1-(*N*-Alkylamino)-11-(*N*-ethylamino)-4,8-diazaundecanes: Simple Synthetic Polyamine Analogues That Differentially Alter Tubulin Polymerization

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Polyamine analogues such as bis(ethyl)norspermine and N^1-(cyclopropylmethyl)-N^{11}-ethyl-4,8-diazaundecane (CPENSpm) act as potent modulators of cellular polyamine metabolism in vitro and possess impressive antitumor activity against a number of cell lines. Some of these polyamine analogues appear to produce their cell-type-specific cytotoxic activity through the superinduction of spermidine/spermine N^1-acetyltransferase (SSAT). However, there are several analogues (e.g., N^1-(cycloheptylmethyl)-N^{11}-ethyl-4,8-diazaundecane (CHENSpm)) which are effective cytotoxic agents but do not superinduce SSAT. We have previously demonstrated that CPENSpm and CHENSpm both initiate the cell death program, although by different mechanisms, and that CHENSpm (but not CPENSpm) induces a G_2/M cell cycle arrest. We now report that one potential mechanism by which some polyamine analogues can retard growth and ultimately produce cytotoxicity is through interference with normal tubulin polymerization. In these studies, we compare the effects of the polyamine analogues CHENSpm, CPENSpm, and (S)-N^1-(2-methyl-1-butyl)-N^{11}-ethyl-4,8-diazaundecane (IPENSpm) on in vitro tubulin polymerization. These spermine analogues behave very differently from spermine and from each other in terms of tubulin polymerization rate, equilibrium levels, and time of polymerization initiation. These results demonstrate that structurally similar polyamine analogues with potent antitumor effects can produce significantly different cellular effects. The discovery of polyamine analogues that can alter tubulin polymerization provides a series of promising lead compounds that may have a similar spectrum of activity to more difficult to synthesize compounds typified by paclitaxel.

Introduction

The natural polyamines serve a number of critical functions in mammalian cells and are absolutely required for cellular division and viability.[1] A number of research groups, including our own,[2−6] have described the synthesis and evaluation of a series of bis(alkyl)-substituted polyamine analogues with impressive in vitro antitumor effects. These analogues have been shown to enter the cell by the polyamine transport system[7] and then to disrupt the biosynthesis and metabolic interconversion of cellular polyamines. In various cultured tumor cell types, many of these agents down regulate ODC and AdoMet-DC and superinduce spermidine/spermine N^1-acetyltransferase (SSAT), the rate-limiting step in polyamine catabolism. However, these bis-alkylated analogues do not substitute for the natural polyamines in terms of supporting cell growth. The resultant cell is functionally depleted of polyamines, a situation which results in rapid cytotoxicity in a variety of tumor cell lines. One of the first of these agents to be described, bis(ethyl)norspermine (BENSpm, **1**; Figure 1), exhibits cell-type-specific cytotoxicity in vitro against the human lung cancer cell lines NCI H157 (non-small-cell lung carcinoma) and NCI H82 (small-cell lung carcinoma).[8−11]

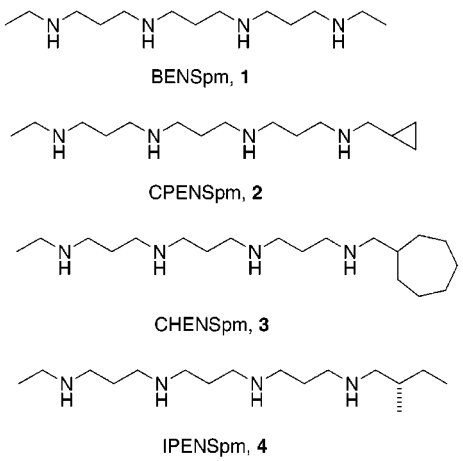

Figure 1. Structures of the alkylpolyamine analogues BENSpm, CPENSpm, CHENSpm, and IPENSpm.

We previously described the synthesis and evaluation of a series of unsymmetrically substituted alkylpolyamine analogues, including CPENSpm (**2**) and CHENSpm (**3**), shown in Figure 1, which exhibit significant antitumor effects in vitro.[2,3] Based on data gathered for these analogues, our initial hypothesis was that the differential induction of SSAT in the H157 line may play a role in determining cell-type-specific sensitivity to the bis-alkylated polyamine analogues. Although CHENSpm treatment is cytotoxic in many of the same cell systems as BENSpm and CPENSpm, CHENSpm cytotoxicity

