Conformationally Restricted Analogues of ^{1}N,^{14}N-Bisethylhomospermine (BE-4-4-4): Synthesis and Growth Inhibitory Effects on Human Prostate Cancer Cells

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Twelve analogues of ^{1}N,^{14}N-bisethylhomospermine (BE-4-4-4) with restricted conformations were synthesized in the search for cancer chemotherapeutic agents with higher cytotoxic activities and lower systemic toxicities than BE-4-4-4. The central butane segment of BE-4-4-4 was replaced with a 1,2-substituted cyclopropane ring, a 1,2-substituted cyclobutane ring, and a 2-butene residue. In each case, the *cis*/*trans*-isomeric pair was synthesized. *Cis*-monounsaturation(s) was also introduced at the outer butane segment(s) of BE-4-4-4. The two possible *cis*-dienes and a *cis*-triene formally derived from the tetraazaeicosane skeleton of BE-4-4-4 were also prepared. Four cultured human prostate cancer cell lines (LnCap, DU145, DuPro, and PC-3) were treated with the new tetramines to examine their effects on cell growth with a MTT assay. One representative cell line (DuPro) was selected to further study the cellular uptake of the novel tetramines, their effects on intracellular polyamine pools, and their cytotoxicity. All tetramines entered the cells, reduced cellular putrescine and spermidine pools while exerting only a small effect on the spermine pool, inhibited cell growth, and killed 2−3 logs of cells after 6 days of treatment at 10 μM. Four new tetramines, the two cyclopropyl isomers, the *trans*-cyclobutyl isomer, and the (5*Z*)-tetraazaeicosene, were more cytotoxic than their saturated counterpart (BE-4-4-4). Their cytotoxicity, however, could not be correlated either with their cellular uptake or with their ability to deplete intracellular polyamine pools. We attribute their cytotoxicity to their specific molecular structures. The cytotoxicity was markedly reduced when the central butane segment was deprived of its rotational freedom by replacing it with a double bond. Introduction of a triple bond or a benzene-1,2-dimethyl residue at the central segment of the polyamine chain, led to complete loss of biological activity. The conformationally restricted alicyclic derivatives were not only more cytotoxic than was the freely rotating BE-4-4-4 by several orders of magnitude but also had much lower systemic toxicities than the latter. Thus, we obtained new tetramines with a wider therapeutic window than BE-4-4-4.

Introduction

N-Alkylated analogues of the natural polyamines (spermine, spermidine, and putrescine) exhibit strong cytotoxic activity against human tumor cell lines.[1] It is by now well-established that $^{\alpha}N$,$^{\omega}N$-bisethyl derivatives of spermine and its higher and lower tetramine homologues show promise as inhibitors of tumor cell proliferation.[2] They are increasingly mentioned in the patent literature on anticancer drugs,[3] and their pharmacological effects have recently been exhaustively discussed.[4] As is the case with most anticancer drugs, bisethyl tetramines are not devoid of systemic toxicity and their efficacious cytotoxic activity has to be balanced against their toxic side effects. Therefore, the search for chemical variants of the bisethyl polyamine structures with broader therapeutic windows is being actively pursued. Hydroxylated polyamine derivatives have recently been prepared in an effort to improve the toxicity profile of the tetramines,[5] conformational restrictions were introduced in the polyamine backbone to enhance their therapeutic activity,[6] and *cis*-unsaturated derivatives have been prepared to facilitate their metabolic breakdown by mixed-function oxidases.[7]

The design of new and more efficacious tetramines poses drawbacks while offering tempting advantages. The main drawback in the search of tetramines with wider therapeutic windows is that the precise mechanisms by which they kill tumor cells and cause systemic toxicity are still not entirely clear.[8] It has been established that polyamines and their analogues bind to nucleic acids and alter their conformations,[9] that they bind to receptor targets,[10] that they drastically reduce the level of ornithine decarboxylase[11,12] (the first enzyme in the pathway that leads to spermine biosynthesis in mammals), that they upregulate the levels of spermidine/spermine *N*-acetyltransferase[13,14] (the enzyme involved in the catabolism and salvage pathways of spermine and spermidine), that they may inhibit the uptake of the natural polyamines by the cells,[15] and that, as a result, they deplete endogenous polyamine pools needed for cell replication.[16] Cell death can result from any one of these effects or from several of them acting in tandem. On the other hand, the main advan-

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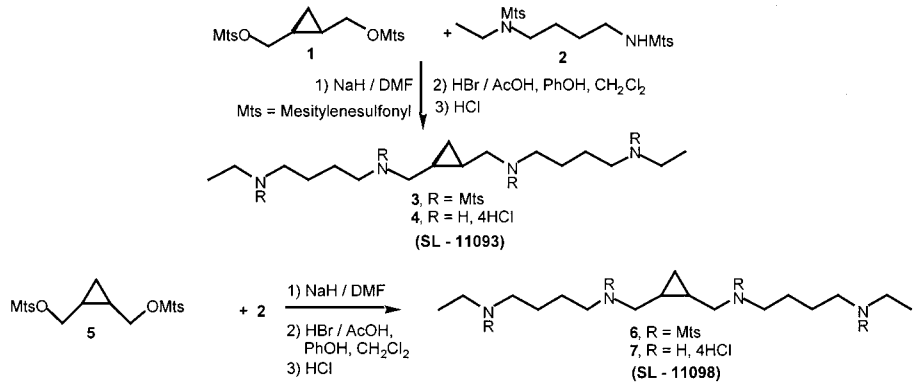
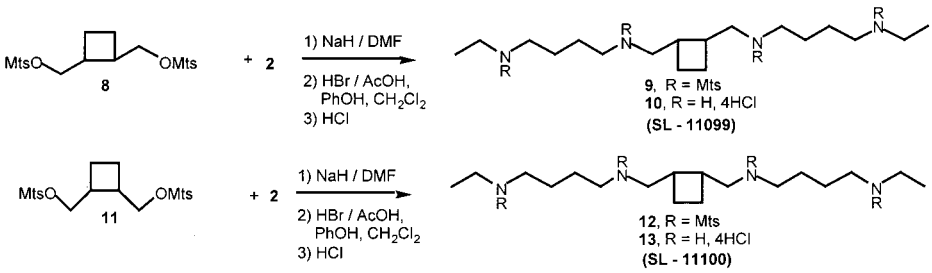
Scheme 1



Scheme 2



tage in the design of new tetramines is that structure–activity studies (SARs) have shown that relatively small structural changes in the aliphatic skeletons of the polyamines can cause pronounced differences in their pharmacological behavior and toxic side effects as well as their antineoplastic activities both at the cellular level as well as in animal models.[17−19]

It has been repeatedly shown that $^1N,^{14}N$-bisethylhomospermine (BE-4-4-4), a higher homologue of bisethylspermine, is a powerful cytotoxic drug but has a narrow therapeutic window.[11,20,21] The results for animal trials reported against tumors such as L1210 leukemia or Lewis lung carcinoma grafted in athymic nude mice are indeed impressive; about a 6-fold increase in lifespan, as compared to control, was achieved.[22] However, maximum tolerated dose (MTD) values for multiple injection (ip) schedules were found to be ca. 6 mg/kg, doses close to the levels necessary to achieve efficient antitumor activity in human tumor xenografts.[20] We, therefore, decided to apply the principle of conformational restriction to the BE-4-4-4 structure, prompted by the promising results obtained with conformationally restricted analogues of bisethylspermine.[6] Since the latter was found to be highly cytotoxic against a line of human prostate cancer cells,[6] we assayed the new tetramines against several lines of human prostate cancer cells: LnCap, DU 145, DuPro, and PC-3. Preliminary results suggest that it is possible to markedly improve the therapeutic efficacy of BE-4-4-4-like compounds against human prostate cancer cells by certain conformational restrictions.

