolamine

analogues with antitumor activity. As shown in Figure 10, seventeen 3-3-3 derivatives (compounds **42-58**) with IC_{50} values of 5 μM of less have been discovered. Subsequent studies have shown that these analogues produce cellular effects (cytotoxocity, apoptosis, induction of SSAT and SMO, and alteration of cellular polyamine levels) in varying degrees. It was also observed that slight changes in chemical structure often effect large changes in biological response in tumor cells in vitro. It is noteworthy that the antitumor alkylpolyamine analogues shown in Figure 10 display a variety of functionalities that have not previously been reported in analogues of this type. Active analogues were identified possessing aralkyl and alkyl substituents on the terminal nitrogens, both symmetrical and unsymmetrical, that in some cases contain sulfur, oxygen or fluorine atoms. A full description of the cellular effects of all of these analogues is not included here, but will be reported in an upcoming manuscript.

Based on a number of factors, including IC_{50}, effects on SSAT and SMO levels and other parameters, three of these analogues, compounds **42** (PG11400), **46** (PG11401) and **50** (PG11402) were selected for scale-up and more extensive pre-clinical trials in vivo. Despite having similar structures, **42, 46** and **50** show different dose-response profiles against four lung tumor cell lines (H157, H82, H69 and A549, data not shown) over a range of doses between 0.1 and 100 μM. Importantly, **42** does not produce significant SSAT induction in any of these lung tumor cell lines, while **46** and **50** produce SSAT induction in the H157 line of the same magnitude as **14**. By contrast, **42** initiates a 30-fold induction of SMO, and is 3-6 times more effective in this regard than **46, 50, 14** and **17**. The in vivo implications of the variation in these cellular effects between analogues and among different cell lines remains to be determined. Compounds **42, 46** and **50** have recently been evaluated in an A549 human lung tumor xenograft model in athymic nu/nu Fox Chase mice. Mice were treated with either 75, 100 or 150 mg/kg of each agent twice a week over a period of 70 days. Compounds **42** and **50** proved to be most effective at inhibiting tumor growth, although **42** produced toxicity at the 100 mg/kg dose. In all three cases, mean body weights of the animals were comparable to control animals. The toxicity observed during treatment with **42** was not observed during treatment with **50**, and as shown in Figure 11, **50** was effective in limiting tumor growth by 65% at both the 75 and 150 mg/kg doses. The reduced in vivo toxicity observed during treatment with **50** may be due to the presence of a hydroxyl moiety in one terminal alkyl substituent, consistent with what was observed for compound **11**.[55] Additional in vivo experiments are being conducted using compounds **42, 46** and **50**, and additional analogues in this series are being synthesized and used to determine the structural requirements for binding at the various alkylpolyamine effector sites. A summary of the antitumor effects of unsymmetrically substituted polyamine analogues selected for pre-clinical studies appears in Table 2.

Structure/Activity Relationships for Alkylpolyamine Analogues

Early structure/activity correlations for alkylpolyamines were based on data from symmetrically alkylated polyamine analogues such as **6-11** (Figure 3). These studies 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.[41] It was further determined that the greatest induction of SSAT was dependent on the presence of "protected" aminopropyl or aminobutyl moieties.[40, 78, 89, 90] Terminal nitrogen bis(alkyl) substituents larger than ethyl led to analogues exhibiting a dramatic reduction in antitumor activity.[43] These studies also suggested that compounds with a 3-3-3 carbon skeleton are more effective that the corresponding 3-4-3 analogues, and that spermine-like compounds are more effective that spermidine-like analogues. Hydroxylation of the central carbon in the terminal aminopropyl chain of spermine analogues afforded alkylpolyamine analogues that retained potency, but had reduced toxicity, presumably due to more rapid clearance.[48, 55] The introduction of conformationally restricted bis(ethyl)polyamines demonstrated the importance of flexibility in the central butane moiety, wherein highly rigid analogues such as **15** and **16**

had poor activity.[56] The somewhat more flexible *cis/trans* isomeric pairs resulting from central cyclopropane- cyclobutane- or ethylene moieties in the central chain were more active than the unrestricted parent compounds, but there was no significant difference in potency between the isomers.[56, 61] Inclusion of multiple *cis* unsaturations in the terminal aminobutyl chains of longer polyamines such as **21-23** (Figures 5 and 6) also produced active analogues.

The structure-activity relationships for unsymmetrically substituted alkylpolyamines appear to be more complex than those for the symmetrically-substituted analogues. This complexity most likely arises from the fact that not all of the effector sites for these analogues have been identified. The result is that seemingly small changes in structure can have dramatic effects of antiproliferative activity, induction of SSAT, down regulation of polyamine biosynthetic enzymes, and other parameters.[1] Unsymmetrically substituted alkylpolyamines can exhibit varying degrees of SSAT and/or SMO induction based on the size of their terminal alkyl substituents, and these analogues can induce these enzymes as well or better than the parent analogues **6-8**. An SAR model for SSAT inducers and non-inducers has been described.[1] Among SSAT inducers, terminal substituents 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 seperated by 5.0-5.8 angstroms, and each terminal nitrogen is 5.0 angstroms from the adjacent central nitrogen. The cytotoxicity of agents (e.g. **25** and **26**, Figure 7) that fit these criteria can be directly related to the superinduction of SSAT, which induces apoptosis through the overproduction of hydrogen peroxide. Among SSAT non-inducers, the requirement for bis-alkyl substitution remains, but the binding pockets for the terminal alkyl substituents seem to be less restrictive. The most active analogues have one small (ethyl) or medium sized alkyl substituent, and one large terminal substituent. Agents with two large terminal alkyl substituents may possess activity, but are not likely to superinduce SSAT. In active analogues, the central nitrogens are separated by 5-10 angstroms, and the terminal nitrogens are roughly 5 angstroms away from the respective internal nitrogens. Steric bulk on the intermediate chain is not well tolerated. Agents that fit these criteria (e.g. **5**, Figure 2 and **30** and **31**, Figure 7) produce apoptosis in the same time frame as the SSAT inducers, but through as yet undetermined pathways. Importantly, expansion of the central carbon chain to a 3-7-3 or 3-8-3 architecture can act as antiparasitic agents that are effective against trypanosomes and *Microsporidia*.[87, 88, 91]

As outlined above, a large number of symmetrically- and unsymmetrically substituted alkylpolyamine analogues for evaluation as antitumor and/or antiparasitic agents. These analogues were designed with varying polyamine backbone structure (3-4, 3-3-3, 3-4-4 and 3-7-3),[75, 79, 83-88] and have a variety of cellular effects, including cytotoxicity, induction of apoptosis, disruption of tubulin polymerization, induction of SSAT and SMO, and inhibition of trypanothione reductase. Analogues of spermidine (3-4 architechture) are capable of producing potent antitumor effects, such as compounds **32-35** shown in Figure 8, although in this series the symmetrically substituted compounds with aralkyl substituents on the terminal nitrogens were much more potent than symmetrically substituted alkylpolyamines or unsymmetrically substituted alkyl- or aralkylpolyamines. Compounds based on the natural polyamine spermine with a 3-4-3, 3-5-3 or 3-4-4 polyamine backbone were also shown to possess IC_{50} values less than 5 μM, as seen in Figures 9 and 10. The SAR for these analogues conforms to that observed for SSAT inducers and non-inducers. It is noteworthy that these antitumor alkylpolyamine analogues display a variety of functionalities that have not previously been reported in analogues of this type. Active analogues were identified possessing aralkyl and alkyl substituents on the terminal nitrogens, both symmetrical and unsymmetrical, that in some cases contain sulfur, oxygen or fluorine atoms. Additional SAR studies are required to determine the optimal structures for interaction with various known and unknown polyamine effector sites.

Polyamine/Metal Complexes

Polyamine/Metal Complexes as Cancer Chemotherapeutics

The serendipitous discovery of cisplatin by Rosenberg in 1969[92] opened a new era for metallopharmaceuticals. The antitumor agents cisplatin **59** and carboplatin **60** (Figure 12) exhibit broad-spectrum activity against epithelial cancers, testicular and ovarian cancers, and cancers of the lung, head and neck, esophagus, bladder, cervix, and endometrium. Compound **59** forms a variety of stable bifunctional adducts with DNA that block replication and inhibit transcription, and treated cells undergo apoptosis[93] characterized by cell volume reduction and chromatin condensation.[94] It is well established that Pt-containing compounds exhibit antitumor activity by forming specific adducts with DNA,[94, 95] binding preferentially to sequences containing two or more adjacent guanosine residues, and form 1,2 or 1,3 intrastrand substitution adducts of the type $(NH_3)_2$-Pt-GpG, $(NH_3)_2$-Pt-ApG, or $(NH_3)_2$-Pt-GpXpG, where A=adenosine, G=guanine and X= any nucleotide residue.[94, 95] Unfortunately, cisplatin, and to a lesser degree carboplatin, have substantial renal, neurologic, and emetogenic toxicities, and resistance to these agents develops through reduction in cellular import, enhanced cytoplasmic detoxification and enhanced repair of DNA adducts. Second generation Pt(IV) and Pt(II) drugs exhibit high therapeutic activity and have somewhat reduced toxicity, but only two additional Pt drugs have joined **59** and **60** in the clinic: oxaliplatin **61** and nedaplatin **62** (Figure 12).[96] More recently, the nucleotide binding properties of transition metal complexes including rhenium (Re), rhodium (Rh) and ruthenium (Ru) that assume a “lantern-type” geometry, such as the dinuclear compounds **63, 64** and **65** (Figure 12), were shown to possess significant antitumor activity in vitro and in vivo. These compounds form unique bonds involving substitutions at the N^7 position of purines, the O^6 position of guanine and the N^6 position of adenine.[97, 98] More recently, it has been demonstrated that dirhodium complexes related to 7 form unprecedented intrastrand DNA adducts, with the dirhodium unit cross-linking in the major groove at residues C5 and A6.[99] Based in part on these findings, there has been renewed interest in the development of metal-based therapy for human cancer.[96]

Early studies indicated that dinuclear bis(platinum) complexes in which the metal centers were separated by diaminoalkanes (compounds **66**, Figure 13) were more potent than **59** against murine leukemia cells, murine solid tumor cells and four human tumor cells in vitro, and had good activity against **59**-resistant cell lines.[100] In the corresponding xenografts, the version of **66** in which n = 3 showed greater activity in the HCT-8 colon adenocarcinoma and H23 non-small cell lung tumor lines, but diminished potency in the AH125 and H520 non-small cell lung tumor lines, when compared to **59**. Complexes between Pt and spermidine were subsequently described[101, 102] that were shown to alter the secondary structure of DNA, but that had unremarkable antitumor effects.[101, 103] However, the trinuclear Pt compound known as BBR 3464 (**67**, Figure 13), in which three Pt centers are separated by diaminohexyl spacers,[104, 105] was shown to produce cytotoxicity in L1210 mouse leukemia cells that was 30 times greater than **59** in vitro, and to bind to DNA in a time-dependent manner[104] that was distinct from the binding pattern of **59**.[105] Continuing studies confirm that **67** is more potent than **59**, forms distinct bifunctional DNA adducts,[106] and induces cell cycle arrest in tumor cells.[107, 108] Clinical trials have now been initiated to evaluate **67** as a treatment for gastric or gastroesophageal adenocarcinoma[109] and non-small cell lung carcinoma.[109, 110] Recently, the compound BBR3610 **68**, a cogener of **67**, was shown to produce cytotoxicity superior to **67** through a caspase 8-dependent mechanism that is enhanced by ERBB1/PI3K inhibitors that activate BAX and caspase 9.[111] In order to increase the therapeutic index of Pt/spermidine complexes, three carbamate prodrugs (**69-71**, Figure 13) were synthesized and evaluated for antitumor activity;[112] only the Fmoc-protected analogue **71** showed any enhanced cytotoxicity. This approach was later extended to acetyl-protected spermidine-bridged dinuclear platinum complexes **72-74** (Figure 13).[113] The rate of hydrolysis of these prodrugs corresponded to the

electronegativity of the amide functional group, and thus **74** (R = trifluoromethyl) produced the greatest effect in vitro. The fact that **74** produces cytotoxicity at micromolar levels suggests that the prodrug approach is valid.

Drugs containing Pt or other transition metals have not found widespread utility in the treatment of advanced breast cancer, but recent data indicate that Pt compounds can be useful. Preclinical studies indicate that a mouse antibody against HER2 synergizes with **59** through a mechanism involving inhibition of the repair of Pt-induced DNA damage.[114, 115] Subsequent phase II clinical trials in patients with HER2-positive breast cancer combining the anti-HER2 humanized antibody trastuzumab in combination with **59** or **60** and docetaxel have shown promising results.[116] In addition, Pt analogues might be particularly useful in breast cancers harboring BRCA1 or BRCA2 mutations, since mouse-derived BRCA1- negative cell lines have been shown to be five-fold more sensitive to **59** when compared to wild-type cells.[117]

Polyamine/Metal Complexes with Pt, Re, Rh and Ru

As outlined above, there are compelling reasons to pursue the discovery and development of new antitumor therapies containing Pt or other transition metals. To date, the structure/activity relationships of polyamine/Pt complexes have not been adequately studied. Further, polyamine metal complexes of Re, Rh and Ru have not been synthesized, and the utility of these metals in cancer chemotherapy is largely unexplored. Based on the promise of the polyamine/Pt complexes described above and the promising antitumor activity of Re, Rh and Ru complexes, compounds containing a metal complex with known DNA binding and antitumor activity, combined with a polyamine sidechain such as those found in **25-58** with demonstrated pro-apoptotic antitumor effects in breast tumor cells,[67, 79, 83, 84, 118, 119] would be expected to be a highly effective for the treatment of breast cancer. It is hypothesized that these complexes would retain the antitumor activity of a mono- or dinuclear metal complex, as well as the pro-apoptotic antitumor effects of the polyamine side chain, as outlined above. In addition, such complexes could, in theory, have three distinct advantages over the individual constituents. Specifically, the polycationic polyamine sidechain could increase the water solubility of the metal complexes, which is usually limited, and could potentially enhance the affinity of the metal for DNA.