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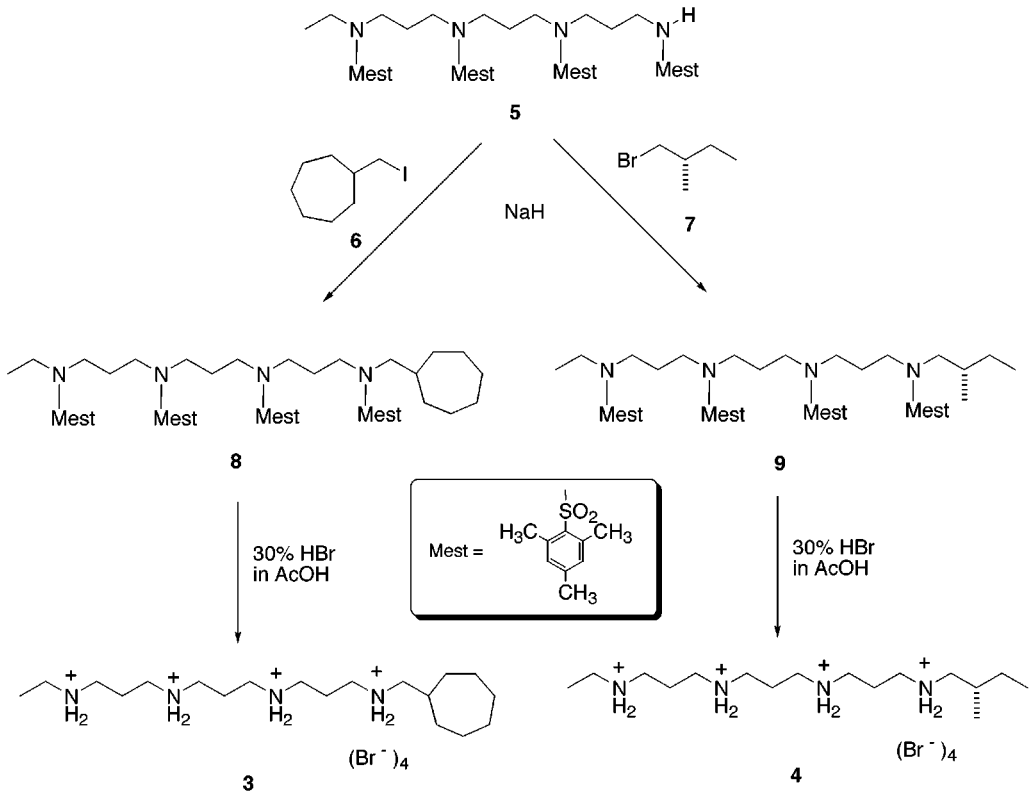
Scheme 1



does not correlate with SSAT superinduction. These data led us to search for the mechanisms of action responsible for the cell-type-specific cytotoxicity caused by **2** and the less cell-type-specific toxicity produced by **3** and to determine whether there are common intermediates or endpoints produced by these agents. The most significant common endpoint produced by **2** and **3** is the ability to induce programmed cell death (PCD). Compound **2** induces PCD in both hormone-dependent and hormone-independent breast tumor lines in vitro,[12] as well as in the H157 non-small-cell lung tumor line.[13] One component of this observed PCD appears to be associated with the production of H_2O_2, secondary to superinduction of SSAT by **2** and polyamine oxidase-dependent oxidation of the resulting acetylated polyamines.[14] PCD induced by **2** is delayed but not abolished in cells overexpressing the Bcl-2 antiapoptotic protein. Although **3** does not cause superinduction of SSAT and resultant overproduction of H_2O_2, it also induces PCD in H157 lung tumor cells in vitro. As was the case for analogue **2**, PCD induced by **3** is delayed but not abolished in cells overexpressing Bcl-2 and appears to proceed by both caspase-3-dependent and -independent mechanisms.[15]

Although both **2** and **3** induce PCD, it is likely that they do so through a combination of potentially dissimilar mechanisms. Consistent with this hypothesis is the observation that they have dramatically different effects on the cell cycle.[14,15] Following 24-h treatment of H157 non-small-lung carcinoma cells with 10 μM **2**, no significant effects on cell cycle are observed by flow cytometric analysis. However, under the same conditions, 10 μM **3** produces a dramatic G_2/M cell cycle block in normal and Bcl-2-overexpressing H157 cells.[15] On the basis of these observations, the potential mechanisms leading to the ability of **3** to produce a G_2/M block were investigated. We now report the synthesis and evaluation of the terminally alkylated polyamines CHENSpm (**3**) and (*S*)-1-[*N*-(2-methyl-1-butyl)mino]-11-(*N*-ethylamino)-4,8-diazaundecane (IPENSpm, **4**). Each of these analogues possesses the ability to significantly and differentially alter tubulin polymerization in human lung cancer cells.

Chemistry

The synthetic route leading to the target analogues **3** and **4** is outlined in Scheme 1. The tetramesitylated N^1-ethylnorspermine **5**, prepared as previously described,[2] was treated with sodium hydride (60% oil dispersion) in DMF, followed by addition of the appropriate alkyl halide (cycloheptylmethyl iodide (**6**) or (*S*)-(+)-1-bromo-2-methylbutane (**7**)), to afford the fully protected precursor molecules **8** and **9**, respectively. Removal of the 2-mesitylenesulfonyl protecting group[2,14,17] then afforded the crude target molecules, which were purified by recrystallization from water/ethanol as the tetrabromide salts **3** and (*S*)-**4**, respectively.

Effects on Cell Growth and Cell Cycle

Each of the three analogues demonstrated similar cytotoxic effects in the human non-small-cell lung carcinoma line, NCI H157. In 96-h dose−response studies each compound was found to be cytotoxic at concentrations greater than 0.1 μM (Figure 2). However, it should be noted that significant induction of SSAT activity was only observed in cells treated with CPENSpm and not in cells treated with CHENSpm or IPENSpm (Figure 3).