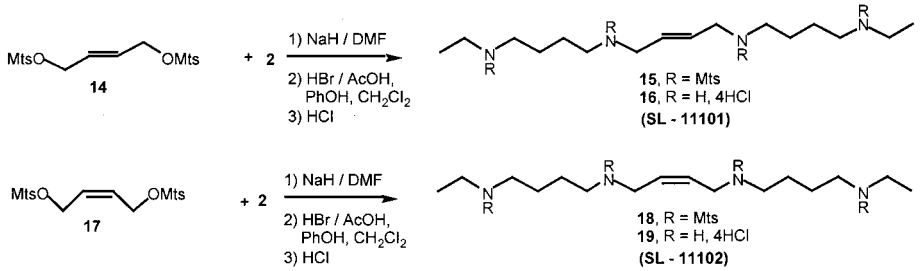
Chemistry

The synthesis of the *trans*- and *cis*-cyclopropane derivatives of bisethylhomospermine made use of the *trans*- and *cis*-cyclopropane 1,2-bis(oxomesitylenesulfonyl) esters **1** and **5** (Scheme 1) whose synthesis we described previously.[6] By condensation of **1** and **5** with the diamide **2** in the presence of NaH, it was possible to secure the tetramides **3** and **6** in good yields. Hydrolysis of the mesitylenesulfonyl protecting groups using hydrogen bromide in glacial acetic acid in the presence of phenol, followed by treatment of the free base with hydrogen chloride, allowed us to obtain **4** and **7**. Using a similar choice of reactions starting with the *trans*- and *cis*-cyclobutane 1,2-bis(oxomesitylenesulfonyl) esters **8** and **11** (Scheme 2), it was possible to obtain the corresponding tetramines **10** and **13** as their hydrochlorides. Unsaturated *cis*- and *trans*-isomers of bisethylhomospermine were obtained starting with the diesters **14** and **17**; on condensation with diamide **2** they gave the tetramides **15** and **18**; deprotection allowed us to obtain **16** and **19** (Scheme 3).

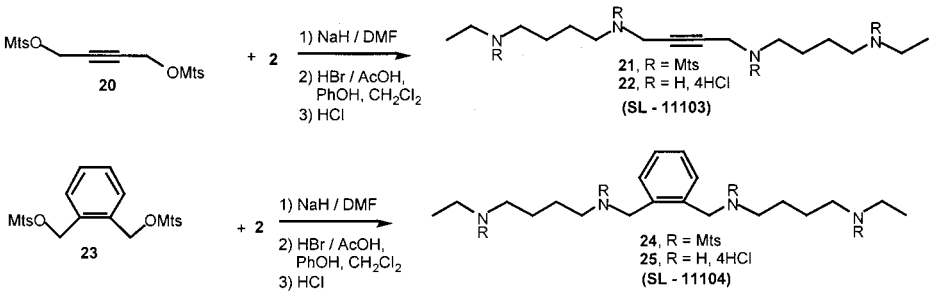
All the aforementioned tetramines had *cis*/*trans*-geometry at the central butane segment of the molecule. To examine the influence of planarity at this segment, we prepared the acetylenic tetramine **22** following the usual reaction pattern and starting with **20** (Scheme 4). In addition, the central butane segment was fixed in the cisoid configuration by making it part of a 1,2-benzenedimethyl structure as in **25**. The synthesis of **25** followed the established reaction pattern (Scheme 4). A fully unsaturated tetramine was prepared where three *cis*-double bonds where introduced in the homospermine skeleton. Starting with the unsaturated diamide **26**[7] it was dimerized by reaction with the diester **17** (Scheme 5). The resulting fully unsaturated tetramide was deprotected to give **28**. A dienic tetramine was prepared by alkylation of **26** with chloride **29**[7] that gave **30**; after deprotection **31** was secured (Scheme 6).

The second possible *cis*-diene formally derived from homospermine was prepared by dialkylation of amide **26** with the bis(mesitylenesulfonyl) ester of 1,4-butanediol **32**. The reaction allowed us to obtain **34** (SL-11114) (Scheme 7). Finally, and by alkylation of amide **2** with the iodoalkylamide **35**,[7] it was possible to secure the