In order to test the hypothesis outlined above, an initial library of 25 polyamine/metal complexes containing Pt, Re, Rh or Ru centers was synthesized. These analogues were synthesized from commercially available metal complexes and polyamine fragments that have been previously described.[75, 86, 87, 120, 121] Eleven of these analogues possess IC_{50} values of <10.0 μM in an H157 non-small cell lung cancer MTT assay (see below), with 3 of the 11 having IC_{50} values of <1.0 μM. Four of these analogues, Pt compound **75**, Ru compound **76** and Re compounds **77** and **78** (Figure 14), were selected for dose-response studies using an MTT assay in the MCF7 breast tumor cell line. All compounds were evaluated over a range of concentrations between 1 and 100 μM. The results of these studies are shown in Figure 15. Polyamine metal complexes **75, 76, 77** and **78** exhibited IC_{50} values of 6.6, 3.8, 9.3 and 6.9 μM, respectively, while the IC_{50} value for **59** could not be determined under these conditions (**59** produced 56% inhibition at 300 μM, data not shown). Importantly, these results demonstrate that polyamine complexes containing 3 distinct (Pt, Ru and Re) metal centers possess antitumor activity against MCF7 breast tumor cells in vitro.

Polyamine metal complexes **75, 76, 77** and **78** were next evaluated for their ability to bind to isolated DNA, as shown in Figure 16. Compound **75** formed more extensive crosslinks with plasmid DNA than cisplatin (Figure 16, Panel A). This DNA binding was found to be concentration dependent between 3 and 300 μM (Figure 16, Panel B) and time dependent over a period of 6 hours (Figure 16, Panel C). Compounds **76, 77** and **78** produced similar results (not shown) and were more effective than cisplatin with respect to DNA crosslink formation.

In each case, the observed DNA binding was concentration- and time-dependent with kinetics similar to **75**. These data clearly demonstrate that polyamine metal complexes represent antitumor agents that are more potent than the cisplatin, and show a greater ability to bind to DNA. It should be noted that 4 reasonable lead compounds for drug discovery have been identified from a library of only 25 analogues, and that active analogues can be identified that incorporate transition metals (Ru, Re) not previously used for cancer chemotherapy.

Polyamine-based Histone Deacetylase (HDAC) Inhibitors

HDACs and HDAC Inhibitors in Normal and Transformed Cells

Chromatin architecture is a key determinant in the regulation of gene expression, and this architecture is strongly influenced by post–translational modifications of histones.[122, 123] Histone protein tails, consisting of up to 40 amino acid residues, contain lysine residues that protrude through the DNA strand, and these residues act as a site for post-translational modification of chromatin, allowing alteration of higher order nucleosome structure.[124] Multiple post-translational modifications of histones[125, 126] mediate epigenetic remodeling of chromatin, with the dynamic process of acetylation/deacetylation being one of the best-characterized processes.[122, 127] Transcriptional repression dominates at times when specific DNA promoter CpG-island methylation occurs, and HDAC activity recruited by specific repressor complexes, which are recruited to these promoters, is an important event in epigenetic silencing.[126, 128] The acetylation status of histones is controlled by a balance between two enzymes, histone acetyltransferase (HAT), which promotes histone hyperacetylation, and the histone deacetylases (HDACs) which catalyze acetyl group cleavage.[127, 129] Acetylation of the ε-amino groups of lysine residues located at N-termini removes the positive charge and reduces the affinity of the histone proteins/nucleosome for the DNA,[124, 127] allowing greater access to DNA by transcription factors and RNA polymerase, thus promoting gene expression.

Normal mammalian cells exhibit an exquisite level of control of chromatin architecture by maintaining a balance between HAT and HDAC activity.[130] There are 11 known zinc-dependent HDAC isoforms[131-133] that can be divided into 4 structural classes: class I (isoforms 1,2,3 and 8),[134] class IIa (isoforms 4, 5, 7 and 9),[131] class IIb (isoforms 6 and 10)[131] and class IV (HDAC 11).[131] An additional class of HDAC, termed Class III, is comprised of enzymes similar to the NAD-dependent yeast transcriptional repressor protein Sir2.[135, 136] These HDACs do not require zinc, and are unresponsive to known Class I/II HDAC inhibitors. Despite extensive study, it is still uncertain which HDAC isoforms play the most important roles in human cancer.

In some tumor cells, hypoacetylation of histones results in aberrant gene silencing leading to the under-expression of growth regulatory proteins such as the cyclin dependent kinase inhibitor $p21^{Waf1}$, and this scenario contributes to the development of cancer. [122, 127] Histone hyperacetylation caused by HDAC inhibitors such as trichostatin A (TSA, **79**, Figure 17), N-(2- aminophenyl)-4-[N-(pyridin-3-ylmethoxycarbonyl)-aminomethyl]benzamide (MS-275, **80**, Figure 17) and suberoylanilide hydroxamic acid (SAHA, **81**, Figure 17) can cause growth arrest in a wide range of transformed cells, and can inhibit the growth of human tumor xenografts.[127, 129, 134, 137] HDAC inhibitors restore quasi-normal cell cycle and apoptotic functions in human tumor cells. Clinical studies indicate that HDAC inhibitors such as **80** and **81** are effective therapies for human cancer,[138] but dose-limiting toxicity remains a problem, and step-down dosing of the HDAC inhibitor is often required to minimize toxicity.[139] Known HDAC inhibitors fall into several structural classes including short chain carboxylic acids (valproate, butyrate), hydroxamic acids (**79, 81**), benzamides (e.g. **80**) and cyclic peptide inhibitors such as apicidin and depsipeptide.[137] Recent studies have identified isoform-selective inhibitors of HDACs 1, 3 and 8[140] and HDAC 6,[141] but do not provide structural information that can aid in the design of more specific inhibitors. Such molecules would be of

great value in elucidating the roles of HDAC isoforms in both normal and cancer cells. In vitro models and phase I clinical trials have demonstrated that synergistic re-expression of genes that are silenced through promoter methylation can be achieved by the sequential application of DNA methyltransferase (DNMT) inhibitors and HDAC inhibitors,[142-145] demonstrating a correlation between chromatin remodeling and clinical efficacy. Although they are effective both in vitro and in vivo, HDAC inhibitors typified by **79-81** suffer from lack of specificity among the various forms of HDAC, including deacetylases that target non-histone proteins, and can produce unacceptable toxicity in non-cancerous cells. Effective HDAC inhibitors with minimal inherent toxicity would be useful for combination therapy with DNA methyltransferase inhibitors such as 5-azacytidine (5-AC) and 5-aza-2'-deoxycytidine.

Polyaminohydroxamic Acid Analogues

HDAC inhibitors such as **79-81** possess three structural features that are required for optimal activity: an aromatic cap group, an aliphatic chain and a metal binding functional group (Figure 17). Structure/activity studies involving analogues of **79-81** have focused largely on modifications to the aromatic ring moiety and aliphatic linker region present in these molecules.[137] Structurally, there is a high degree of sequence homology between Class I and II HDACs in the channel and surface recognition portion of the proteins. However, there is reduced sequence homology in the rim area that lies outside of the binding pocket.[134] One strategy for targeting specific HDAC isoforms is to design molecules that exploit the variation in structure in this rim area. We and others have reported alkylpolyamine analogues related to **2** and **3** (see Figures 1 and 7-10) that enter cells using a specific polyamine transporter[1, 7] and exert antitumor effects due to their high affinity for DNA and other effector sites.[1, 7, 59, 66, 75, 86, 87, 146, 147] Thus, a series of polyaminohydroxamic acid (PAHA) derivatives was proposed that incorporate structural features of alkylpolyamines such as **4-58**, and the metal binding moiety, linker and cap groups found in molecules such as **80** and **81**. It was postulated that these compounds could enter cells using the polyamine cellular transporter,and be selectively directed to DNA and the associated HDACs by the positively charged polyamine portion of the structure. An additional advantage to this approach is that it may ultimately be possible to produce isoform specific inhibitors for individual HDACs by altering the polyamine chain composition and the terminal alkyl group on that chain, taking advantage of the fact that histone deacetylases differ in primary sequence at specific residues in the rim region.[134]

An initial series of 16 PAHA analogues was synthesized and screened for inhibitory activity against a mixture of HDACs derived from HeLa cell lysates.[120] Two of these compounds, **82** and **83** (Figure 17), inhibited HDAC in this assay by 75% at a 1.0 μM concentration. Compound **83** at 1.0 μM was subsequently found to produce significantly greater induction of acetylated histone H3 (AcH3) and acetylated histone H4 (AcH4), and was markedly more effective at promoting re-expression of the cyclin dependent kinase inhibitor $p21^{Waf1}$, in the ML-1 mouse leukemia cell line. However, **83** exhibited an IC_{50} value of 10 μM in ML-1 leukemia cells following a 7-day treatment, as compared to 0.25 μM and 0.75 μM for **79** and **80**, respectively. Following the synthesis of additional PAHAs, 6 new analogues were found that inhibited HDACs in a HeLa cell lysate by 50% or greater. Among these derivatives, compound **84** (Figure 17) inhibited the HDAC mixture by 51.5%, but caused a 253-fold induction of acetylated α-tubulin while only causing a minimal increase in AcH3, AcH4 and $p21^{Waf1}$.[148] These data strongly suggest that **84** shows a marked selectivity towards HDAC6, which is known to deacetylate α-tubulin.[149]

Polyaminobenzamide Analogues

Using the same design strategy, a series of 40 polyaminobenzamides (PABAs) and their homologues were synthesized and evaluated as inhibitors of HDAC.[148] Representative structures for this class are shown in Figure 18. Compound **85**, one of the first entries in the

series, exhibits an IC_{50} value against global HDAC from the HeLa cell lysate of 4.9 μM, which is comparable to the reported IC_{50} value for **80** (4.8 μM).[150] When the purified HDAC isoforms became available, **85** was evaluated as an inhibitor of the class I HDAC 3, and the class II HDAC 6. Although **85** was an effective inhibitor of global HDAC, it had little activity against either HDAC 3 or 6, suggesting that the observed inhibition of global HDAC was due to inhibition of one or more of the remaining HDACs. Additional analogues in the PABA series (for example **86-96**) were evaluated against 4 HDAC isoforms representing Class I (HDAC 1, 3 and 8) and Class II (HDAC 6). In these analogues, structural modifications were made in the linker chain length, in the polyamine substituent and in some cases in the metal binding moiety (**87, 90, 92** and **95**). As can be seen in Table 3, isoform selectivity among the 4 HDACs evaluated varied significantly, demonstrating that global percent HDAC inhibition is a composite of strong and weak inhibition at different isoforms. For example, **87** produced a 72.6% inhibition of global HDAC at a 5 μM concentration, and inhibited the Class II HDAC 6 by 97.9%, but acted as a significantly weaker inhibitor against the Class I HDACs 1, 3 and 8. By contrast, **91**, which inhibited global HDAC by a moderate 42.9%, was selective for HDAC 1 (78.7% inhibition), but has almost no effect against HDACs 3, 6 and 8. In our hands, the traditional HDAC inhibitor **81** at 5μM produced a 97.1% inhibition of global HDAC (Table 3), but exhibited no selectivity for HDACs 1, 3, 6 or 8. These data suggest that the observed HDAC selectivity with PAHAs such as **84**, and PABAs such as **87** and **91** may be in part due to structural variations in their polyamine side chains. The synthesis of additional analogues and accompanying molecular modeling studies are being conducted to determine if interaction of the polyamine side chain with the rim region of HDAC 8[151] provides a basis for isoform selectivity.

As mentioned above, one of the potential advantages of incorporating polyamine side chains into PAHA and PABA HDAC inhibitors was that the resulting molecules could utilize the polyamine transport system. Cellular uptake of polyamine-based drug molecules presents a particular advantage in most tumor cells, where the polyamine transport system is highly up regulated as compared to normal cells.[8] Although polyamine transport proteins have been characterized for bacteria, yeast and protozoans, an analogous transport system in mammalian cells has not been identified. There is recent evidence that mammalian polyamine transport is mediated through caveolin 1-dependent endocytosis,[152, 153] and that this process may involve electrostatic attraction of polyamines by the glypican subfamily of glycosylphosphatidylinositol-anchoredcell-surface heparan sulfate proteoglycans (HSPG).[152] These data are consistent with the finding that hydroxamic acid-containing PAHAs, with pKa values of approximately 9, are not substrates for the mammalian transporter, while PABAs such as **85** are effectively imported using the polyamine transport system.[148]

PABA Analogues in Breast Cancer

The most active global HDAC inhibitors in the PABA series were evaluated against MCF7 wild type tumor cells in vitro, and variable growth inhibitory activity was observed. Although PABA analogues **85-96** show interesting selectivity among HDAC isoforms 1, 3, 6 and 8, only 2 of these analogues produced significant cytotoxicity in tumor cells in vitro. The antitumor activity of compounds **91, 92** and **93** are shown in Figure 19. Over a range of concentrations between 0.3 and 30 μM, benzamide **91** appeared to stimulate tumor cell growth, while **92**, which features a shorter linker chain region and an α-ketoamide metal-binding moiety, was cytostatic. However, benzamide **93**, featuring a shorter polyamine substituent, was cytotoxic in the MCF7 cell line, with an IC_{50} of 0.9 μM. In the MCF10A normal breast epithelial cell line, **93** exhibited an IC_{50} of 24 μM, and thus demonstrated significant selectivity for tumor cells. Under the same conditions, the marketed inhibitor **81** (also known as vorinostat) exhibited IC_{50} values of 8.5 μM (MCF7) and 31 μM (MCF10A), and thus **93** compares favorably to known HDAC inhibitors that are currently in use in the clinic. Microscopic examination of

treated cells revealed that cytotoxicity was mediated by apoptosis in MCF7 cells treated with **93** (data not shown). Subsequent experiments showed that **93**, but not **91**, promoted the induction of annexin A1 (Figure 20), which is considered an early intermediate in the apoptosis pathway.[154] HDAC inhibitors have been shown to promote apoptosis through induction of annexin A1, and recent studies suggest that breast tumors expressing high levels of annexin I are more likely to respond to neoadjuvant chemotherapy than cells with low levels of the protein.[155]