We have previously reported a differential effect on the cell cycle by CHENSpm and CPENSpm.[14,15] To confirm these results and to compare the effects of the newly synthesized IPENSpm, the effects of all three compounds on the cell cycle progress were analyzed

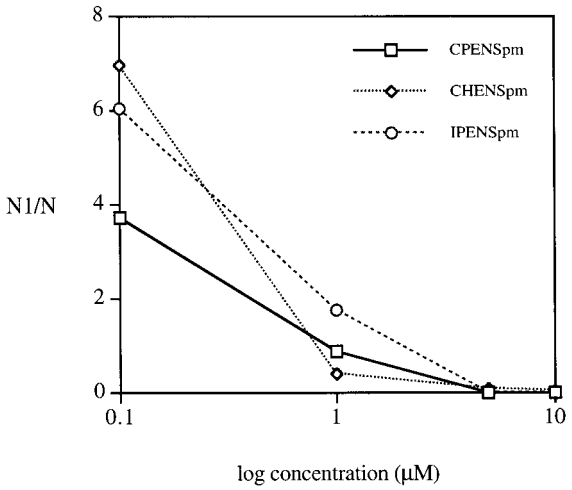


Figure 2. Dose response to unsymmetrically substituted polyamine analogues **2**−**4**. NCI H157 cells were exposed to increasing concentrations of the indicated compounds for 96 h. The growth effects are expressed as a ratio of cells at 96 h (N_1) versus the number of cells seeded at time zero (N). Cell toxicity is defined as a N_1/N ratio < 1. This figure is representative of three separate experiments run in duplicate with variation of 10% or less.

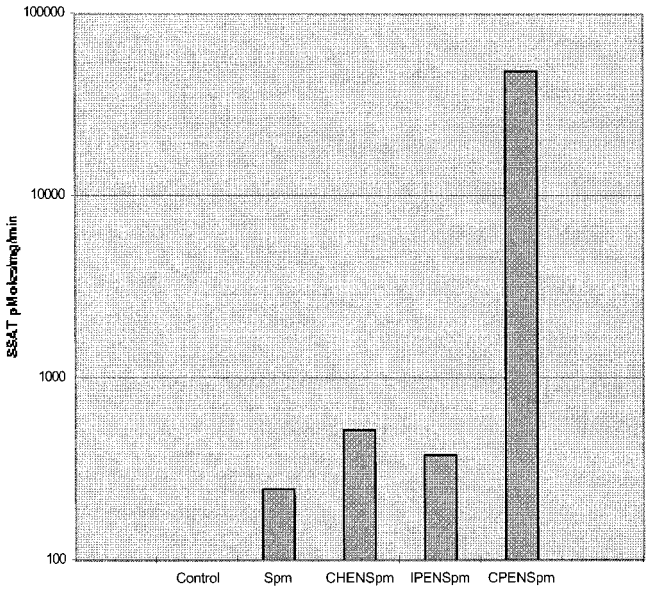


Figure 3. SSAT induction by polyamine analogues **2**−**4**. NCI H157 cells were treated with 10 μM of the indicated compounds for 24 h followed by analysis of their SSAT activity. Note the ordinate is a log scale. Only CPENSpm treatment was found to superinduce SSAT. The graph depicts the results of a representative experiment performed in triplicate, within which the standard deviation was uniformly less than 10%.

after a 24-h exposure to 10 μM of each compound (Figure 4). As previously observed, CPENSpm treatment had no significant effect on the cell cycle. However, both CHENSpm and IPENSpm had a profound effect, each treatment resulting in a significant G_2/M block and a concurrent decrease in the G_1 fraction (Figure 4).

Effects on Tubulin Polymerization

Analogues **2**−**4**, as well as the natural tetraamine spermine, stimulate tubulin polymerization in the absence of microtubule-associated proteins (MAPs) and other polymerization stimulants (Figure 5 and Table 1). In the absence of the analogues or spermine, there was

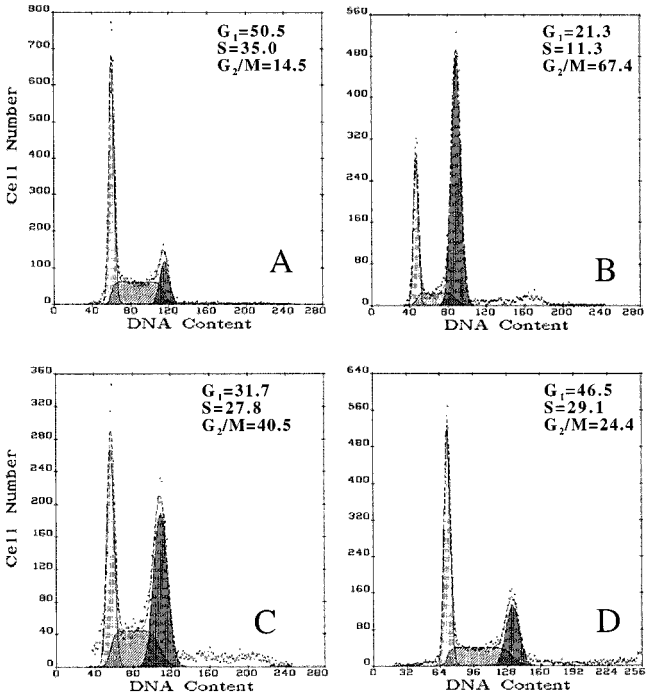


Figure 4. Effects of polyamine analogue treatment on the cell cycle in NCI H157 cells. Subconfluent H157 cells were exposed to A, no treatment; B, 10 μM CHENSpm; C, 10 μM IPENSpm; D, 10 μM CPENSpm for 24 h. Cell cycle histograms were derived as previously described.[13] This figure depicts the results of a minimum of three trials, each with nearly identical results.