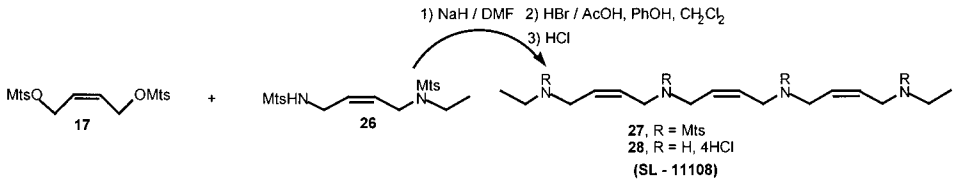
Scheme 3



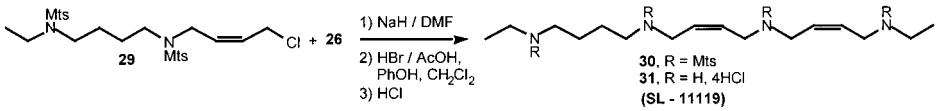
Scheme 4



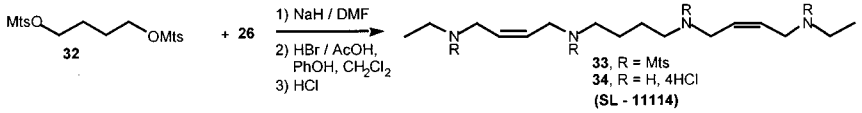
Scheme 5



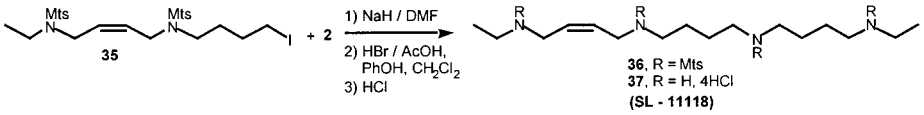
Scheme 6



Scheme 7



Scheme 8



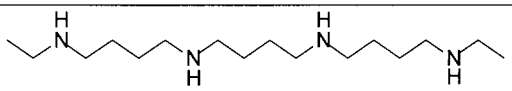
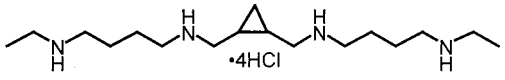
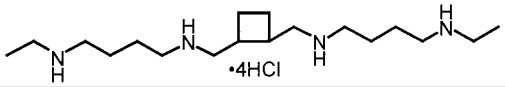
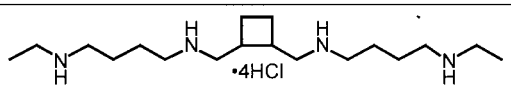
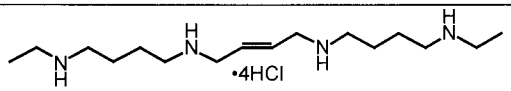
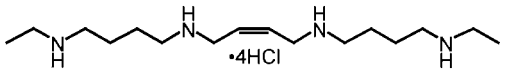
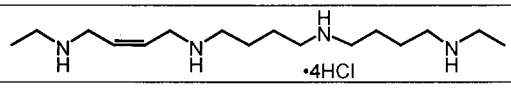
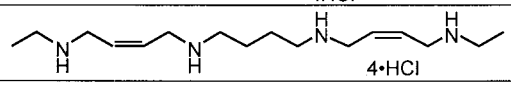
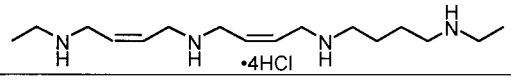
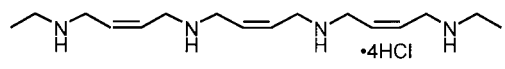
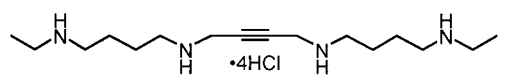
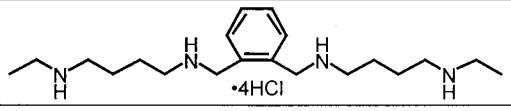
second possible *cis*-monounsaturated isomer of BE-4-4-4, namely, **37** (Scheme 8). The new synthetic tetramines allowed us to revisit the cytotoxic behavior of the higher homologues of bisethylspermine in the search for more efficacious and less toxic drugs.

Biological Results and Discussion

The concentration of each new tetramine required to inhibit 50% cell growth (ID_{50}) on day 6 of treatment was determined by a MTT assay. The ID_{50} values for each tetramine in the human prostate cancer cell lines LnCap, DU145, DuPro, and PC-3 along with their SLIL identification numbers and chemical structures are shown in Table 1. Each cell line showed a slightly different sensitivity when treated with the new tetramines. LnCaP, DU145, and DuPro were more sensitive than was PC-3 to all but one tetramine (**25**); **25** was inactive and **22** was less active in all four cell lines. Ten out of 12 new tetramines had ID_{50} values less than 1 μM in all but PC-3 cells, and several of them had ID_{50} values in the range 10−100 nM. PC-3 cells are in general more resistant to the polyamine analogues than are the other three cell lines, possibly because of decreased polyamine transport in these cells.

We selected the DuPro cell line that exhibits an intermediate level of sensitivity to the tetramines (Table 1) as a representative cell line for further studies. All new tetramines plus BE-4-4-4 at 1 and 10 μM were assayed for their effects on changes in cell number and cellular polyamine levels over time. The effects of the

Table 1. Effect of Polyamine Analogues on Human Prostate Tumor Cell Growth by MTT Assay

PA analogs	Structures of PA analogs of restricted chain flexibility	ID_{50} (μM) values for Human Prostate Tumor Cell Lines			
		LNCAP	DU145	DUPRO	PC-3
BE-4-4-4		0.1	0.03	0.2	0.7
4		0.21	0.016	0.059	1.6
7		0.17	0.02	0.08	1.4
10		0.21	0.014	0.08	2.5
13		0.7	0.021	0.3	4.7
16		0.497	0.22	0.85	7.7
19		0.065	0.03	0.15	>31.25
37		0.21	0.05	0.25	1.65
34		3.0	0.13	0.64	0.7
31		0.69	0.08	0.44	>31.25
28		0.77	0.13	0.98	2.2
22		2.2	2.8	3.2	>31.25
25		>31.25	9.4	>31.25	>31.25

tetramines on DuPro cell growth along with the cellular polyamine levels are shown in Figures 1−6. At 1 μM, most tetramines were ineffective in growth inhibition (Figures 1−3). Only **31** and **25** inhibited cell growth by approximately 50% at 1 μM (Figure 3A). At 10 μM, however, all but two tetramines exhibited significant growth inhibition (Figures 4−6). The exceptions were **22** and **25** that showed almost no detectable growth inhibition (Figure 4A). One tetramine **37** was better than BE-4-4-4 in inhibiting cell growth (Figure 6A). At 1 μM, none of the tetramines were able to deplete cellular polyamine pools even on day 6 after treatment (Figures 1−3). At 10 μM, most tetramines except **25**, markedly depleted cellular putrescine and spermidine levels within day 4 of treatment without appreciably changing the cellular spermine level even by day 6 of treatment (Figures 4−6). The abilities of various tetramines to deplete cellular putrescine and spermidine levels, however, could not be correlated with their growth inhibitory effects. For example, **10** at 10 μM had much less effect on cellular putrescine and spermidine pools as compared to five other tetramines: BE-4-4-4, **4**, **7**, **16**, and **19** (Figures 4B,C and 5B,C). However **10** inhibited cell growth to the same extent as did the other five tetramines (Figures 4 and 5). On the other hand, on day 6 of treatment with 10 μM, **22** depleted the polyamine pool to the same extent as did **10**. However, **22** had no observable effect on cell growth, while **10** showed marked growth inhibition (Figures 4A and 5A).

Variations in intracellular tetramine levels were also observed; **25** was poorly taken up by the cells and, as mentioned above, exhibited no cell growth inhibition (Figures 1 and 4). Except for this tetramine, however, no reasonable correlation could be established between intracellular tetramine levels and their growth inhibitory activities. For example, on day 6 of treatment at 10 μM, the intracellular level of **13** was 10-fold higher than that of **10**, while both were equally efficient in