Polyamine-based HDAC Inhibitors in Other Diseases

It is tempting to assume that HDAC inhibitors that do not produce frank cytotoxicity cannot be used clinically, but such is not the case. In cancer chemotherapy, traditional HDAC inhibitors are used clinically in combination with other classes of antitumor compounds that promote apoptosis, and thus their tumor cell cytotoxicity is acceptable. Combination therapy is based on the supposition that inhibition of HDAC restores tumor cells to a pseudo-normal cell cycle, making them susceptible to agents that cause apoptosis. However, aberrant HDAC activity plays a role in other diseases where a non-toxic HDAC inhibitor would be of great value. Recent studies have suggested that histone acetylation status plays a role in the development of diabetes mellitus,[156, 157] and that HDAC inhibitors may serve as a novel class of agents for the treatment of diabetes and its complications.[158, 159] As described above, a number of PAHA and PABA analogues produce significant epigenetic effects in cells (e.g. re-expression of tumor suppressor factors and increased acetylation of histones) with little or no cytotoxicity. We thus evaluated PABA HDAC inhibitors for their cytoprotective effects against IL-1β-induced damage to isolated β-cells. Three PABAs, **94**-**96** (Figure 18) significantly inhibited HDAC activity and increased the acetylation of histone H4 in isolated β-cells. These compounds further exerted no toxic effects on metabolic cell viability in these cells. However, among the three compounds tested only **94** protected against IL-1β-mediated loss in β-cell viability.[160] Remarkably, compound **94** differs from **96**, which does not protect against this loss in β-cell viability, by a single carbon in the linker chain region. Compound **94** was also able to attenuate IL-1β-induced iNOS expression and subsequent NO release. These data suggest that the cytoprotective properties of **94** against IL-1β-mediated effects may, in part, be due to inhibition of IL-1β-induced transactivation of NFκB in these cells. A novel HDAC inhibitor such as **94** that can prevent IL-1β -mediated effects on isolated β-cells supports the development of non-toxic PABAs to prevent deleterious effects of cytokines and the onset of autoimmune diabetes.

Polyamines and LSD1 Inhibition

Epigenetic Control of Gene Expression by Lysine-Specific Demethylase 1

As stated above, in cancer, DNA promoter hypermethylation in combination with other chromatin modifications, including decreased activating marks and increased repressive marks on histone proteins 3 and 4, have been associated with the silencing of tumor suppressor genes.[161] The important role of promoter CpG island methylation and its relationship to covalent histone modifications has recently been reviewed.[162] As was mentioned above, the N-terminal lysine tails of histones can undergo numerous post-translational modifications, including phosphorylation, ubiquitination, acetylation and methylation.[127, 163, 164] To date, 17 lysine residues and 7 arginine residues on various histone proteins have been shown to undergo methylation,[165] and lysine methylation on histones can signal transcriptional activation or repression, depending on the specific lysine residue involved.[166-168] All known histone lysine methyltransferases contain a conserved SET methyltransferase domain, and it has been shown that aberrant methylation of histones due to SET domain deregulation is linked to carcinogenesis.[169] Histone methylation, once thought to be an irreversible process, has recently been shown to be a dynamic process regulated by the addition of methyl groups by histone

methyltransferases and removal of methyl groups from mono- and dimethyllysines by lysine specific demethylase 1 (LSD1), and from trimethyllysines by specific Jumonji C (JmjC) demethylases.[163, 164, 170, 171] Additional demethylases in the JmjC methylase class are continuing to be identified. It has recently been demonstrated that LSD1 demethylates K370me1 and K370me2 on the p53 tumor suppressor and transcriptional activator protein, indicating that LSD1 also plays a role in demethylation of non-histone proteins.[172] A key positive chromatin mark found associated with promoters of active genes is histone 3 dimethyllysine 4 (H3K4me2).[173, 174] LSD1, also known as BHC110,[163, 175] catalyzes the oxidative demethylation of histone 3 methyllysine 4 (H3K4me1) and H3K4me2, and thus is associated with transcriptional repression. H3K4me2 is a transcription-activating chromatin mark at gene promoters, and demethylation of this mark by LSD1 may prevent expression of tumor suppressor genes important in human cancer.[176] Thus, LSD1 is emerging as an important new target for the development of specific inhibitors as a new class of antitumor drugs.[177]

The X-ray structure and mechanism of LSD1 have recently been reported. As shown in Figure 21, the LSD1 reaction is an FAD-dependent oxidative demethylation that most likely proceeds through a protonated imine intermediate.[178] The K_m for H3K4me2 has been determined to be 30 μM,[163] which is comparable to substrate K_m values for other histone-modifying enzymes. To date, only a few compounds have been shown to act as inhibitors of LSD1. The active site structure of LSD1 has considerable sequence homology to monoamine oxidases A and B (MAO A and B), and to acetylpolyamine oxidase (APAO) and spermine oxidase (SMO).[163, 179, 180] The MAO inhibitors nialamide, clorgyline, deprenyl, and pargyline were devoid of any inhibitory activity, while phenelzine and tranylcypromine effectively inhibited LSD1 at 1 mM. The most potent inhibitor, tranylcypromine was titrated to reveal an IC_{50} of < 2 μM. LSD1 inhibition by tranylcypromine was subsequently shown to be irreversible and mechanism-based, and it increased global levels of H3K4me2 in the P19 cell line. However, no evidence was presented to show whether tranylcypromine caused the re-expression of silenced tumor suppressor genes. It has recently been demonstrated that the polyaminoguanidine guazatine (**97**, Figure 21) is a non-competitive inhibitor of maize polyamine oxidase.[181] Importantly, guazatine can discriminate between purified recombinant murine APAO and SMO, suggesting that it is possible to selectively inhibit these enzymes.[182] Recently, a series of N,N'-bis [(pyridinyl) methyl]-diamines typified by BPOD (**98**, Figure 21) were shown to produce competitive inhibition of purified plant polyamine oxidase (PAO) in vitro.[183]

Polyamine Analogues, LSD1 Inhibition and Re-expression of Silenced Gene Products

We reported the synthesis of a novel series of polyamino(bis)guanidines and polyaminobiguanides with potent trypanocidal activity in vitro,[88] and subsequently found these analogues to be effective inhibitors of APAO and SMO (unpublished observations). Because LSD1 is a similar flavin-dependent oxidase, we evaluated small library of these bisguanidine (**99-105**, Figure 22) and biguanide (**106-111**, Figure 22) polyamine analogues for the ability to inhibit recombinant LSD1 in vitro. Nine of the 13 compounds tested were found to non-competitively inhibit recombinant LSD1 activity by >50% at 1 μM.[176] Although these analogues also inhibit APAO and SMO to some degree, they are significantly less potent in that regard. The most potent LSD1 inhibitor, **109** (90.9% inhibition), was chosen for further study. In many cancer cell types, H3K4me2 is depleted in the promoters of several epigenetically silenced, and aberrantly DNA-hypermethylated genes that are important in tumorigenesis.[184] In HCT116 human colon tumor cells, exposure to 1 μM **109** produced significant increases in both H3K4me1 and H3K4me2, while not affecting the global histone 3 dimethyllysine 9 (H3K9me2) levels. Following 48-hour treatment of HCT116 cells with 1.0 μM **109**, four members of the secreted frizzle-related protein family (SFRP1, SFRP2, SFRP4, and SFRP5)[185] and two GATA family transcription factors (GATA4 and GATA5)[186] were re-expressed. Compound **109** produced similar effects on re-expression of SFRP4 and SFRP5,

and global H3K4me2 levels in RKO colon cancer cells. Treatment with **109** resulted in ~20-35% of the gene re-expression produced by 1 μM of the DNA methyltransferase inhibitor 5-AC, whereas no measurable expression was seen after treatment with the HDAC inhibitor **79** (300 nM), or 1 μM of the related analogues **102** or **107**. These results demonstrate that **109**, although not as potent as 5-AC, is effective at re-expressing epigenetically silenced genes. Importantly, compound **109** and its cogeners are the first examples of synthetic LSD1 inhibitors that produce epigenetic changes leading to re-expression of silenced tumor suppressor genes. Promising in vivo effects of treatment with **109** alone and in combination with 5-AC have recently been observed in an HCT166 human colon tumor xenograft model in athymic nu/nu Fox Chase mice. The results of these studies are being reported in an upcoming primary publication. Taken together, these data support the contention that LSD1 inhibitors that promote re-expression of aberrantly silenced tumor suppressor genes can be of benefit when used in combination with established antitumor agents. Additional analogues in this series are being synthesized and evaluated as a means to develop structure/activity relationships for this novel class of LSD1 inhibitors.

Conclusion

Prior to 1990, antitumor drug discovery research in the polyamine area was characterized by the design, synthesis and development of polyamine biosynthesis inhibitors. Despite a great deal of research, development of specific inhibitors as antitumor agents has only resulted in advancing one compound to the market. The discovery of the bis(ethyl)polyamines in the early 1990's made it clear that the natural polyamines have numerous effector sites in tumor cells, and that therapeutic agents designed to interact with effector sites distinct from the biosynthetic enzymes can be developed for use in chemotherapy. The latest trend, the inclusion of polyamine-like features in drug candidates for the purpose of enhancing transport or DNA affinity, has opened the door to new targets and new avenues of investigation. Thus, polyamine research in drug development remains a robust area of exploration, with great potential for the identification of new antitumor agents.

Acknowledgments

Portions of the work described in this manuscript were supported by NIH RO1 CA85509 (PMW), and by a generous gift from Progen Pharmaceuticals, Toowong, Queensland, Australia (PMW, RAC).

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Abbreviations

DFMO

α-difluoromethylornithine

ODC

ornithine decarboxylase

AdoMet-DC
S-adenosylmethionine decarboxylase

BENSpm
bis(ethyl)norspermine

BESpm
bis(ethyl)spermine

BEHSpm
bis(ethyl)homospermine

BE-4444
1,20-(ethylamino)-5,10,15-triazanonadecane

PENSpm
N^1-propargyl-N^{11}-ethylnorspermine

CPENSpm
N^1-cyclopropylmethyl-N^{11}-ethylnorspermine

MTT
(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

SSAT
spermidine/spermine-N^1-acetyltransferase

SMO
spermine oxidase

HDAC
histone deacetylase

HAT
histone acetyltransferase

SAHA
suberoylanilide hydroxamic acid

DNMT
DNA methyltransferase

5-AC
5-azacytidine

PAHA
polyaminohydroxamic acid

PABA
polyaminobenzamide

HSPG
heparan sulfate proteoglycans

LSD1
lysine-specific demethylase 1

JmjC
jumonji C demethylases

H3K4me1
: histone 3 methyllysine 4

H3K4me2
: histone 3 dimethyllysine 4

MAO
: monoamine oxidase

APAO
: acetylpolyamine oxidase

SFRP
: secreted frizzle-related protein

SAR
: structure/activity relationships

CNS
: central nervous system

Biographies

Robert A. Casero, Jr., Ph.D. is a Professor of Oncology in the Johns Hopkins University School of Medicine. Dr. Casero is a molecular pharmacologist who has spent most of the last 30 years studying the role of polyamines in normal and tumour cell growth, and devising strategies to target polyamine function and metabolism for therapeutic benefit. His laboratory was responsible for cloning several genes involved in human polyamine catabolism; genes whose expression are thought to play a role in determining cellular responses to specific polyamine analogues. Dr. Casero is also a scientific advisor to Progen Pharmaceuticals.

Patrick M. Woster, Ph.D. is Professor of Pharmaceutical Sciences in the Eugene Applebaum College of Pharmacy and Health Sciences at Wayne State University. Dr. Woster is a medicinal chemist with an interest in the synthesis of molecules that modulate polyamine metabolism as potential antitumor agents. He has produced a number of enzyme inhibitors that target enzymes in the polyamine biosynthetic pathway, and synthesized the first unsymmetrically substituted alkylpolyamine analogues. Molecules developed in the Woster laboratory have been shown to produce dramatic effects on a variety of tumor cells by initiating apoptosis, binding to DNA and by producing epigenetic changes in gene expression. Dr. Woster currently serves as a consultant to Progen Pharmaceuticals.