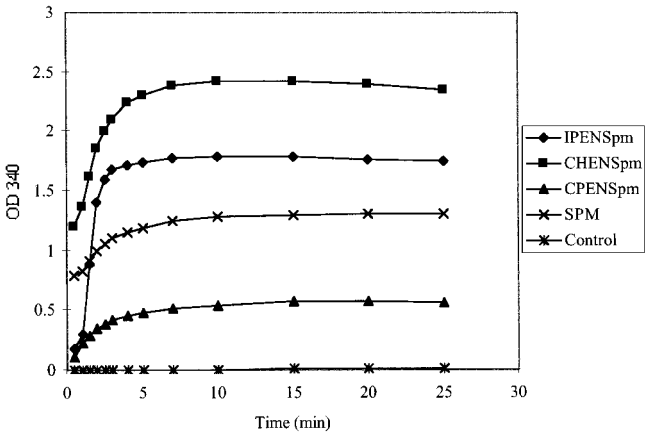


Figure 5. Effects of CPENSpm (**2**), CHENSpm (**3**), IPENSpm (**4**), and spermine on tubulin polymerization. Buffer containing the polyamine was equilibrated at 37 °C. The polymerization was initiated by the 10-fold dilution of 10 mg/mL tubulin which had been thawed immediately before the reaction. The first OD_{340} reading was taken at 30 s. Each point represents the mean of 2−3 polymerization reactions; the values generally varied <10%.

no detectable polymerization, as determined either by increase in absorbance or by observable microtubule formation (37 °C, $MgCl_2$, without GTP or organic solvent). In experiments run in the presence of spermine or analogues **2**−**4**, the three phases of tubulin polymerization (initiation, polymerization, and equilibrium) were different. Initiation of polymerization was observable following treatment with spermine and **4**. Both initiated polymerization approximately 1 min after mixing tubulin with the buffer and the analogue. The rate of polymerization was greatest in the case of **4** and

Table 1. Effects of Spermine, CPENSpm, CHENSpm, and IPENSpm on Tubulin Polymerization in the Presence and Absence of MAPs[a]

compound	rate (ΔOD_{340}/min)	equilibrium OD_{340} (10-min reading)	rate (ΔOD_{340}/min) with MAPS	equilibrium OD_{340} (20-min reading) with MAPS
SPM (1 mM)	0.14	1.310	0.055	0.624
CPENSpm (1 mM)	0.096	0.563	0.036	0.098
IPENSpm (1 mM)	0.69	1.791	0.021	0.247
CHENSpm (1 mM)	0.37	2.422	0.25	1.302
CPENSpm (1 mM) + SPM (1 mM)	0.131	1.083	0.031	0.514
IPENSpm (1 mM) + SPM (1 mM)	0.205	1.088	0.069	0.392
CHENSpm (1 mM) + SPM (1 mM)	0.131	1.714	0.16	1.007

[a] Each reported data point is the result of at least three determinations which in all cases varied by 10% or less.

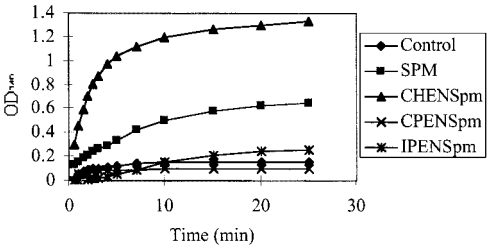


Figure 6. Effects of CPENSpm (**2**), CHENSpm (**3**), IPENSpm (**4**), and spermine on the polymerization of MAP-rich tubulin. The buffer containing polyamine was equilibrated at 37 °C. The polymerization was initiated by 7.5-fold dilution of 7.5 mg/mL MAP-rich tubulin which had been thawed just prior to the reaction. The first OD_{340} reading was taken at 30 s. Each point represents the mean of 2−3 polymerization reactions; the values generally varied <10%.

proceeded 4.9 times faster than that of spermine. The analogues altered the equilibrium OD_{340} from the control in the following order: **3** > **4** > spermine > **2**. The samples with OD_{340} values of 0.7 or greater showed that an increasing portion of the material causing turbidity was nonmicrotubule aggregates and bundled microtubules. These were identified when the polymerization was performed with rhodamine-modified tubulin and observed by fluorescent microscopy.

To determine if the presence of spermine would mitigate the effects of analogues, the effect of spermine on the polymerization reaction was observed (Table 1). Polymerization rates are significantly enhanced when **3** or **4** is added to the polymerization reaction, while **2** has an effect similar to that of spermine. An analogous effect is seen on the equilibrium value, since **3** and **4** enhance tubulin polymerization, while **2** diminishes the effect with respect to control. When analogues **2**−**4** are added to the mixture in combination with 1 mM spermine, the rate and equilibrium values for tubulin polymerization did not differ significantly from control values. This strongly suggests that analogues **2**−**4** are competing for spermine at some polymerization-inducing site on tubulin. Since the concentration of polycations is double in each of the combination reactions, the increase in polymerization is likely a specific effect rather than the result of simply increasing polycations in the reaction.