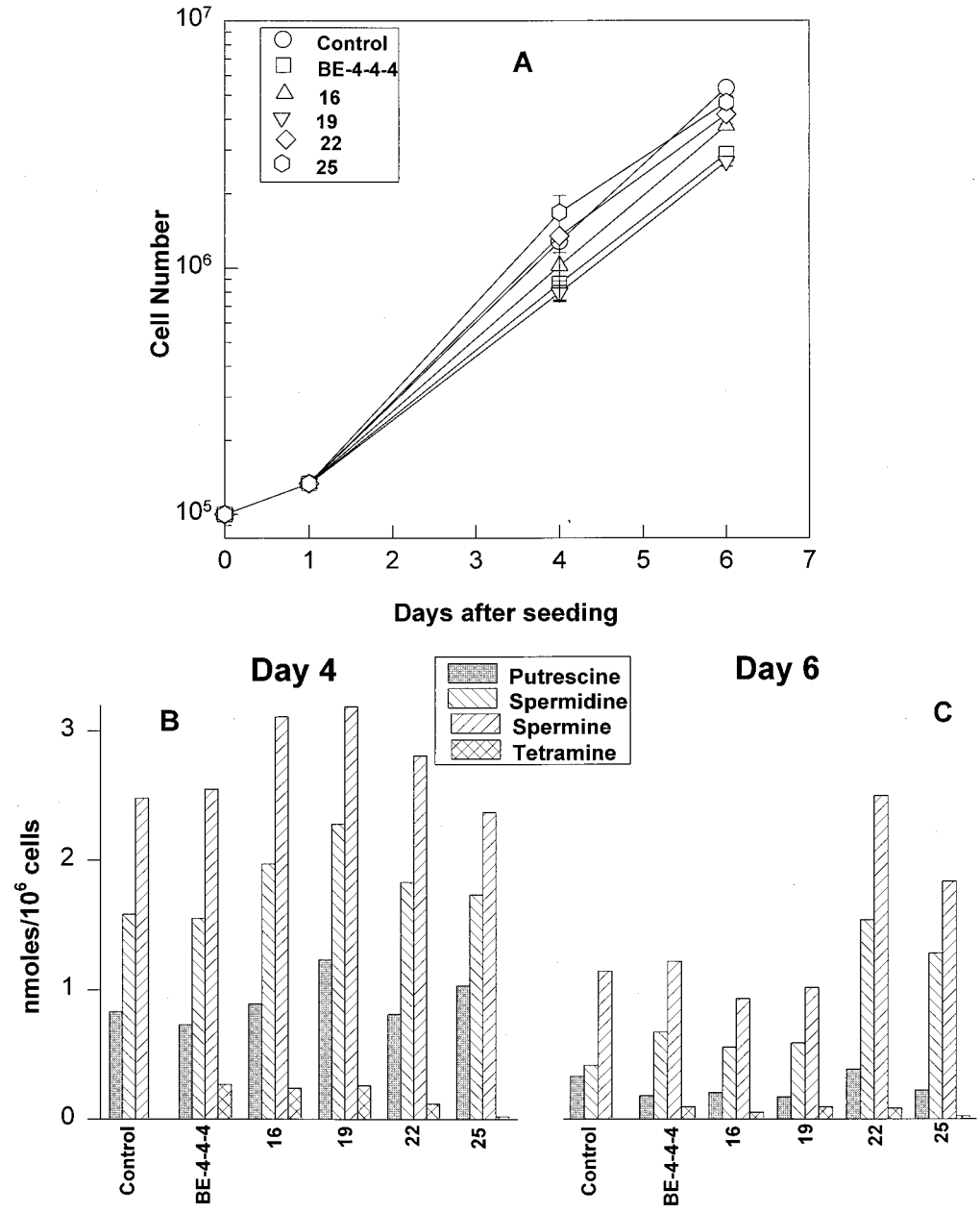


Figure 1. (A) Effects of 1 μM BE-4-4-4, **16**, **19**, **22**, and **25** on the growth of DuPro cells. Symbol for each tetramine is shown in the inset. Each data point is an average of at least three separate experiments run in duplicate. Error bars where not shown are smaller than the symbol size. (B) Effects of 1 μM BE-4-4-4, **16**, **19**, **22**, and **25** on the polyamine levels of DuPro cells on day 4 of treatment. Symbol for each polyamine is shown in the inset. Each data point is an average of two separate experiments. (C) Effects of 1 μM BE-4-4-4, **16**, **19**, **22**, and **25** on the polyamine levels of DuPro cells on day 6 of treatment. Symbol for each polyamine is shown in the inset. Each data point is an average of two separate experiments.

inhibiting cell growth (Figure 5). On the other hand, on day 6 of 10 μM treatments, the intracellular level of **10** was similar to that of **22**, while **10** was a much better growth inhibitor than was **22** (compare Figures 4 and 5). It is evident that in most cases, the growth inhibitory activities of the tetramines cannot be correlated either to their intracellular levels and/or to their abilities to deplete intracellular polyamine pools.

Cytotoxicities of 10 active tetramines were further studied by examining their effects on the colony-forming efficiency (CFE) of cells on day 6 of treatment. The results are shown in Figures 7 and 8. Cytotoxicity of BE-4-4-4 is included in both figures for comparison. *cis*-Dehydro-BE-4-4-4 (**19**) was as cytotoxic as BE-4-4-4. At 10 μM, it was 15-fold more cytotoxic than the *trans*-isomer **16** (Figure 7). When the *cis*-double bond was placed at the outer segment of the molecule (as in **37**), cytotoxicity was greatly enhanced and the resultant tetramine was 1 order of magnitude more cytotoxic than the saturated tetramine BE-4-4-4 (Figure 8). Introduction of additional double bonds, as in **34** and **31**, reduced cytotoxicity. These results parallel those observed for unsaturated pentamines, and a possible rationale for these changes in cytotoxicities has previously been discussed.[7]

Restricting rotational freedom of the central butane chain by the introduction of alicyclic groups, however, increased the cytotoxicity of the tetramines. Three of the four tetramines containing alicyclic groups (**4**, **7**, and **10**) at 10 μM concentrations were 10−50-fold more