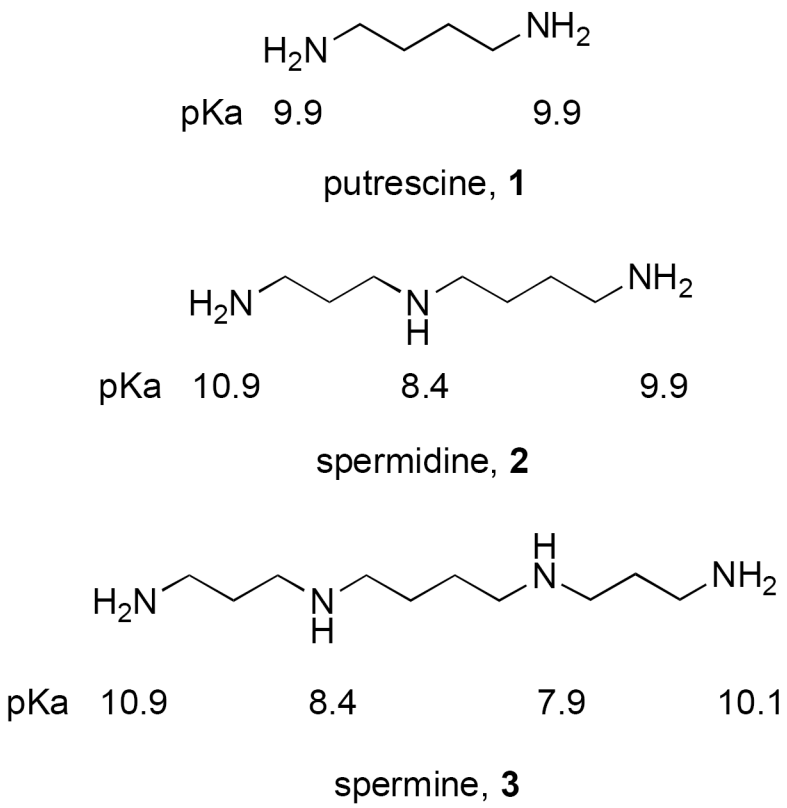
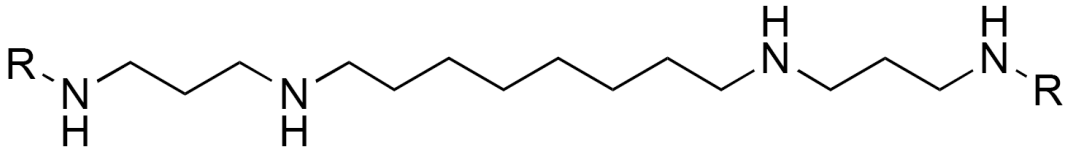
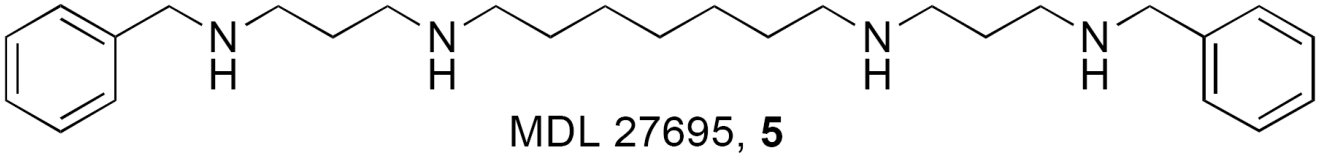


Figure 1.
Structures and pKa values of the polyamines putrescine, spermidine and spermine.



4a, tetraamine A (R = H)
4b, tetraamine B (R = methyl)
4c, tetraamine C (R = ethyl)



MDL 27695, **5**

Figure 2.
Early examples of polyamine analogues with biological activity in vitro.

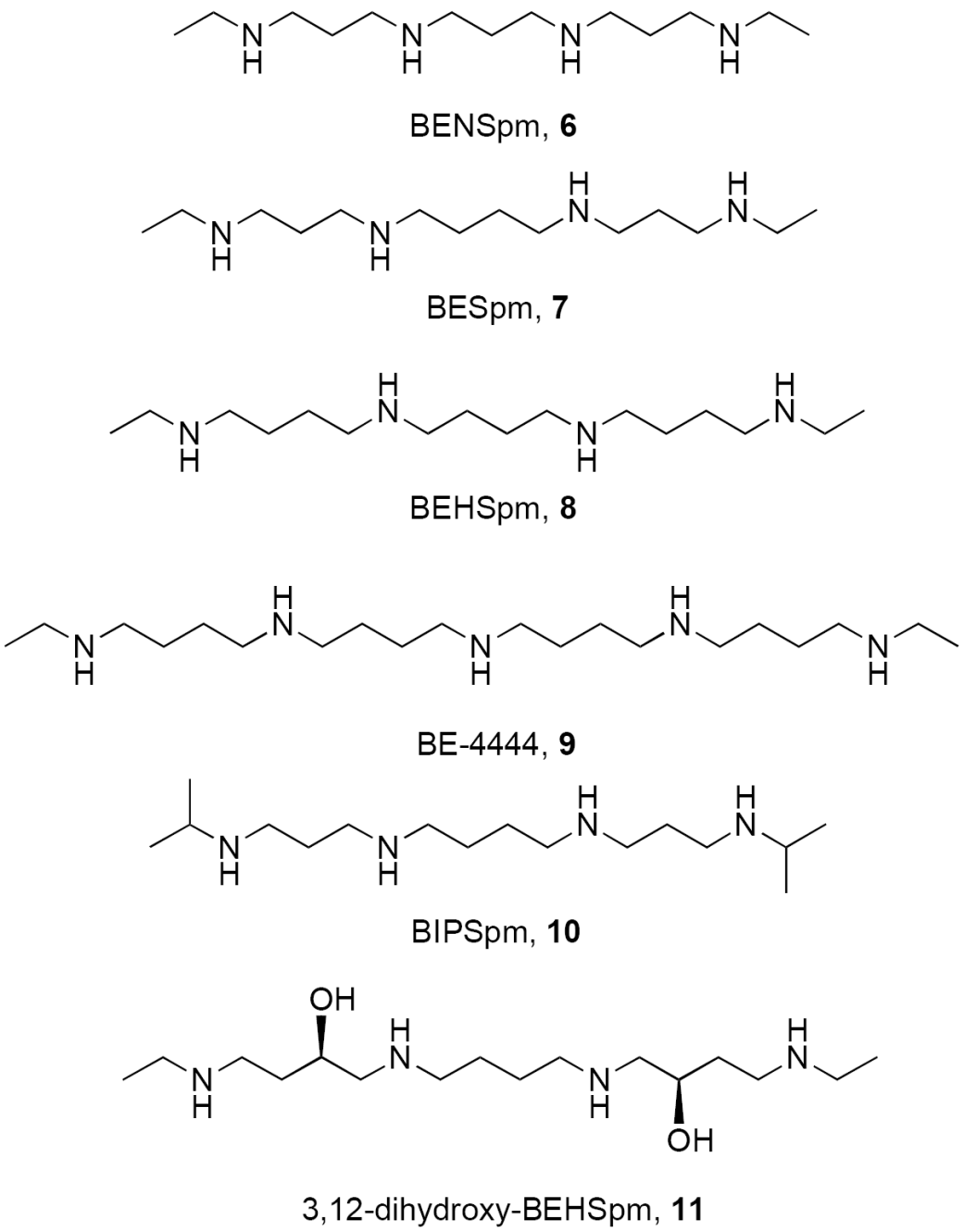


Figure 3.
Structures of bis(ethyl)polyamines **6-11**.

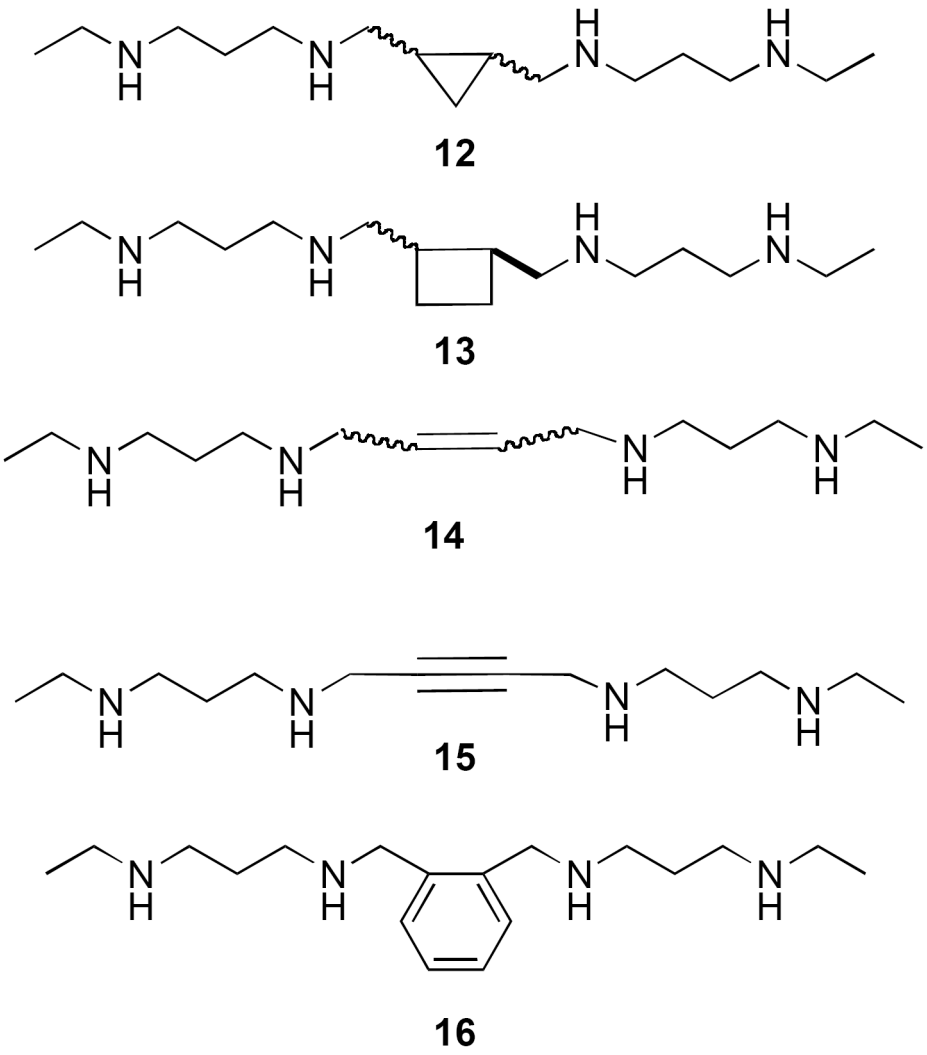


Figure 4.
Conformationally restricted analogues of BESpm.

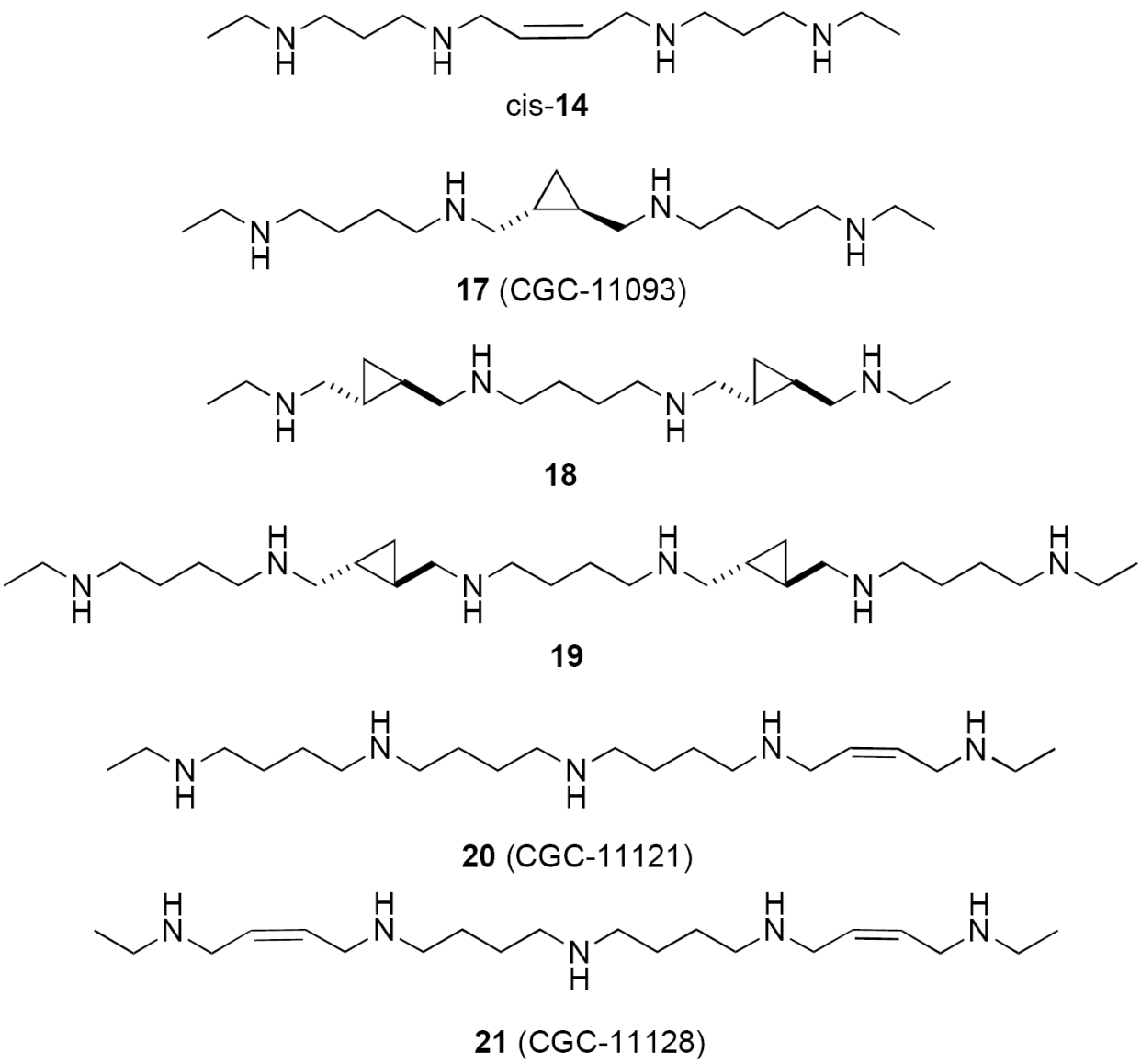
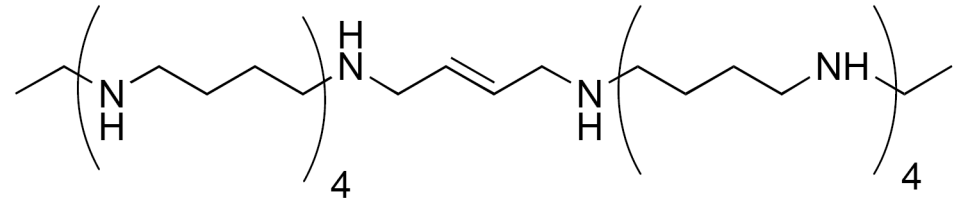
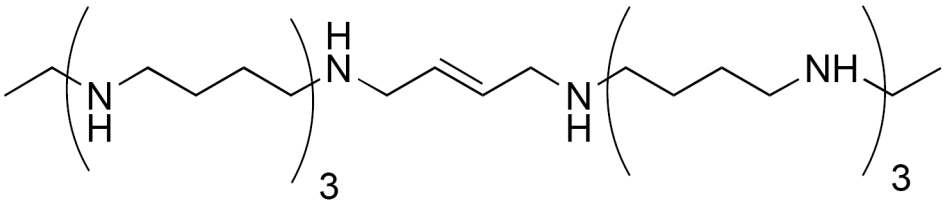


Figure 5.
Conformationally restricted bis(ethyl) polyamine analogues with enhanced antitumor activity against prostate tumor cells in culture.