To further investigate elements that could compete for interaction with tubulin in the intact cell, the polymerization assay was repeated with MAP-rich tubulin (Figure 6 and Table 1). MAPs are believed to interact with the tubulin at least in part through their polylysine cationic regions. A direct comparison of the OD_{340} values is not valid since the concentration of tubulin and the diameter of the microtubules are

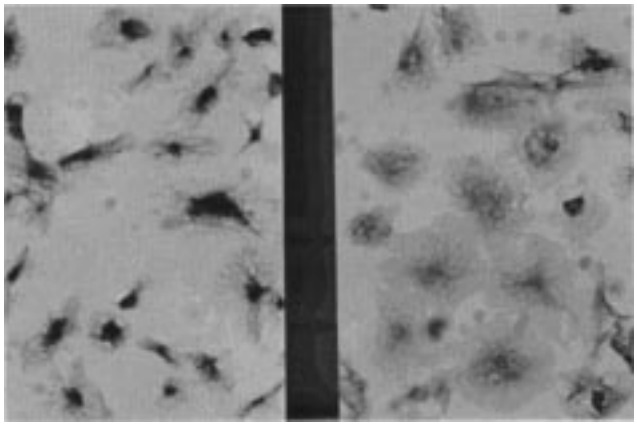


Figure 7. Immunohistochemistry of NCI H157 cells probed using anti-tubulin monoclonal antibody. The nucleus was stained with methyl green. These are representative pictures of three separate experiments. The DAB staining was performed simultaneously to ensure comparable results: left, control; right, 24-h treatment with 10 μM CHENSpm (**3**).

different in the presence of MAPs. Under the conditions of this assay, again with $MgCl_2$ and without GTP or organic solvent, there is observable microtubule formation. The polymerization profile changed most significantly for **4** relative to the reaction without MAPs. Compound **3** and spermine showed decreases in their ability to effect the tubulin polymerization. The initiation of polymerization was delayed substantially, and the rate of polymerization was the most significantly decreased by the presence of MAPs. Compound **3** was most effective at altering the tubulin dynamics in the presence of both MAPs and spermine and increased both the rate and the equilibrium relative to the reaction with spermine and MAPs only.

Compound **3** altered the microtubule density in the putative centrosome adjacent to the nucleus when analyzed by immunohistochemical staining of tubulin (Figure 7). The cytoplasmic microtubules do not appear to be affected by treatment. However, the strong staining of the microtubules emanating from the centrosomal area is absent or significantly reduced. Higher concentrations of **3** caused rapid apoptosis and thus were not amenable to immunohistochemistry. Compound **4** was also tested for its ability to alter the cellular microtubules. There were no observable changes in the density or distribution of the microtubules after treatment with compound **4**.

Discussion

Interference with polyamine metabolism has been established as a rational target for antineoplastic intervention. Much work has been performed aimed at

the inhibition of polyamine biosynthesis and, more recently, to induce polyamine catabolism. The net effect of these strategies has been to suggest that growth inhibition is concurrent with the depletion of natural polyamines. However, the underlying mechanisms involved in the inhibition of growth by the disturbance of polyamine metabolism are only now being elucidated. We have demonstrated that in some cases, the superinduction of SSAT and the subsequent production of H_2O_2 lead to the induction of programmed cell death.[14,15] However, it is clear that polyamine analogues can kill cells through other mechanisms. In the current work, we present evidence that two new cytotoxic antitumor polyamine analogues that do not superinduce SSAT produce a significant G_2/M block and effect the process of tubulin polymerization. These results underscore the observation that small changes in analogue structure can have profound effects on their activity. It should be noted that Kramer et al. have reported a G_1 block produced by several symmetrically substituted polyamine analogues.[18] However, the underlying mechanisms causing the observed G_1 block are currently not known.

Both α- and β-tubulin have an acidic carboxy terminus which has been proposed to inhibit polymerization.[19] Natural cations serve to affect the microtubule polymerization dynamics, both enhancing and inhibiting microtubule formation. The effects of Ca^{2+} and Mg^{2+} on microtubule dynamics are well-documented; however, there are other natural cations that may play a role in microtubule dynamics. Deprivation of polyamines has been shown to have significant effects on the cytoskeleton in some cells.[20−22] In 1985, it was observed that natural polyamines enhance tubulin polymerization in the presence and absence of MAPs.[22] Recently, Wolff proposed that spermidine and spermine may affect the state of tubulin polymerization and promote microtubule assembly.[23] Using a modification of the polymerization assay described here, in which DMSO or taxol and 0.8 mM GTP are used to promote polymerization, Wolff observed that 100 μM spermine tripled the rate of polymerization. The stabilization of microtubule formation by the natural polyamines, spermine and spermidine, may be part of the natural dynamics of microtubules and represents part of the influence of polyamines on cell cycle.

The series of polyamine analogues presented here indicate that there is a more specific interaction of polyamines with tubulin than a simple electrostatic interaction, since there are significant changes in the initiation of polymerization, polymerization rate, and equilibrium OD_{340} when different alkyl groups are appended to the analogues. The three analogues CHENSpm, IPENSpm, and CPENSpm have identical point charge distributions along their alkyl backbone. The only structural differences between these analogues is the alkyl modification at the one terminal nitrogen. Though IPENSpm and CHENSpm both induce a G_2/M arrest, only CHENSpm shows an alteration in the immunohistochemical results. This is consistent with the observation that IPENSpm is not inducing an increase in polymerization rate and equilibrium values in the in vitro polymerization assay in the presence of MAPs. IPENSpm also elicits a less pronounced effect on cell cycle. Taken together, these results suggest that CHENSpm and IPENSpm could be affecting tubulin polymerization by different mechanisms.