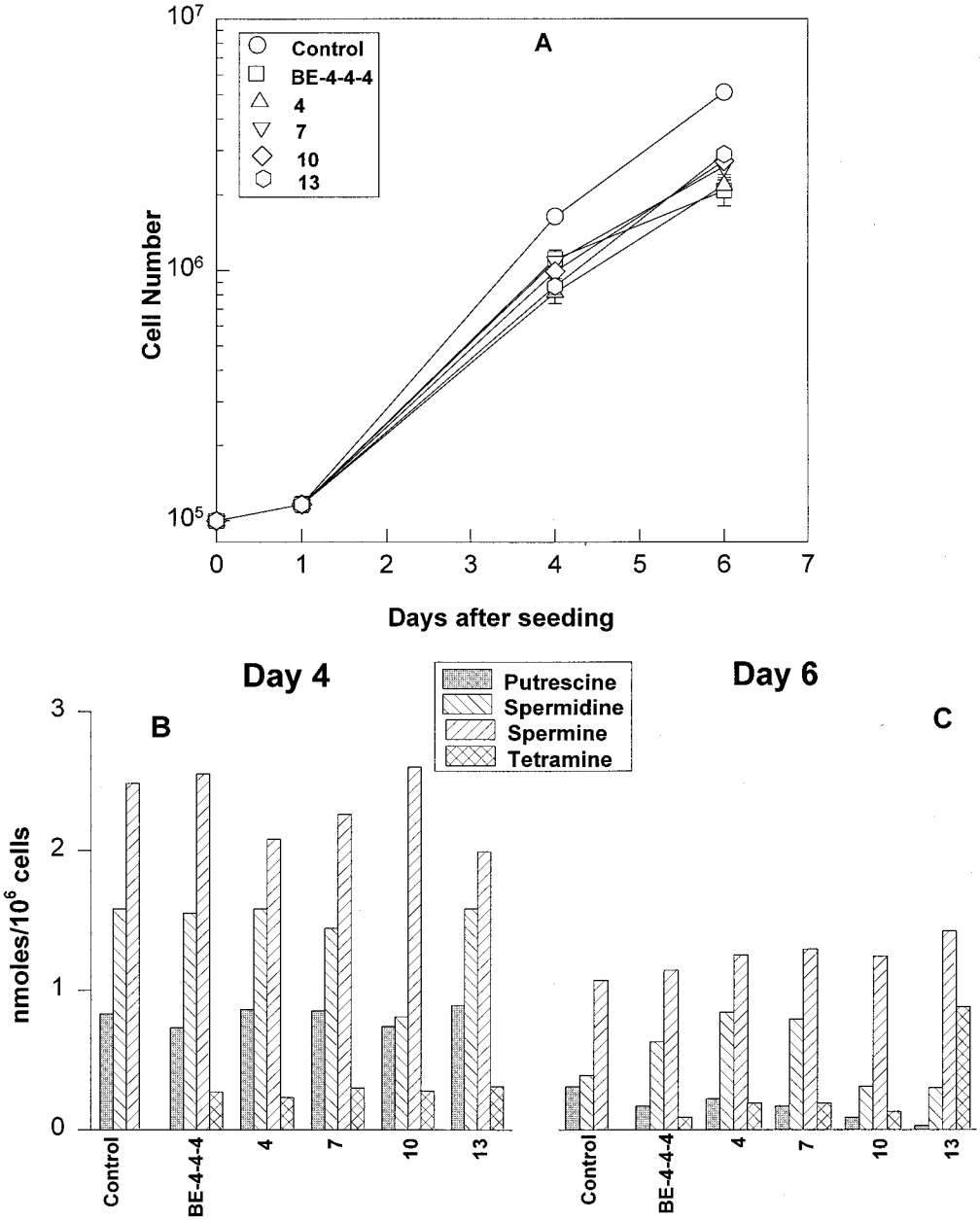


Figure 2. (A) Effects of 1 μM BE-4-4-4, **4**, **7**, **10**, and **13** on the growth of DuPro cells. Symbol for each tetramine is shown in the inset. Each data point is an average of at least three separate experiments run in duplicate. Error bars where not shown are smaller than the symbol size. (B) Effects of 1 μM BE-4-4-4, **4**, **7**, **10**, and **13** on the polyamine levels of DuPro cells on day 4 of treatment. Symbol for each polyamine is shown in the inset. Each data point is an average of two separate experiments. (C) Effects of 1 μM BE-4-4-4, **4**, **7**, **10**, and **13** on the polyamine levels of DuPro cells on day 6 of treatment. Symbol for each polyamine is shown in the inset. Each data point is an average of two separate experiments.

cytotoxic than was BE-4-4-4 (Figures 7 and 8). The tetramines with cyclopropyl groups were more cytotoxic than were the tetramines with cyclobutyl groups. Although no significant difference was observed between the cytotoxicity of the *cis*- and *trans*-cyclopropyl isomers **4** and **7** (Figure 7), for the cyclobutyl isomers, the *trans*-isomer **10** was approximately 20-fold more cytotoxic than was the *cis*-isomer **13** (Figure 7). These differences in cytotoxicities of the isomeric pairs **16/19** and **10/13** were not detected in the cell growth inhibition assays (Figures 1−6). This could be due to the shorter time span of the cell growth assay (6 days) relative to the CFE assay (14−16 days). The *cis*-cyclobutyl isomer **13** is in that conformation where the pharmacophores, i.e. the 1,2-NH_2^+R chains, are in an eclipsed conformation. Therefore, this conformer is at a higher energy state than is the *trans*-isomer **10**, where the pharmacophores are in a staggered conformation. The lower cytotoxicity of the former could result from this unfavorable steric effect that in turn may affect the compound's interactions with DNA and other cellular macromolecules. We have now initiated a detailed nucleic acid binding study of these tetramines to better understand this effect. Interestingly, the presence of an alicyclic ring reduced the systemic toxicity of the tetramines; thus, **4**, **7**, and **10** displayed 10-fold higher host tolerance than did BE-4-4-4 (C. Bacchi, personal communication). Therefore, conformational restriction by alicyclic groups enhances cytotoxicity while lowering systemic toxicity, thus wid-