22 (CGC-11144)



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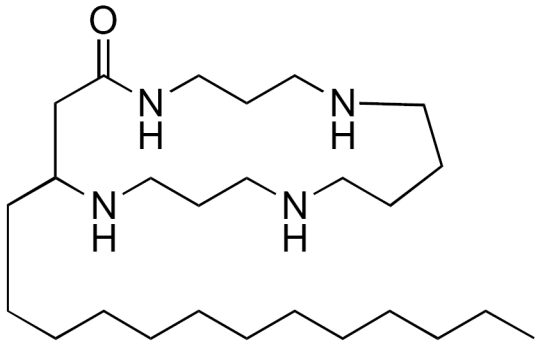

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Figure 6.
Structures of oligamines and macrocyclic polyamines with antitumor activity.

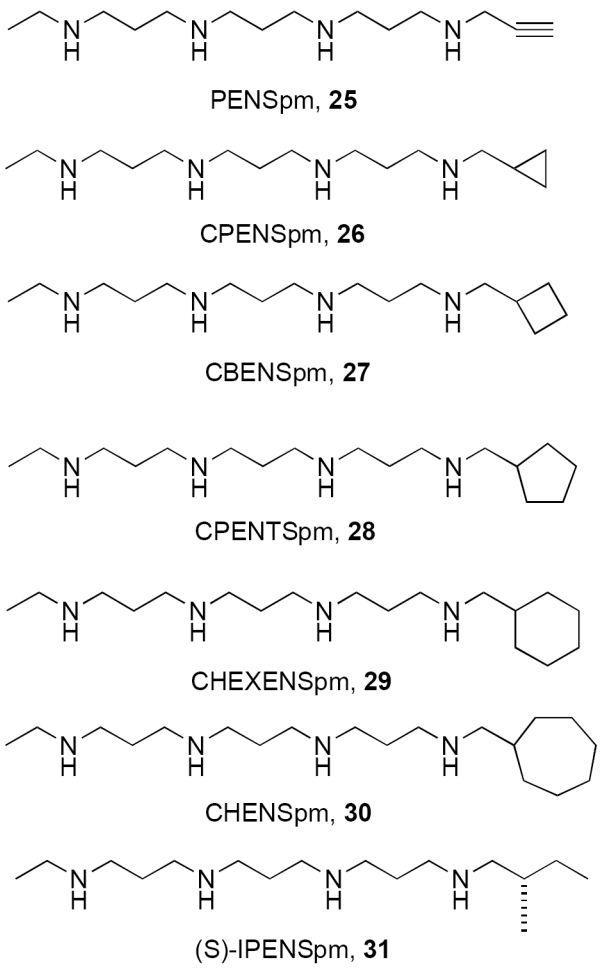


Figure 7.
The unsymmetrically substituted alkylpolyamines PENSpm, CPENSpm, CBENSpm, CPENTSpm, CHEXENSpm, CHENSpm and IPENSpm.

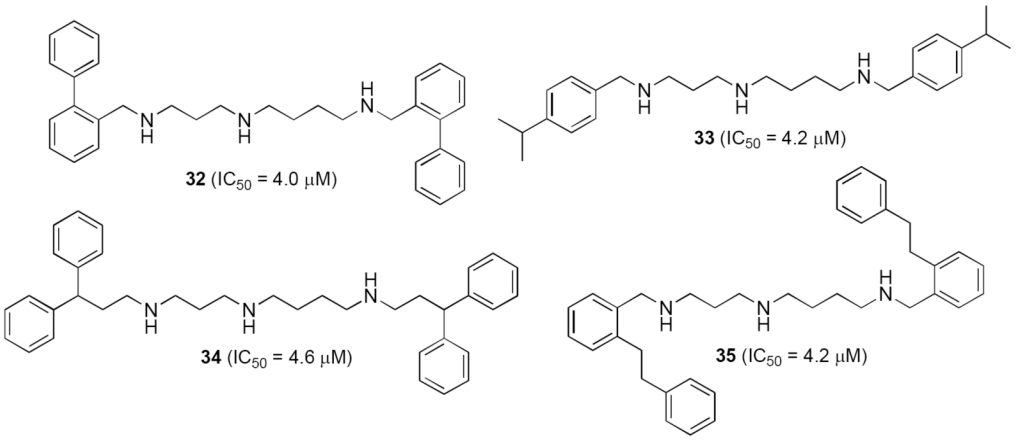


Figure 8.
Alkypolyamine analogues with a 3-4 architecture that possess antitumor activity in vitro.

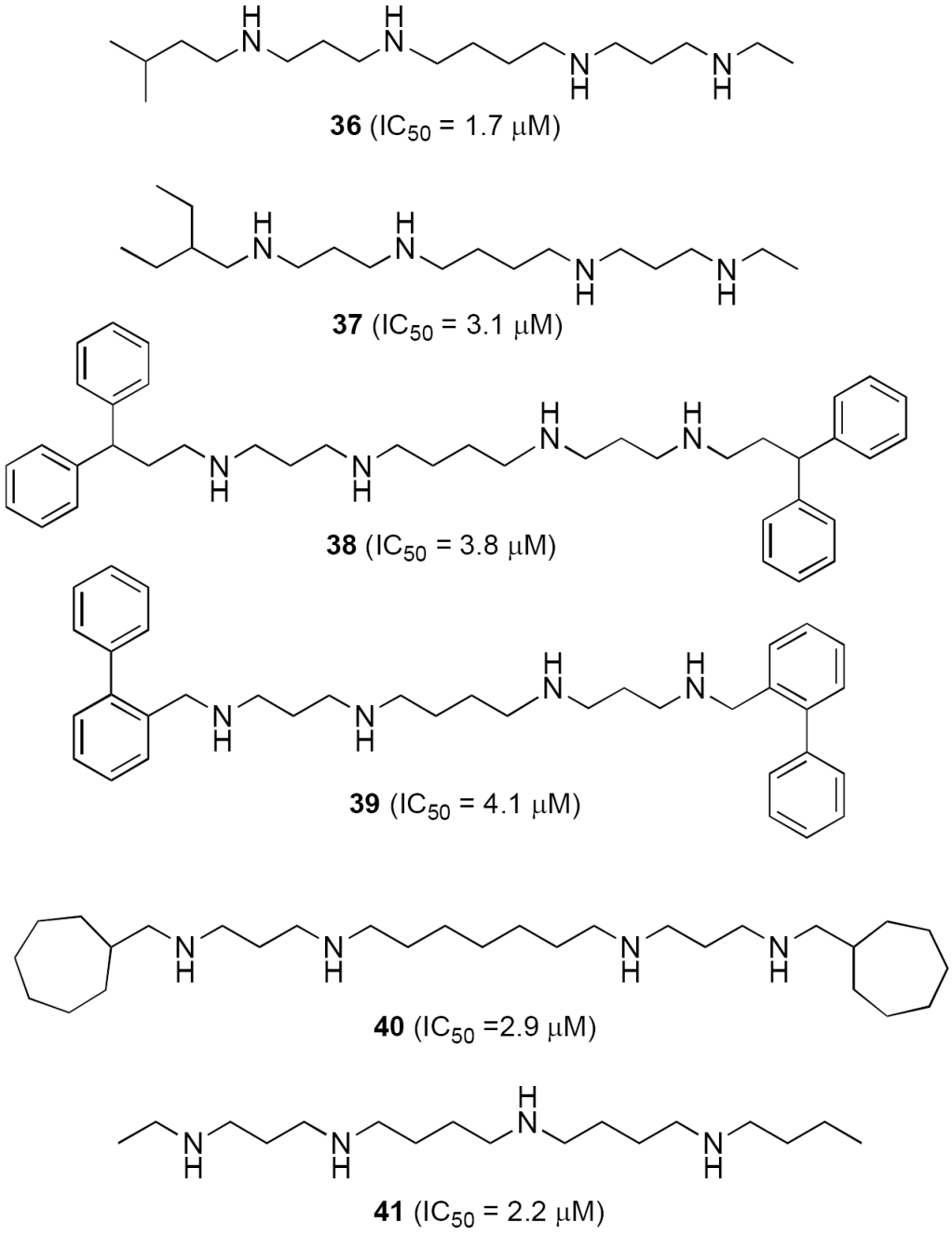


Figure 9.
Alkypolyamine analogues with a 3-4-3, 3-5-3 or 3-4-4 architecture that possess antitumor activity in vitro.

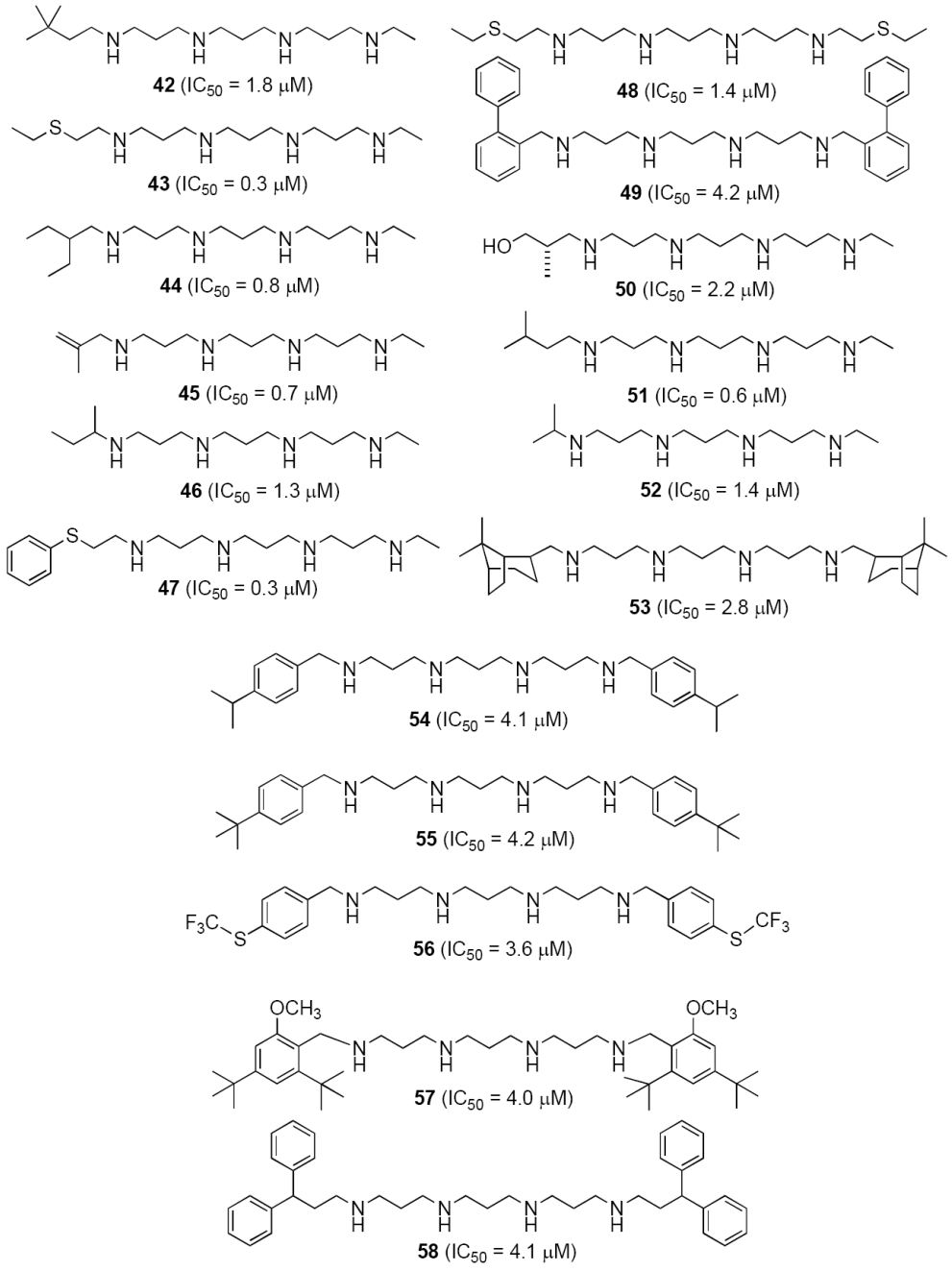


Figure 10.
Alkypolyamine analogues with a 3-3-3 architecture that possess antitumor activity in vitro.