In the in vitro tubulin polymerization assay, microtubules do not spontaneously form without the addition of a stimulant such as paclitaxel or, in this case, a polyamine or MAPs. However, the final optical density at the equilibrium stage with 1 mM polyamine analogue added is higher than would be expected for normal helical microtubules. Other forms of polymerized tubulin have been found to form in the presence of cations, such as "C" tubules, crenelated ribbon, and "σ" forms.[19] These forms of tubulin defract light more effectively than regular microtubules. We are unable to distinguish these forms using fluorescence microscopy of rhodamine-modified tubulin. However, we can observe linear forms of tubulin polymer and the associated increase in the amount of these linear tubulin forms observed in bundles with time and polyamine concentration. The observed increase in bundling with time occurs whether the polymerization reaction takes place at 25 or 4 °C.

The observable effects of polyamine analogues on tubulin dynamics in a cuvette are more dramatic than the effects observed in the cell, although this is probably due to the higher (1 mM) concentrations used for these assay procedures. However, significant effects on tubulin polymerization can be detected at levels of the analogues that are closer to cytotoxic concentrations. Pohjanpelto et al. observed a large decrease in the total microtubule content of polyamine-deficient CHO cells using immunohistochemistry.[21] Following treatment with 10 μM analogue concentrations, immunohistochemical studies indicate that these polyamine analogues do not appear to decrease the quantity of microtubules but rather decrease the association of microtubules with the centrosomes. This is consistent with G_2/M arrest, since abnormal association of microtubules with the centrosomes could prevent spindle formation and mitosis. IPENSpm did not appear to alter cellular microtubule structure despite the observed G_2/M arrest, again suggesting that IPENSpm and CHENSpm have differential mechanisms for inducing tubulin polymerization changes. It is also possible that alterations in the microtubules were not observable due to the competing apoptotic mechanisms induced by IPENSpm.

The results of the current study demonstrate that small changes in analogue structure can have profound consequences on observed cellular effects. Compound **2**-induced toxicity appears to be, at least in part, associated with its induction of SSAT[14] without obvious effects on cell cycle progression. By contrast, neither **3** nor **4** superinduce SSAT but both produce a profound G_2/M block and subsequent cytotoxicity by an apparent PCD pathway.[15] Although the observed changes in tubulin polymerization induced by **3** and **4** suggest a direct effect on the polymerization process, the possibility that these effects are caused by a combination of other mechanisms, including an analogue-induced alteration in specific gene expression, cannot be excluded.[15] Further study will be necessary to determine what properties of terminally alkylated polyamine analogues should be synthetically exploited to improve tumor-specific activity. The synthesis and evaluation of additional analogues, as well as additional experiments

aimed at elucidation of the cellular effects of these analogues, are ongoing concerns in our laboratories.

Experimental Section

All reagents were purchased from Aldrich Chemical Co. (Milwaukee, WI) or Sigma Chemical Co. and were used without further purification except as noted below. Triethylamine was distilled from potassium hydroxide and stored in a nitrogen atmosphere. Methanol was distilled from magnesium and iodine under a nitrogen atmosphere and stored over molecular sieves. Methylene chloride was distilled fron phosphorus pentoxide, and chloroform was distilled from calcium sulfate. Dimethyl formamide was dried by distillation from anhydrous calcium sulfate and was stored under nitrogen. Preparative scale chromatographic procedures were carried out using E. Merck silica gel 60, 230−440 mesh. Thin-layer chromatography was conducted on Merck precoated silica gel 60 F-254. All ^{1}H and ^{13}C NMR spectra were recorded on a General Electric QE-300 spectrometer, and all chemical shifts are reported as δ values referenced to TMS or DSS. Infrared spectra were recorded on a Nicolet 5DXB FT-IR spectrophotometer and are referenced to polystyrene. In all cases, ^{1}H NMR, ^{13}C NMR, and IR spectra were consistent with assigned structures. Melting points were recorded on a Thomas-Hoover capillary melting point apparatus and are uncorrected. Microanalyses were performed by Galbraith Laboratories, Knoxville, TN, and were within 0.4% of calculated values.