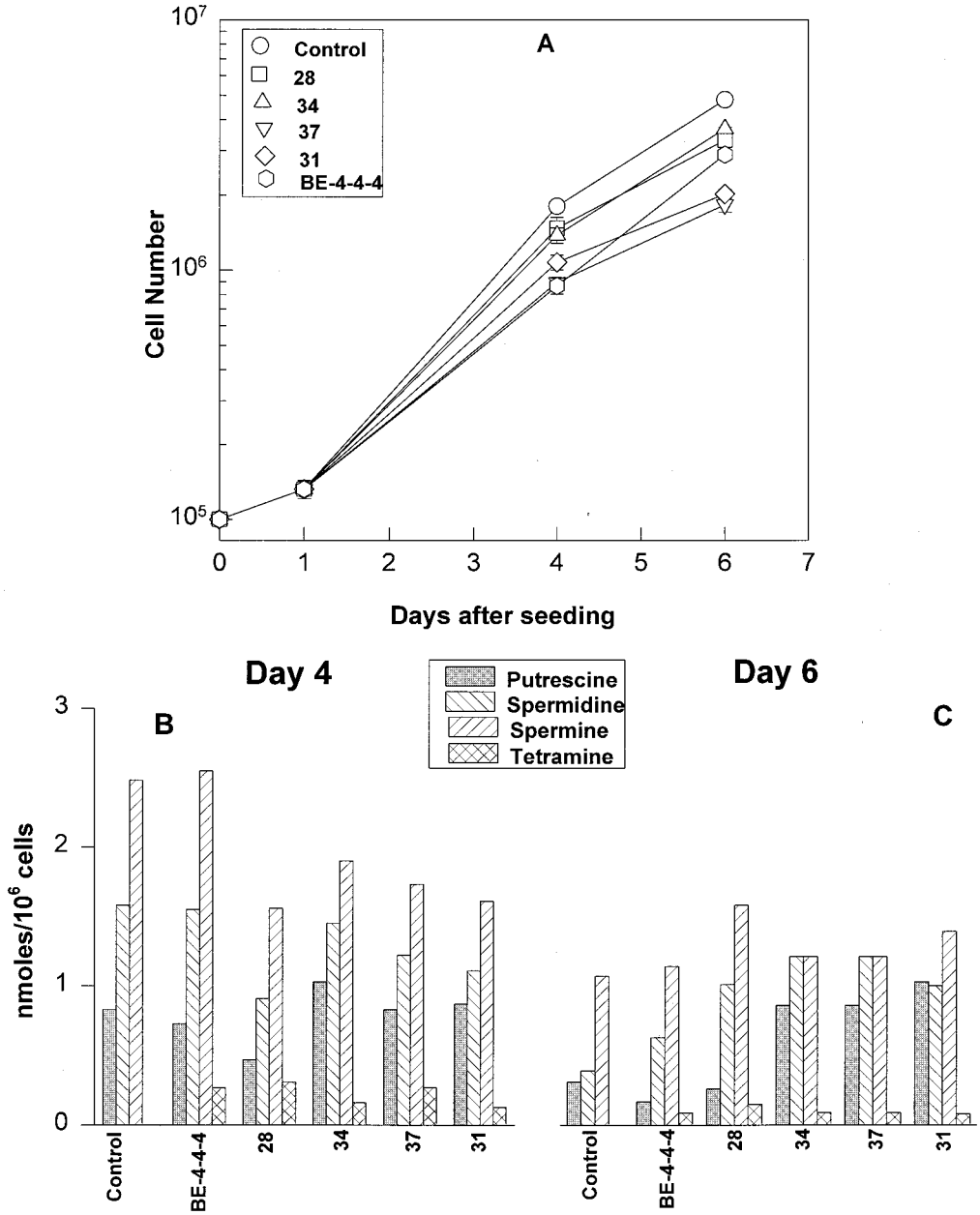


Figure 3. (A) Effects of 1 μM BE-4-4-4, **28**, **34**, **37**, and **31** on the growth of DuPro cells. Each data point is an average of at least three separate experiments run in duplicate. Symbol for each tetramine is shown in the inset. Error bars where not shown are smaller than the symbol size. (B) Effects of 1 μM BE-4-4-4, **28**, **34**, **37**, and **31** on the polyamine levels of DuPro cells on day 4 of treatment. Symbol for each polyamine is shown in the inset. Each data point is an average of two separate experiments. (C) Effects of 1 μM BE-4-4-4, **28**, **34**, **37**, and **31** on the polyamine levels of DuPro cells on day 6 of treatment. Symbol for each polyamine is shown in the inset. Each data point is an average of two separate experiments.

ening the therapeutic window of the new derivatives (Frydman et al., unpublished results).

Conclusions

The most revealing conclusion to be drawn from the data reported above is that by introducing conformational restrictions in the otherwise freely rotating BE-4-4-4 structure, it is possible to enhance the cytotoxic effect of the tetramine. The growth inhibitory activity is highly enhanced by the introduction of a cyclopropyl ring into the central segment of the BE-4-4-4 chain. Both the *cis*-isomer **4** and *trans*-isomer **7** of the cyclopropyl tetramine are highly cytotoxic. When the larger cyclobutyl ring is introduced, cytotoxicity of the *cis*-isomer **13** drops below that of BE-4-4-4 while cytotoxicity of the *trans*-isomer **10** goes above that of BE-4-4-4. When a cyclopentyl ring is introduced, an inactive tetramine is obtained (data not shown). To the best of our knowledge, the bisethyl *trans*-cyclopropyl tetramine **4** is the most cytotoxic polyamine analogue yet reported in human prostate tumor cell lines. These results fall in line with a well-known tenet of drug design. When a rigid analogue of a flexible drug molecule is constructed by adding atoms and/or bonds to the original molecule, the rigid analogue should be as close to the parent molecule in size and shape as possible to be biologically active.

Cytotoxicity of the BE-4-4-4 molecule was also enhanced by introducing a *cis*-double bond in the outer segment of the tetramine (as in **37**). This supports our

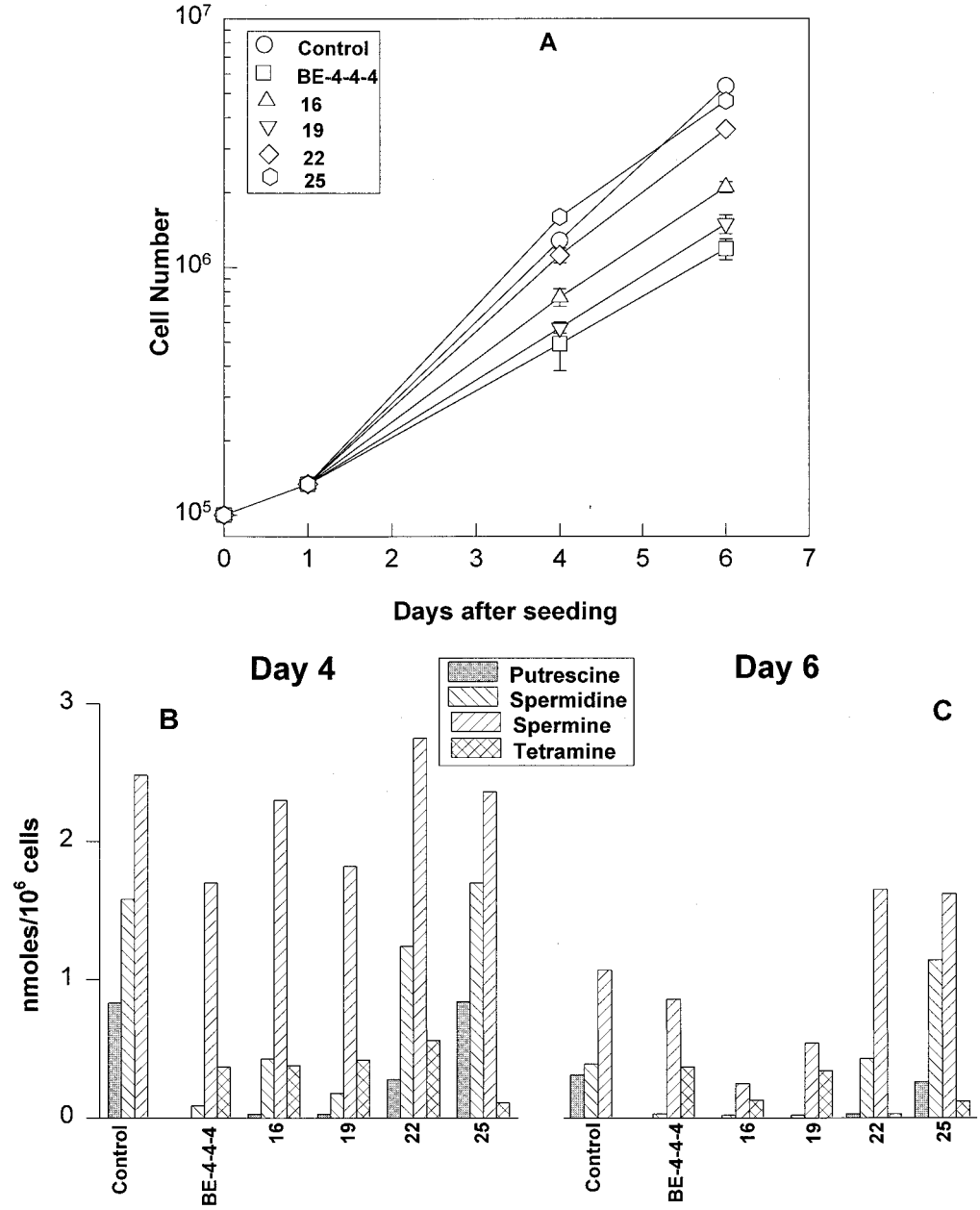


Figure 4. (A) Effects of 10 μM BE-4-4-4, **16**, **19**, **22**, and **25** on the growth of DuPro cells. Symbol for each tetramine is shown in the inset. Each data point is an average of at least three separate experiments run in duplicate. Error bars where not shown are smaller than the symbol size. (B) Effects of 10 μM BE-4-4-4, **16**, **19**, **22**, and **25** on the polyamine levels of DuPro cells on day 4 of treatment. Symbol for each polyamine is shown in the inset. Each data point is an average of two separate experiments. (C) Effects of 10 μM BE-4-4-4, **16**, **19**, **22**, and **25** on the polyamine levels of DuPro cells on day 6 of treatment. Each data point is an average of two separate experiments.

previous suggestions that a cisoid bending at one extreme of the polyamine molecule will favor its interaction with DNA and increase its cytotoxicity by displacing natural polyamines from their DNA binding site(s).[9] If the central butane segment is made very rigid by introducing two double bonds, as in **34** and **31**, or a triple bond, as in **4**, the growth inhibitory activity is either reduced or completely abolished. Introduction of a 1,2-benzenedimethyl residue, as in **25**, made the compound unacceptable for cellular uptake. The lack of correlation for the new tetramines between the depletion of the endogenous cellular polyamine pools or the cellular uptake of the most of the tetramines and their cytostatic effects suggests that the latter is a result of their specific structural features. This again points in the direction of their specific binding to larger molecules. The enhanced host tolerance to the alicyclic tetramines is undoubtedly related to pharmacokinetic and metabolic factors that are currently under investigation.