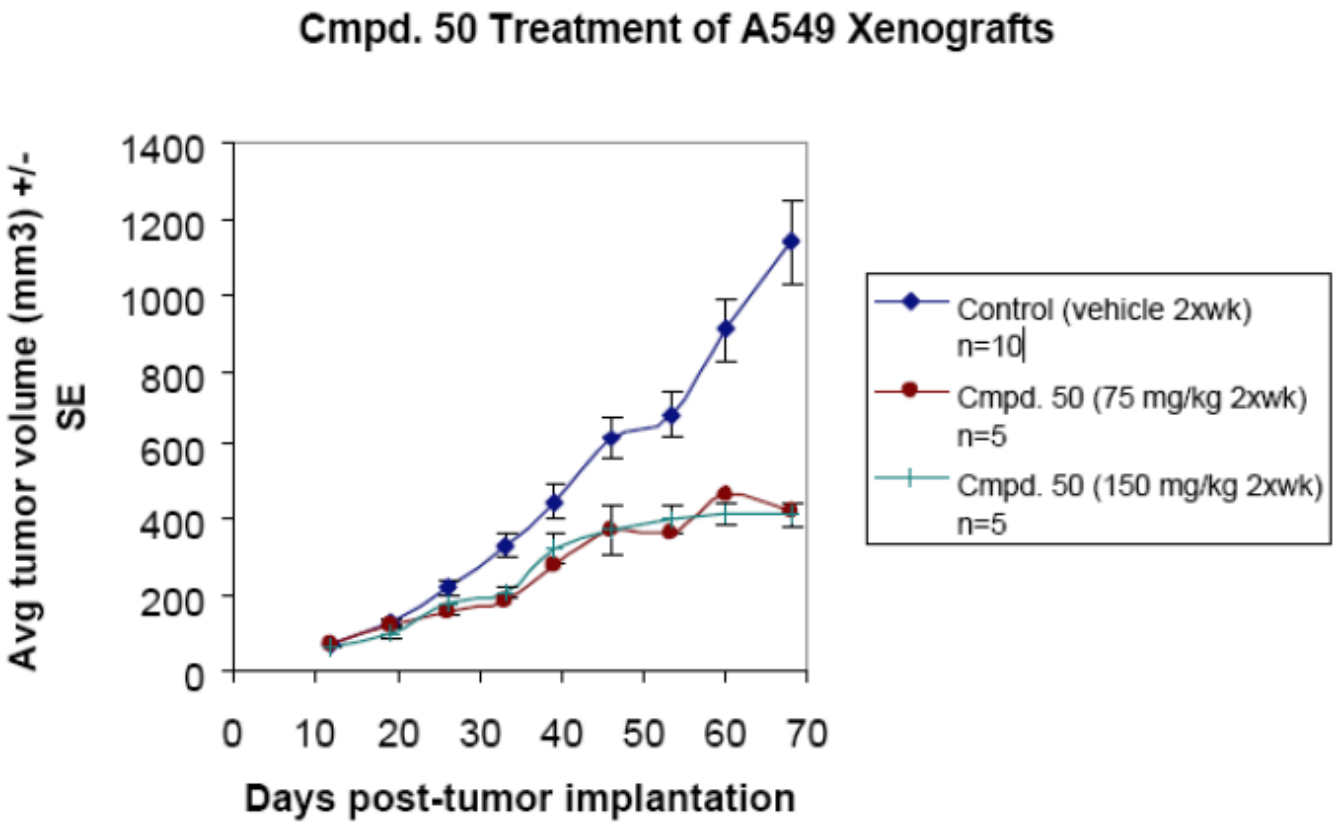


Figure 11.
Antitumor effects of compound **50** in an A549 human lung tumor xenograft model. Animals were given either 75 or 150 mg/kg of **50** twice weekly by i.p. injection.

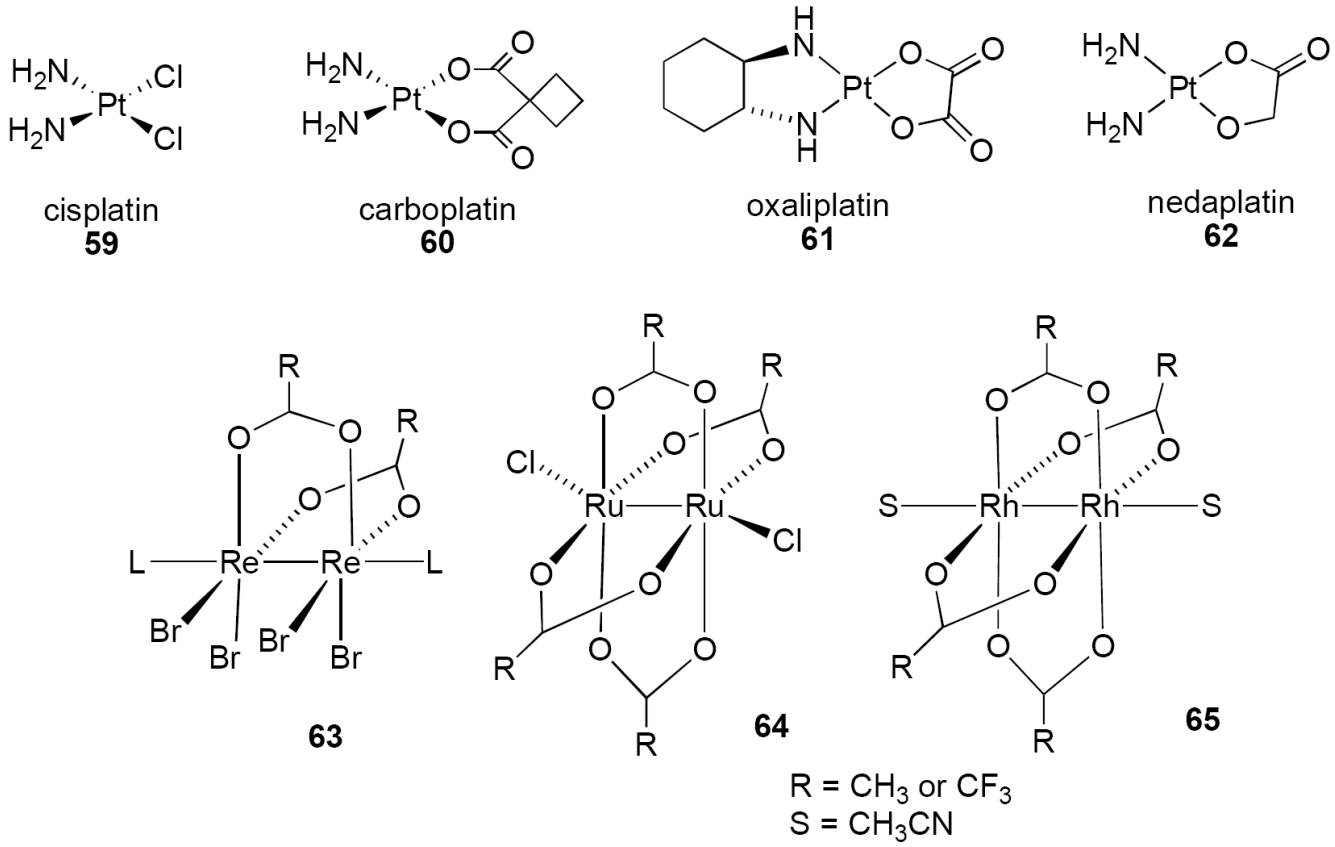


Figure 12.
Platinum, rhenium, rhodium and ruthenium compounds with demonstrated antitumor activity.

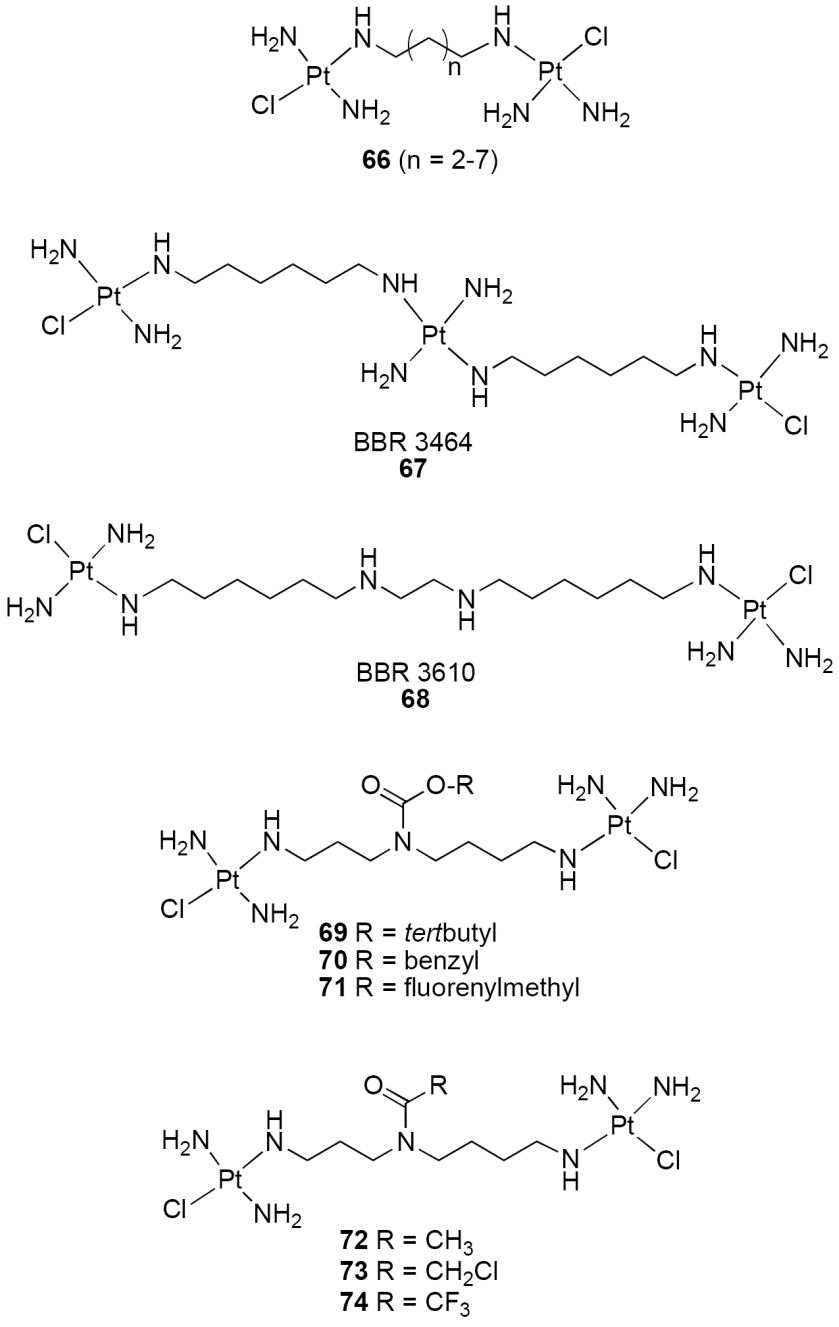


Figure 13.
Dinuclear and trinuclear Pt/polyamine complexes with significant antitumor activity.

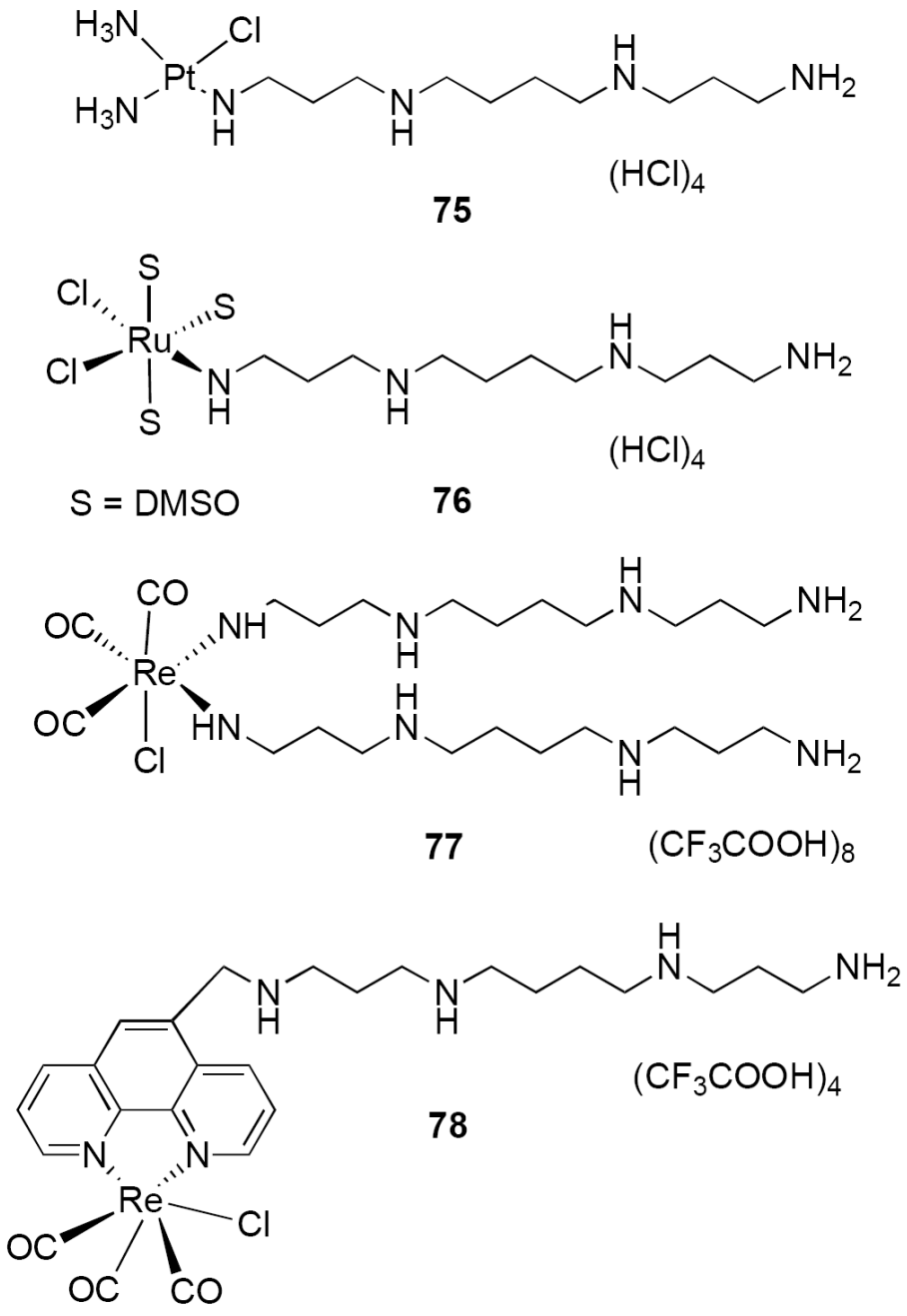


Figure 14.
Representative polyamine/metal complexes with antitumor activity in vitro. S = solvent (DMSO).