Cycloheptylmethyl Iodide, 6. A 5.0-g portion of cycloheptanemethanol (0.039 mol) was dissolved in 100 mL of dry CH_2Cl_2 along with 4.76 g (0.047 mol) of triethylamine and 5.38 g (0.047 mol) of methanesulfonyl chloride, and the mixture was allowed to stir at room temperature overnight. The reaction mixture was washed with two 100-mL portions of 1 N HCl, 100 mL of water, and 100 mL of saturated NaCl, and the organic layer was then dried over $MgSO_4$. Filtration and removal of solvent afforded the crude mesylate (7.88 g, 97.9%) as a colorless oil. The crude product was sufficiently pure to use in the subsequent reaction without further purification. A 7.88-g portion of the crude mesylate (0.038 mol) was dissolved in 100 mL of acetone, and 86.20 g (0.575 mol) of sodium iodide was added. The reaction mixture was allowed to reflux for 3 h, after which the solution was evaporated to dryness and redissolved in 100 mL of ethyl acetate. The organic layer was washed with 100 mL of water and 100 mL of saturated $NaHCO_3$ and then dried over $MgSO_4$. Filtration and removal of the solvent in vacuo afforded the crude iodide **6**, which was chromatographed on silica gel (hexanes) to produce 7.05 g (77.5%) of the desired iodide as a pale pink oil: ^{1}H NMR ($CDCl_3$) δ 3.13 (d, 2H, exocyclic CH_2), 1.78 (m, 2H, H_2 and H_7 cis to I), 1.62 (m, 3H, H_1 methyne, H_2 and H_7 trans to I), 1.49 (m, 6H, H_3, H_6 and H_4 and H_5 cis to I), 1.26 (m, 2H, H_4 and H_5 trans to I); ^{13}C NMR ($CDCl_3$) δ 41.39 (C_1), 34.67 (C_2 and C_7), 28.13 (C_4 and C_5), 26.08 (C_3 and C_6), 18.04 (exocyclic CH_2).

1-[*N*-(Cycloheptylmethyl)-*N*-(2-mesitylenesulfonyl)-amino]-4,8-*N*-(2-mesitylenesulfonyl)-11-[*N*-ethyl-*N*-(2-mesitylenesulfonyl)amino]-4,8-diazaundecane (8). A 0.053-g portion of sodium hydride (60% mineral oil dispersion, 0.0022 mol) in 10 mL of dry DMF was added slowly via dry syringe to a solution of **5** (1.0 g, 0.0011 mol) and cycloheptylmethyl iodide (**6**) (0.786 g, 0.0033 mol) in 20 mL of dry DMF at 0 °C under a nitrogen atmosphere. The reaction was allowed to stir at 0 °C for 30 min, and then cold water (10 mL) and chloroform (30 mL) were added with stirring. Removal of the solvent in vacuo (35 °C at 0.2 mmHg) and chromatography of the residue on silica gel (hexane/ethyl acetate, 5:5) then afforded pure **8** (0.978 g, 84%) as a yellow oil: ^{1}H NMR ($CDCl_3$) δ 6.93 (s, 8H, mesityl aromatic H-3 and H5), 3.18 (q, 2H, ethyl CH_2), 3.00 (m, 13H, H_1, H_3, H_5, H_7, H_9, H_{11} and cycloheptylmethyl H_1 methyne), 2.77 (m, 2H, cycloheptylmethyl exocyclic methylene), 2.56 (m, 24H, mesityl 2-CH_3 and 6-CH_3), 2.29 (s, 12H, mesityl 4-CH_3), 1.63 (m, 6H, H_2, H_6 and H_{10}), 1.45 (m, 4H, cycloheptylmethyl H_2 and H_7), 1.19 (m, 8H, cycloheptylmethyl H_3, H_4, H_5 and H_6), 0.9 (t, 3H, ethyl CH_3). Anal. ($C_{55}H_{82}N_4O_8S_4$) C, H, N.

(*S*)-1-[*N*-(2-Methyl-1-butyl)-*N*-(2-mesitylenesulfonyl)-amino]-4,8-*N*-(2-mesitylenesulfonyl)-11-[*N*-ethyl-*N*-(2-mesitylenesulfonyl)amino]-4,8-diazaundecane (9). A 0.053-g portion of sodium hydride (60% mineral oil dispersion, 0.0022 mol) in 10 mL of dry DMF was added slowly via dry syringe to a solution of **5** (1.0 g, 0.0011 mol) and (*S*)-2-methylbromobutane (**7**) (0.498 g, 0.0033 mol) in 20 mL of dry DMF at 0 °C under a nitrogen atmosphere. The reaction was conducted exactly as described above for the synthesis of compound **8** to afford pure **9** (0.815 g, 73%) as a yellow oil: ^{1}H NMR ($CDCl_3$) δ 6.90 (s, 8H, mesityl aromatic H_3 and H_5), 3.25−3.03 (br m, 17H, H_1, H_3, H_5, H_7, H_9, H_{11}, 2-methylbutyl methyne, 2-methylbutyl N−CH_2 and ethyl CH_2), 2.31 (s, 24H, mesityl 2-CH_3 and mesityl 6-CH_3), 2.27 (s, 12H, mesityl 4-CH_3), 1.67 (m, 8H, H_2, H_6, H_{10} and 2-methylbutyl CH_2), 1.21 (s, 6H, 2-methylbutyl CH_3), 1.03 (t, 3H, ethyl CH_3). Anal. ($C_{52}H_{78}N_4O_8S_4$) C, H, N.