Experimental Section

NMR spectra were obtained using a Bruker AM-250 spectrometer. Reactions were monitored using TLC on silica gel plates (0.25 mm thick). Flash chromatography was performed on columns packed with EM Science silica gel, 230−400 mesh ASTM. Melting points were determined on an Electrothermal IA 9100 digital melting point apparatus. Mass spectra (ESI) were obtained on a PE Sciex API 365 electrospray triple quadrupole spectrometer; matrix-assisted laser desorption ionization (MALDI) mass spectra were obtained using a Bruker Biflex III spectrometer operating in the time-of-flight

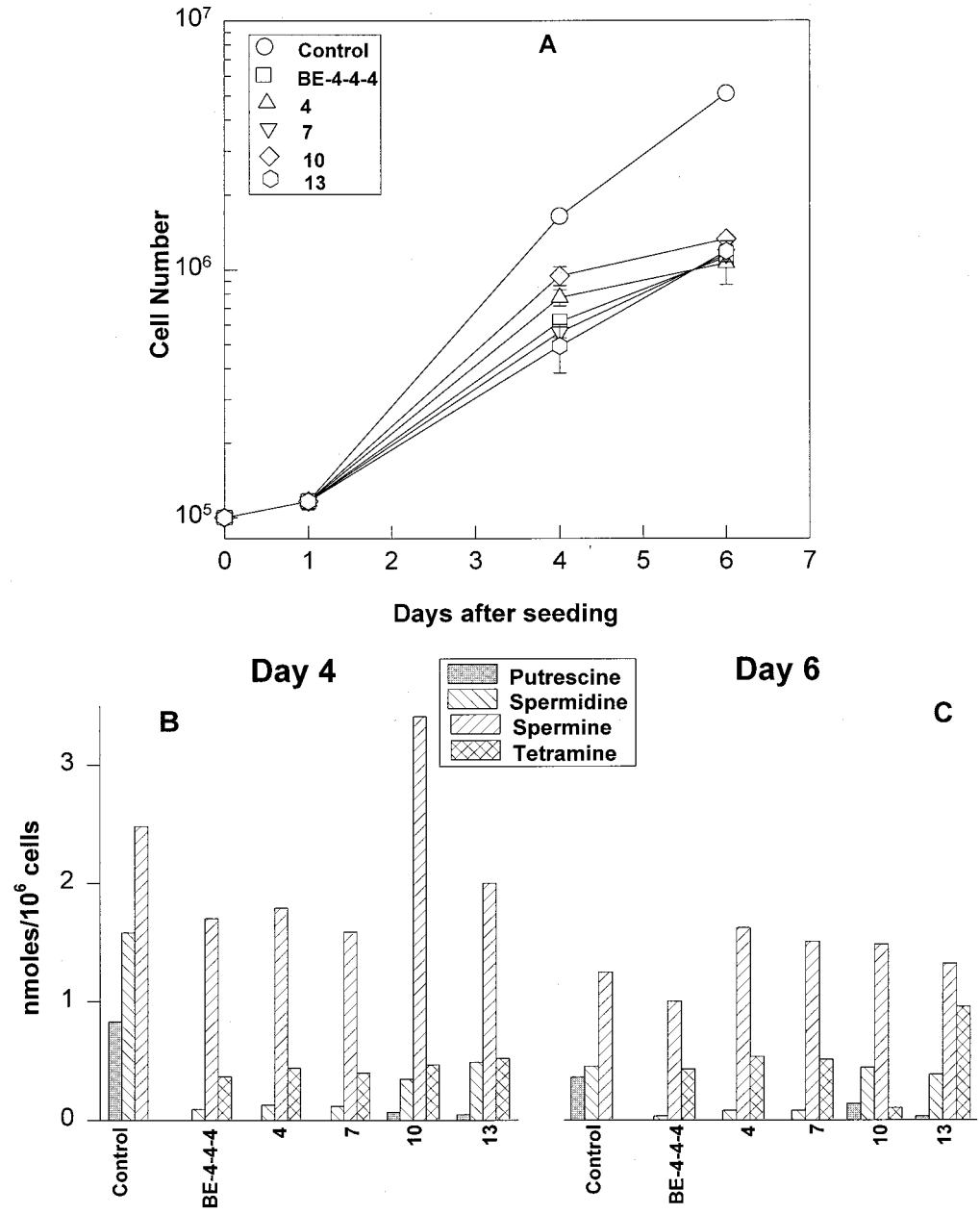


Figure 5. (A) Effects of 10 μM BE-4-4-4, **4**, **7**, **10**, and **13** on the growth of DuPro cells. Symbol for each tetramine is shown in the inset. Each data point is an average of at least three separate experiments run in duplicate. Error bars where not shown are smaller than the symbol size. (B) Effects of 10 μM BE-4-4-4, **4**, **7**, **10**, and **13** on the polyamine levels of DuPro cells on day 4 of treatment. Symbol for each polyamine is shown in the inset. Each data point is an average of two separate experiments. (C) Effects of 10 μM BE-4-4-4, **4**, **7**, **10**, and **13** on the polyamine levels of DuPro cells on day 6 of treatment. Symbol for each polyamine is shown in the inset. Each data point is an average of two separate experiments.

mode; mass spectra (FAB) were obtained on an Autospec (VG) spectrometer. HPLC analyses of the dansyl derivatives of the polyamine tetramines were routinely performed to check the purity of the samples. A Vydac C-18 (300-μm pore) column for separations and a fluorescence spectrometer (340-nm excitation, 515-nm emission) for detection were used.

3,8,13,18-Tetrakis(mesitylenesulfonyl)-10,11-[(*E*)-1,2-cyclopropyl]-3,8,13,18-tetraazaeicosane (3). Amide **2**[23] (2.0 g, 4.16 mmol) and diester **1**[6] (0.92 g, 1.98 mmol) were dissolved in 40 mL of anhydrous DMF under argon, the solution was cooled to 5 °C, and NaH (85%, 0.13 g) was slowly added with continuous stirring. After 4 h the solid disappeared, the reaction was then quenched with water (5 mL), the solution was evaporated to dryness and the resulting slurry was partitioned between ethyl acetate (20 mL) and water (3 × 15 mL). The organic layer was evaporated and the residue purified by flash chromatography using hexane−ethyl acetate (4:1) as eluant; 1.2 g (58%) of **3** was obtained: mp 55−56 °C; ^{1}H NMR ($CDCl_3$) δ 0.04−1.13 (m, 2H), 0.74 (m, 2H), 0.98 (t, 6H), 1.37 (m, 8 H), 2.29 (s, 12H), 2.56 (s, 24H), 2.81 (m, 2H), 3.13 (m, 14H), 6.93 (s, 8H); ^{13}C NMR ($CDCl_3$) δ 2.72,13.82, 20.93, 22.75, 24.55, 24.80, 40.04, 44.53, 44.90, 45.22, 131.90, 131.96, 133.15, 133.43, 140.04, 140.11, 142.23, 142.44; MS-FAB (*m*/*z*) calcd 1027.46 (M^+), found 1027.50.