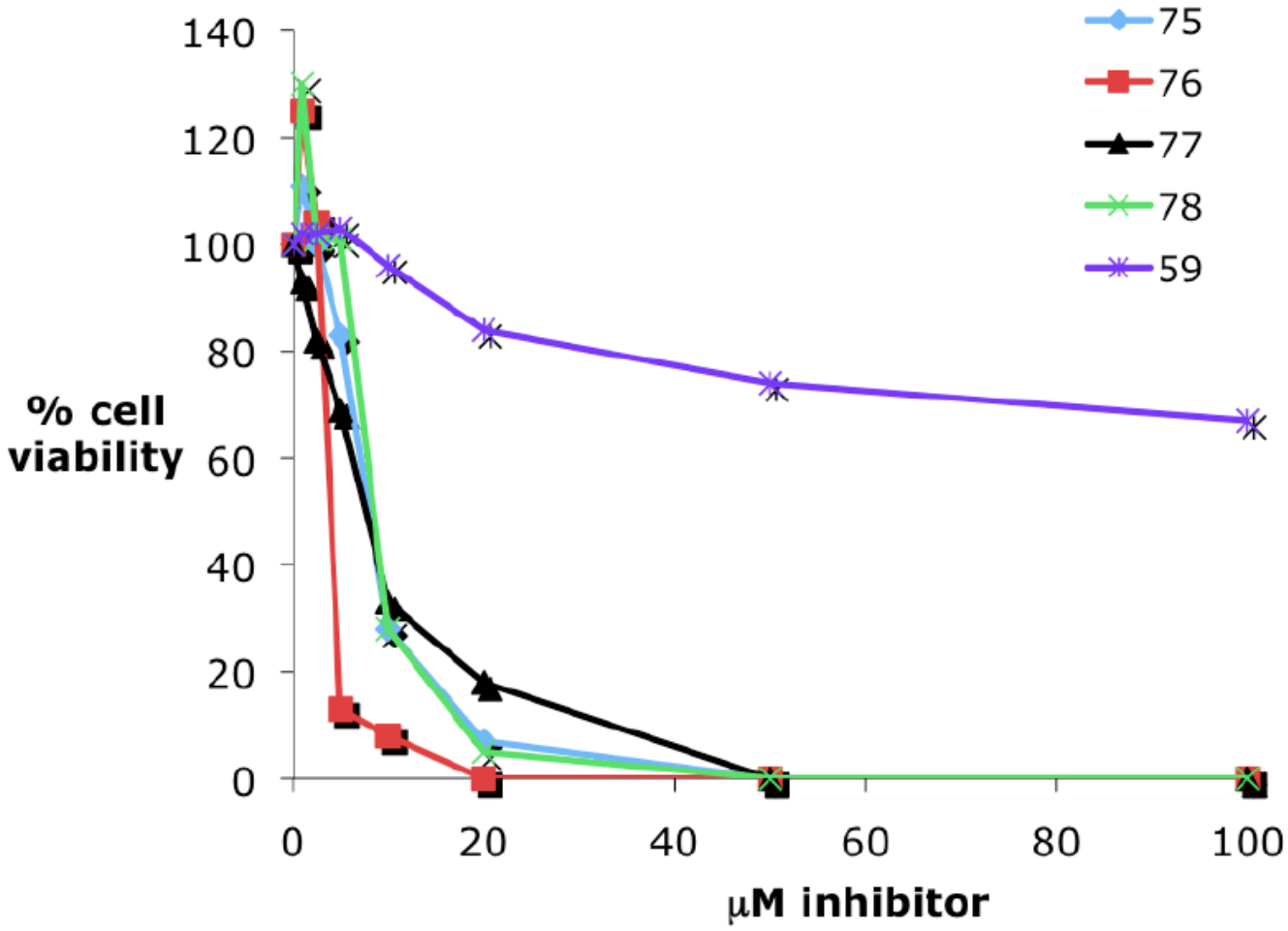


Figure 15.
MTS assay demonstrating growth inhibition caused by **75, 76, 77** and **78** in cultured MCF7 breast tumor cells.

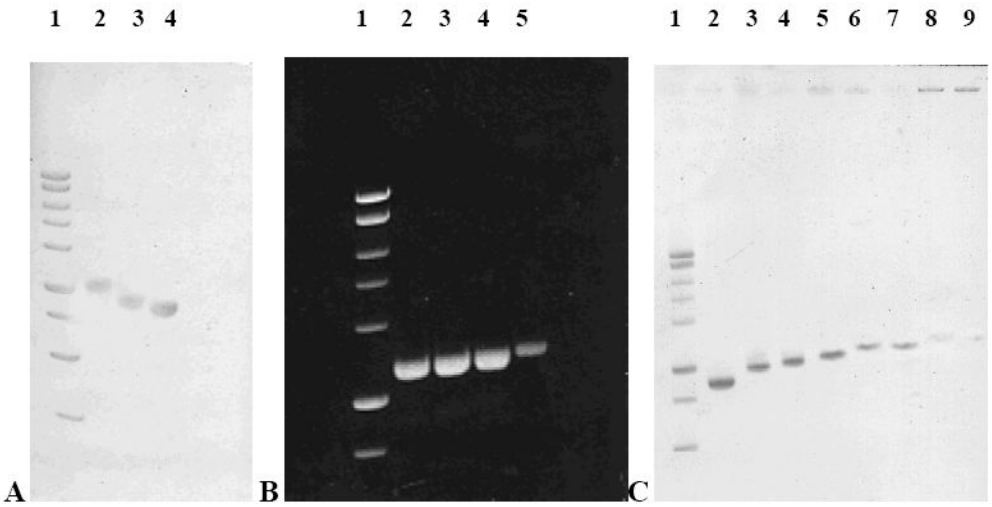


Figure 16.

DNA binding characteristics of compound **75**. Linearized plasmid DNA (1.5μg) was incubated with appropriate concentrations of metal complexes at 37°C for the indicated time. Samples were analyzed on 0.8% agarose gel. **Panel A**. Comparison of Lane 1: MW standard; Lane 2: DNA + 30 μM **75**; Lane 3: DNA + 30 μM cisplatin; Lane 4: plasmid DNA control. **Panel B**. Concentration dependence of DNA binding to **75**. A 1.5μ G sample of plasmid DNA was treated with 3, 10, 30, 100 and 300 μM **75** at 37°C for 4 hours. Lane 1: MW standard; Lane 2: 10 μM **75**; Lane 3: 30 μM **75**; Lane 4: 100 μM **75**; Lane 5: 300 μM **75. Panel C**. Time-dependence of DNA binding to **75**. From left to right - Lane 1: MW standard; Lane 2: Plasmid DNA control; Lane 3: 5 min; Lane 4: 15 min; Lane 5: 30 min; Lane 6: 1 h; Lane 7: 2 h; Lane 8: 4 h; Lane 9: 6 h.

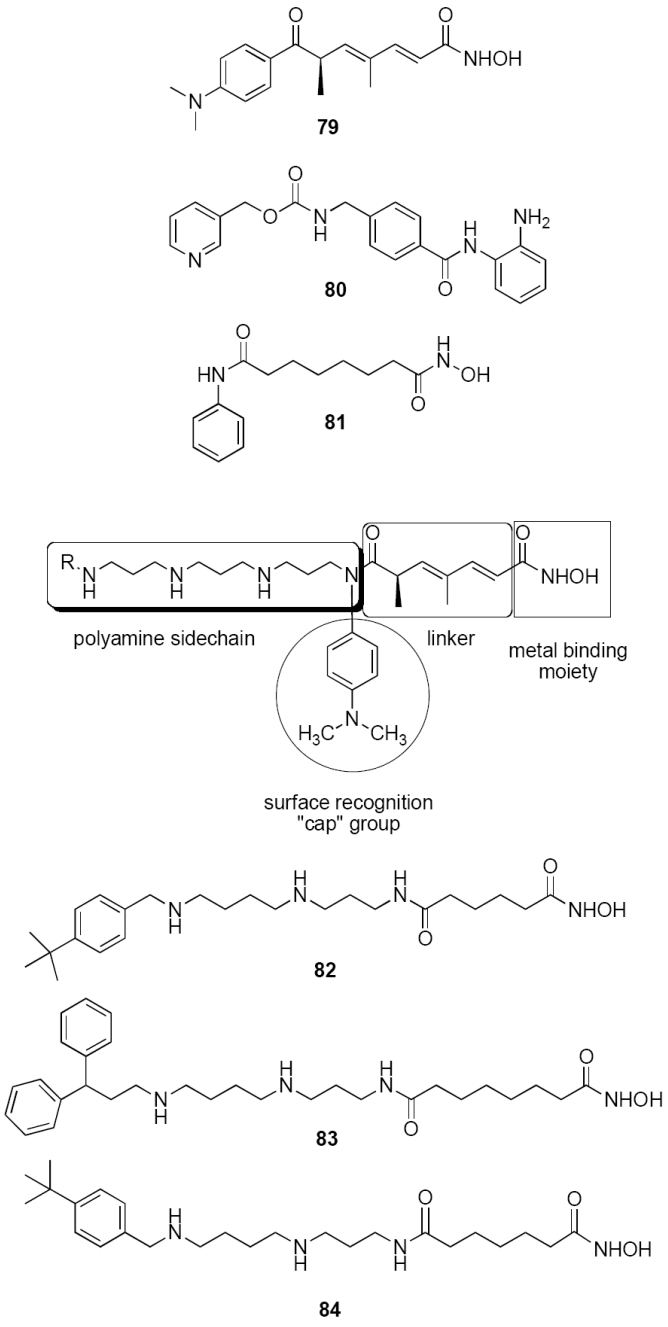


Figure 17.
Structures of the classical HDAC inhibitors **79-81**, the general structure for polyamine-based HDAC inhibitors, and PAHAs **82-84**.

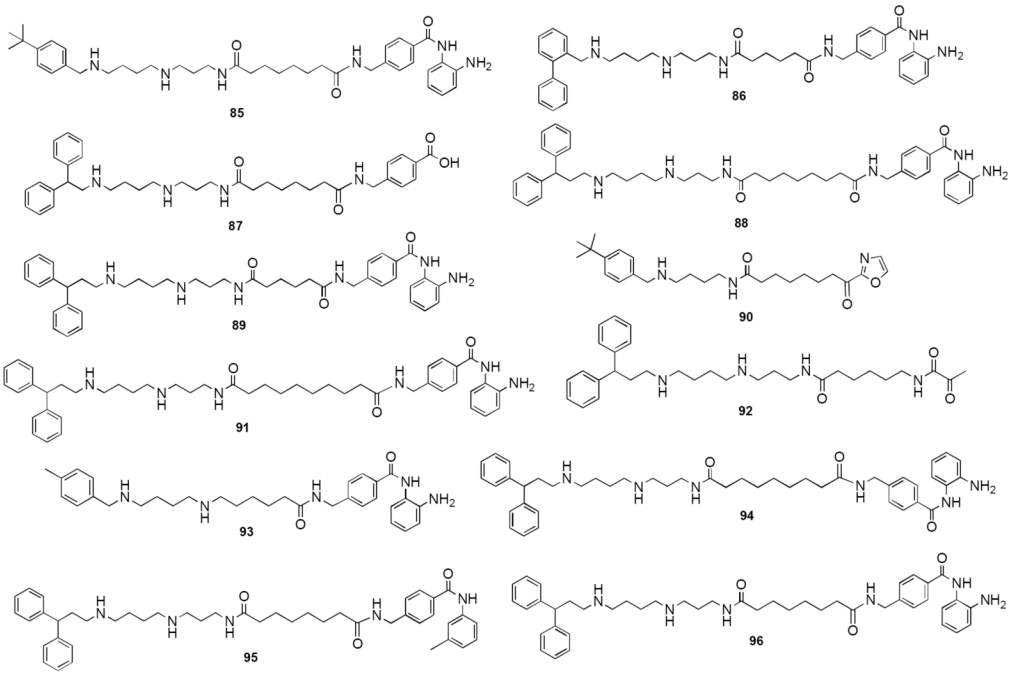


Figure 18.
Structures of PABA analogue inhibitors of HDAC **85-95**.

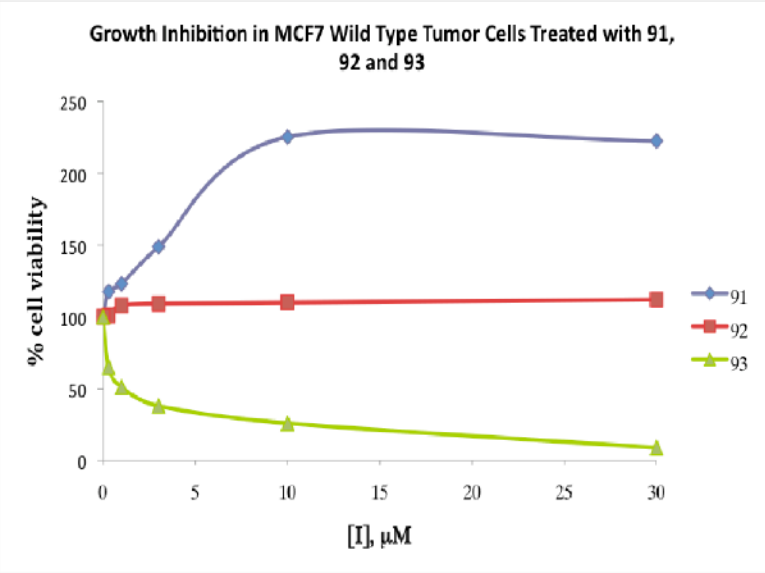


Figure 19.

Dose-response curves following 96 hour treatment of MCF7 wild type tumor cells with **91, 92** or **93**. Data points are the average of three determinations that in all cases differ by < 5%.