1-[*N*-(Cycloheptylmethyl)amino]-11-(*N*-ethylamino)-4,8-diazaundecane Tetrahydrobromide, 3. An 11.01-g portion of phenol (0.117 mol) was dissolved in 50 mL of 30% HBr/HOAc in a stoppered flask, and to this mixture was added a solution of **8** (0.978 g, 0.00093 mol) in 20 mL of ethyl acetate in three portions over a period of 3 h. After the addition was complete, the reaction mixture was stirred for an additional 15 h at room temperature, then cooled to 0 °C, and diluted with 100 mL of water. The aqueous phase was washed with two 100-mL portions of ethyl acetate before being lyophilized to give the crude product as dark yellow solid. This crude product was washed with methanol and filtered to yield the tetrahydrobromide salt of **3** (0.349 g, 57.8%) as a white solid. An analytical sample of **3** was prepared by recrystallization from aqueous ethanol: ^{1}H NMR (D_2O) δ 3.14 (m, 14H, H_1, H_3, H_5, H_7, H_9, H_{11} and ethyl CH_2), 2.93 (d, 2H, cycloheptylmethyl methylene), 2.09 (m, 8H, H_2, H_6, H_{10} and cycloheptylmethyl methylene), 1.41−1.76 (br m, 12H, remaining cycloheptyl protons), 1.25 (t, 2H, ethyl CH_3). Anal. ($C_{19}H_{46}N_4Br_4$) C, H, N.

(*S*)-1-[*N*-(2-Methyl-1-butyl)amino]-11-(*N*-ethylamino)-4,8-diazaundecane Tetrahydrobromide, 4. A 9.51-g portion of phenol (0.101 mol) was dissolved in 50 mL of 30% HBr/HOAc in a stoppered flask, and to this mixture was added a solution of **9** (0.815 g, 0.0008 mol) in 20 mL of ethyl acetate in three portions over a period of 3 h. The reaction was conducted exactly as described for the synthesis of **3** to yield the tetrahydrobromide salt of **4** (0.462 g, 85%) as a white solid. An analytical sample of **4** was prepared by recrystallization from aqueous ethanol: ^{1}H NMR (D_2O) δ 3.08 (m, 15H, H_1, H_3, H_5, H_7, H_9, H_{11}, ethyl CH_2 and 2-methylbutyl methyne), 2.95 and 2.82 (pair of m, 2H, diastereotopic2-methylbutyl N−CH_2), 2.04 (m, 6H, H_2, H_6, and H_{10}) 1.72 and 1.36 (pair of m, 2H, diastereotopic 2-methylbutyl C−CH_2), 1.21 (t, 3H, 2-methylbutyl C−CH_3), 0.90 (d, 3H, 2-methylbutyl CH_3), 0.83 (t, 3H, ethyl CH_3). Anal. ($C_{16}H_{42}N_4Br_4$) C, H, N.

Cell Culture Studies. The NCI H157 non-small-cell lung carcinoma line was maintained in culture as previously described.[24] This line was refed with fresh medium every 3 days to maintain log phase growth. Cells were exposed to the polyamine analogue N^1,N^{12}-bis(ethyl)spermine and the potential SSAT inhibitor. Effects on growth and SSAT activity were then determined as previously published.[3] The SSAT activity of these cell lysates was determined by a previously published method.[10]

Tubulin Polymerization Assay. Tubulin polymerization was monitored by the change in optical density at 340 nm using a modification of the method described by Jordan et al.[19] Purified bovine brain tubulin with or without MAPs or glycerol was purchased from Cytoskeleton (Denver, CO). The final buffer concentrations for tubulin polymerization were 0.1 M 2-(*N*-morpholino)ethanesulfonic acid (MES) (pH 6.7), 1.0 mM $MgCl_2$, 0.5 mM ethylene glycol bis(β-aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) (buffer A). Test analogues were added in 1 mM concentration, and then all components except the purified tubulin were warmed to 37 °C. The reaction was initiated by the addition of tubulin to a final concentration of 1.0 mg/mL (10 μM heterodimer for pure tubulin and approximately 7 μM for the MAP-rich tubulin). The optical

density was measured by a Perkin-Elmer Lambda 4B UV/VIS spectrophotometer with a temperature-controlled cuvette holder.

Microtubule Microscopy. Purified bovine brain tubulin without MAPs or glycerol and modified with rhodamine was purchased from Cytoskeleton (Denver, CO). The rhodamine-modified bovine brain tubulin was diluted to a stoichiometry of 0.04 rhodamine modifications per heterodimer. A solution was prepared with buffer A. The tubulin was added, and the solution was incubated at room temperature for 1 h. The resulting microtubules were observed using fluorescence microscopy.

Immunohistochemistry. NCI H157 cells were grown on a single-well glass slide in RPMI 1640 with 9% fetal calf serum, 100 units/mL penicillin, and 100 units of streptomycin. The cells were then exposed to 1.0 mM aminoguanidine with and without 10 μM **3** or **4** for 24 h. The cells were washed with PBS, fixed with 70% ethanol at −20 °C, and permeablized with 0.1% Triton X-100 and 0.1% BSA in PBS. The slides were then covered with the monoclonal TUB 2.1 anti-β-tubulin (Sigma, St. Louis, MO). The slides were washed with PBS again and covered with the secondary antibody, a rabbit anti-mouse HRP conjugate antibody (Sigma). The tubulin antibody was then visualized by Elite Universal kit Vectastain ABC peroxidase staining method (Vector Laboratories, Inc., Burlingame, CA) followed by Sigma FAST DAB (3,3′-diaminobenzidine tetrahydrochloride) staining. All slides were stained for 1 min to ensure comparable results.

Cell Cycle Analysis. NCI H157 cells (0.5×10^6) were plated in a 25-mm^2 flask and allowed to adhere. The cells were then treated with 10 μM of each analogue in the presence of 1 mM aminoguanidine for 24 h before harvesting. Flow cytometric analyses of cells treated for 24 h with the indicated compounds were performed as previously described.[25]

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