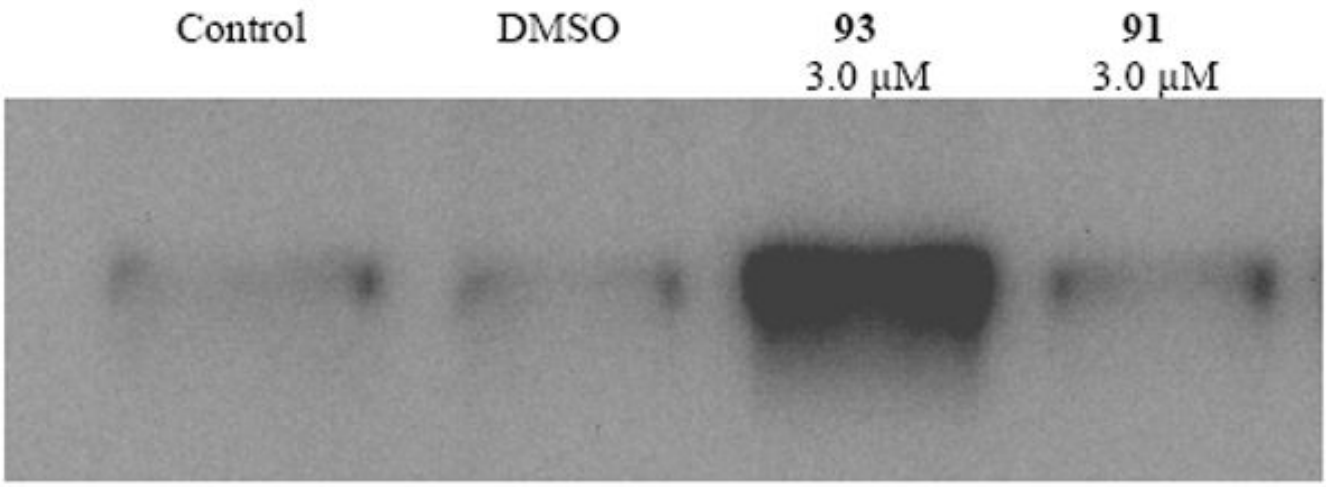


Figure 20.
Induction of Annexin I by PABA HDAC inhibitors **91** and **93**.

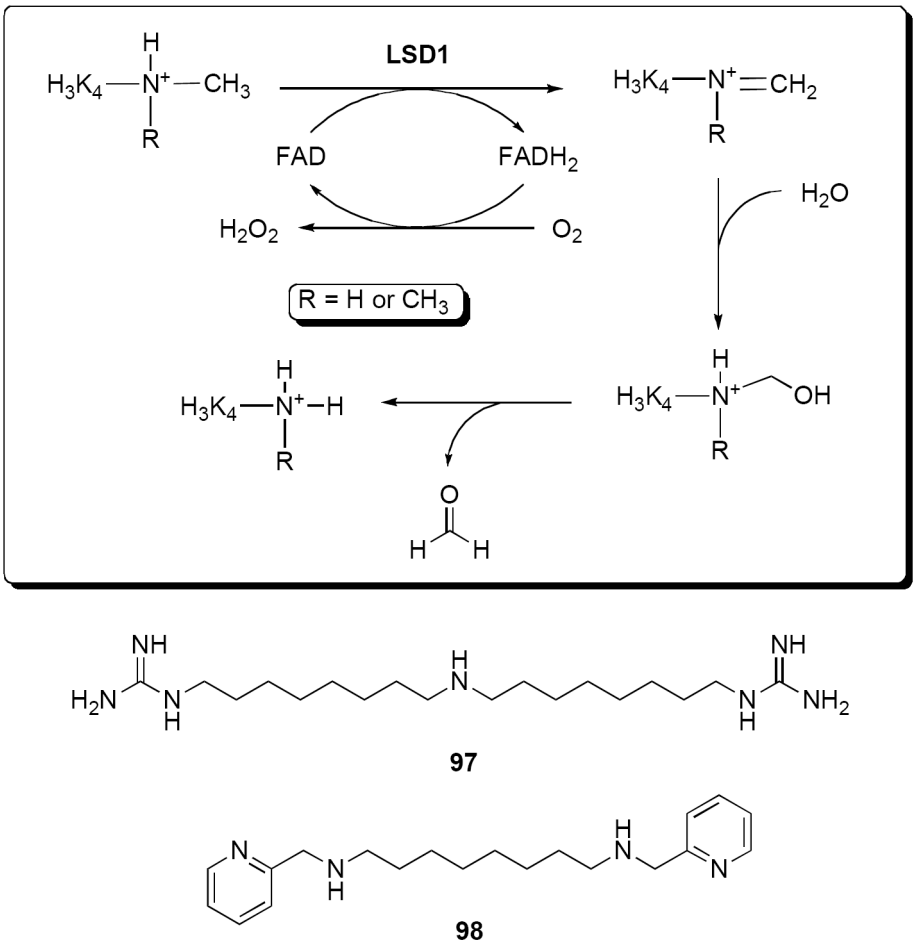
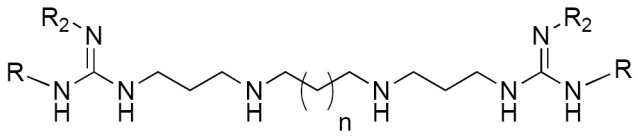
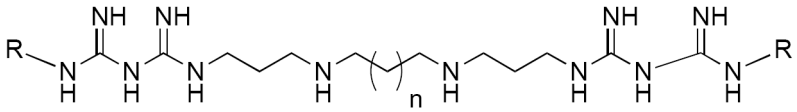


Figure 21.
The catalytic mechanism of LSD1, and the amine oxidase inhibitors **97** and **98**.



99 (n = 1, R = CH_3, R_2 = H)
100 (n = 5, R = CH_3, R_2 = H)
101 (n = 1, R = CH_3, R_2 = CH_3)
102 (n = 1, R = phenyl, R_2 = phenyl)
103 (n = 5, R = phenyl, R_2 = phenyl)
104 (n = 2, R = R_2 = 3,3-diphenylpropyl, R_2 = H)
105 (n = 2, R = R_2 = 2,2-diphenylethyl, = H)



106 (n = 1, R = 2,2-diphenylethyl)
107 (n = 5, R = 2,2-diphenylethyl)
108 (n = 1, R = 3,3-diphenylpropyl)
109 (n = 5, R = 3,3-diphenylpropyl)
110 (n = 2, R = 3,3-diphenylpropyl)
111 (n = 2, R = 2,2-diphenylethyl)

Figure 22.
Structures of polyamino(bis)guanidines **99**-**105** and polyaminobiguanides **106**-**111**.

Table 1

Summary of the antitumor effects of symmetrically substituted polyamine analogues 4-24. References for these data can be found in the text.

Compound	Target	IC_{50} (μM)	Biological Effect	Cell Line(s)	Therapeutic Area	Status
4c	unknown	not determined	cytotoxicity	L 1210	antleukemic	abandoned
5	unknown	3.0	antiparasitic	*Plasmodium falciparum*	antimalarial	abandoned
6	SSAT, mitochondrial DNA synthesis	0.2 – 1.0	cytotoxicity; apoptosis	L 1210; MALME-3M	lung tumor; breast tumor; melanoma; antidiarrheal	Phase II trials terminated (lack of efficacy)
7	SSAT, mitochondrial DNA synthesis	0.5 – 1.0	cytotoxicity; apoptosis	L1210	lung tumor; melanoma; antidiarrheal	abandoned
8	SSAT, mitochondrial DNA synthesis	1.0 – 5.0	cytotoxicity; apoptosis	MALME-3M	lung tumor; melanoma; antidiarrheal	abandoned
9	DNA	0.1 – 1.0	cytotoxicity; apoptosis	DU145; LnCaP; PC3	prostate tumor; brain tumor	abandoned
10	SSAT, mitochondrial DNA synthesis	3.0 – 5.0	cytotoxicity; apoptosis	MALME-3M	lung tumor; melanoma; antidiarrheal	abandoned
11	SSAT, mitochondrial DNA synthesis	0.6	cytotoxicity	L 1210	lung tumor; melanoma; antidiarrheal	abandoned
12	DNA	0.3 - > 30	cytotoxicity	A549, HT-29, U251MG, DU145, PC-3 and MCF7	prostate tumor; breast tumor	abandoned
13	DNA	0.1 - > 30	cytotoxicity	A549, HT-29, U251MG, DU145, PC-3 and MCF7	prostate tumor; breast tumor	abandoned
***cis*-14**	DNA	0.25 – 3.6 (prostate) and 9.25 (breast)	cytotoxicity	A549, HT-29, U251MG, DU145, PC-3 and MCF7; *Cryptosporidium parvum*	prostate tumor; breast tumor, lung tumor; antiparasitic macular degeneration	Phase I trials
15	DNA	> 30	growth inhibition	A549, HT-29, U251MG, DU145, PC-3 and MCF7	prostate tumor; breast tumor	abandoned
16	DNA	> 30	growth inhibition	A549, HT-29, U251MG, DU145, PC-3 and MCF7	prostate tumor; breast tumor	abandoned
17	DNA	0.25 – 12.4 (prostate) and > 30 (breast)	cytotoxicity; apoptosis	A549, HT-29, U251MG, DU145, PC-3 and MCF7	lung tumor; prostate tumor; myeloma; macular degeneration	Phase I trials
18	DNA	0.06 – 0.26	cytotoxicity; apoptosis	DuPro; DU145	prostate tumor	Phase I trials
19	DNA	0.12 – 0.38	cytotoxicity; apoptosis	DuPro; DU145	prostate tumor	Phase I trials
20	DNA	0.08 - 0.52	cytotoxicity; apoptosis	LnCaP; DU145; DuPro; PC-3	prostate tumor	Phase I trials
21	DNA	0.14 – 0.5	cytotoxicity; apoptosis	LnCaP; DU145; DuPro; PC-3	prostate tumor	Phase I trials
22	DNA	0.09 – 0.47	cytotoxicity; DNA aggregation	LnCaP; DU145; DuPro; PC-3; MDA-MB-435	prostate tumor	advanced pre-clinical trials

Compound	Target	IC_{50} (μM)	Biological Effect	Cell Line(s)	Therapeutic Area	Status
23	DNA	0.13 – 0.41	cytotoxicity; DNA aggregation	LnCaP; DU145; DuPro; PC-3; MDA-MB-435; *Enterocytozoon bieneusi*	prostate tumor, chronic diarrhea	advanced pre-clinical trials
24	ATP	0.6 – 0.83	ATP depletion	DuPro; PC-3	prostate tumor	unknown

Table 2

Summary of the antitumor effects of selected unsymmetrically substituted polyamine analogues. References for these data can be found in the text.

Compound	Target	IC_{50} (μM)	Biological Effect	Cell Line(s)	Therapeutic Area	Status
26	SSAT	0.2 - 1.3	SSAT induction, production of peroxide, apoptosis	H157; MCF-7; T47D; ZR-75-1; MDA-MDB-468; MDA-MB-231; Hs578T	lung tumor; breast tumor	advanced pre-clinical trials
30	unknown; polyamine metabolic enzymes	0.7	apoptosis; disruption of tubulin depolymerization	H157; H82	lung tumor	not currently in development
(S)-31	unknown; polyamine metabolic enzymes	1.3	apoptosis; disruption of tubulin depolymerization	H157; H82	lung tumor	not currently in development
32	polyamine metabolic enzymes	4.0	apoptosis	H157	lung tumor	not currently in development
33	polyamine metabolic enzymes	4.2	apoptosis	H157	lung tumor	not currently in development
34	polyamine metabolic enzymes	4.6	apoptosis	H157	lung tumor	not currently in development
35	Polyamine metabolic enzymes	4.2	apoptosis	H157	lung tumor	not currently in development
36	polyamine metabolic enzymes	1.7	apoptosis	H157	lung tumor	not currently in development
37	polyamine metabolic enzymes	3.1	apoptosis	H157	lung tumor	not currently in development
38	polyamine metabolic enzymes	3.8	apoptosis	H157	lung tumor	not currently in development
39	polyamine metabolic enzymes	4.1	apoptosis	H157	lung tumor	not currently in development
40	polyamine metabolic enzymes	2.9	apoptosis	H157	lung tumor	not currently in development
41	polyamine metabolic enzymes	2.2	apoptosis	H157	lung tumor	not currently in development
42	polyamine metabolic enzymes	0.9 – 9.1	induction of SMO (A549); polyamine depletion; apoptosis	H157; A549; H69; H82	lung tumor	pre-clinical
46	polyamine metabolic enzymes	0.8 – 3.6	induction of SSAT (H157); polyamine depletion; apoptosis	H157; A549; H69; H82	lung tumor	pre-clinical
50	polyamine metabolic enzymes	0.7 – 7.1	induction of SSAT (H157); polyamine depletion; apoptosis	H157; A549; H69; H82	lung tumor	pre-clinical
58	unknown; polyamine metabolic enzymes	4.1	apoptosis; anoikis	H157	lung tumor	abandoned

Table 3

IC_{50} values (MCF 7 and MCF10A breast tumor cells) and percent inhibition of global HDAC, and of HDAC 1,3,6 and 8 by compounds 81 and 85-96. ND = not determined

Compound	IC_{50} in MCF7 cells (μM)	IC_{50} in MCF10A cells (μM)	Global HDAC % inhibition	HDAC 1 % inhibition	HDAC 3 % inhibition	HDAC 6 % inhibition	HDAC 8 % inhibition	Status
81	8.5	31	97.2	91.40	97.0	97.20	94.20	marketed (vorinostat)
85	ND	ND	51.2	ND	1.3	11.7	ND	pre-clinical trials
86	> 30	> 30	60.1	68.6	72.9	3.8	14.8	not currently under development
87	> 30	> 30	72.6	45.6	35.4	97.9	48.6	not currently under development
88	> 30	> 30	47.0	79.9	25.0	17.5	13.4	not currently under development
89	> 30	> 30	48.4	61.9	0.3	3.6	10.4	not currently under development
90	27	> 30	43.7	19.5	0.3	9.7	18.9	not currently under development
91	> 30	> 30	42.9	78.7	13.2	0.3	11.9	not currently under development
92	> 30	> 30	30.2	0.5	ND	ND	0.00	not currently under development
93	0.9	24.0	48.1	73.5	ND	ND	8.6	pre-clinical trials
94	> 30	> 30	47.0	79.9	25.0	17.5	13.4	pre-clinical trials
95	> 30	> 30	42.6	0.0	3.0	3.0	0.0	pre-clinical trials
96	> 30	> 30	66.9	65.2	18.6	15.0	13.9	pre-clinical trials