3,8,13,18-Tetraaza-10,11-[(*E*)-1,2-cyclopropyl]eicosane Tetrahydrochloride (4). Phenol (1.94 g, 20.66 mmol) and 33% hydrogen bromide in glacial acetic acid (9.9 mL) were added in tandem to solution of **3** (0.53 g, 0.52 mmol) in 6 mL of methylene chloride at 25 °C. Stirring was kept on for 18 h, water (5 mL) was then added, the aqueous layer separated, reextracted with methylene chloride (3 × 8 mL), and the aqueous solution was evaporated to dryness. The residue was dissolved in 10 N sodium hydroxide (3 mL), the solution extracted with chloroform (10 × 6 mL), the pooled organic

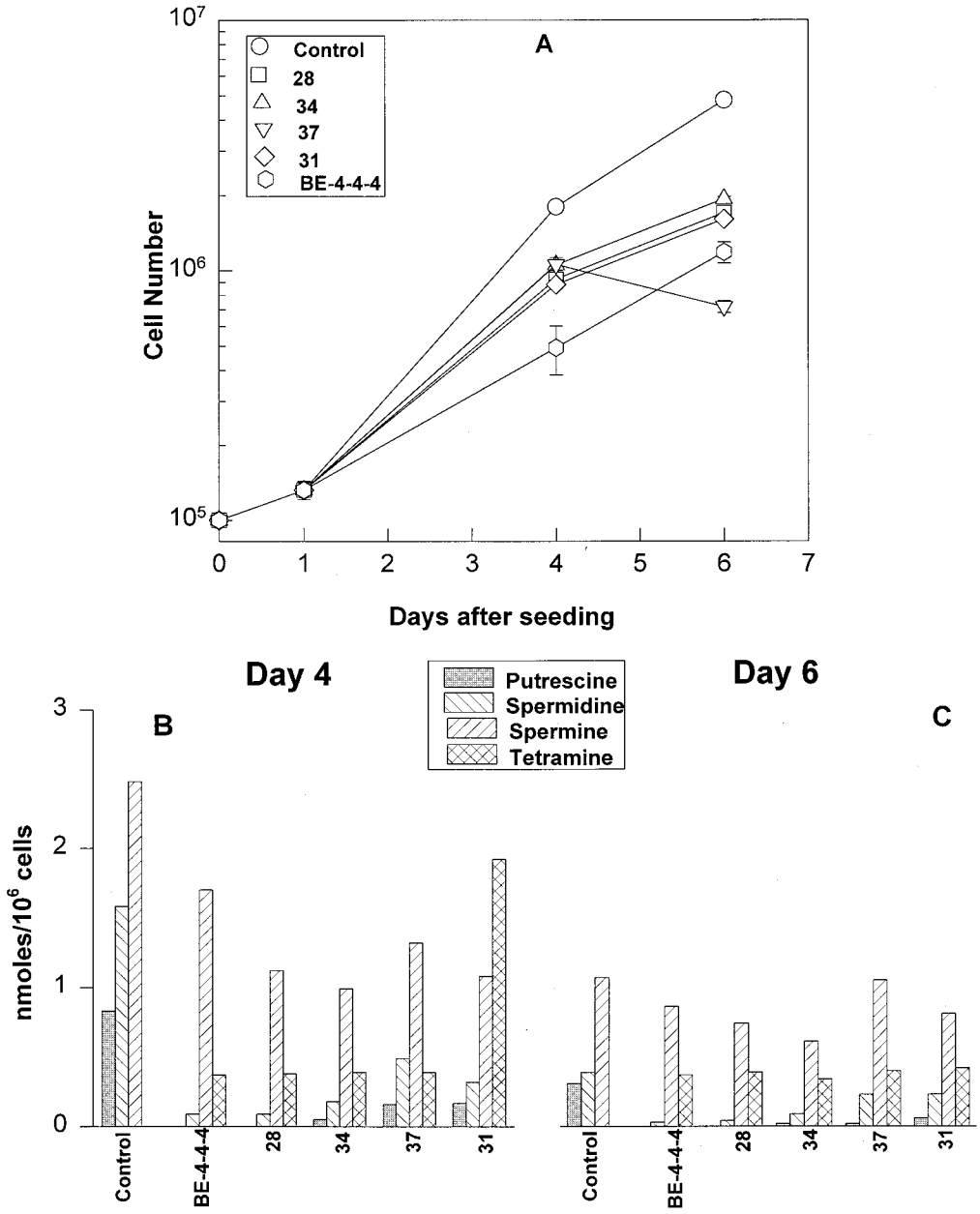


Figure 6. (A) Effects of 10 μM BE-4-4-4, **28**, **34**, **37**, and **31** on the growth of DuPro cells. Symbol for each tetramine is shown in the inset. Each data point is an average of at least three separate experiments run in duplicate. Error bars where not shown are smaller than the symbol size. (B) Effects of 10 μM BE-4-4-4, **28**, **34**, **37**, and **31** on the polyamine levels of DuPro cells on day 4 of treatment. Symbol for each polyamine is shown in the inset. Each data point is an average of two separate experiments. (C) Effects of 10 μM BE-4-4-4, **28**, **34**, **37**, and **31** on the polyamine levels of DuPro cells on day 6 of treatment. Symbol for each polyamine is shown in the inset. Each data point is an average of two separate experiments.

extracts were dried (Na_2SO_4), evaporated to dryness, the residue dissolved in dry ether, and hydrogen chloride was passed through the solution kept at 5 °C. The white precipitate was filtered and crystallized from ethanol−ether; 0.14 g (60%) of **4** was obtained: mp 250 °C dec; ^{1}H NMR (D_2O) δ 0.57 (m, 1H), 1.14 (m, 1H), 1.29 (6H), 1.40 (m, 2H), 1.80 (m, 8H), 2.92 (m, 2H), 3.12 (m, 12H), 3.41 (m, 2H); ^{13}C NMR (D_2O) δ 12.47, 13.31, 14.92, 25.61, 45.69, 49.04, 49.44, 50.12; MS-ESI (*m*/*z*) calcd 299.52 ($M^+ + 1$), found 299.54. Anal. ($C_{17}H_{42}Cl_4N_4$) C, H, N.

3,8,13,18-Tetrakis(mesitylenesulfonyl)-10,11-[(*Z*)-1,2-cyclopropyl]-3,8,13,18-tetraazaeicosane (6) was prepared (71%) following the procedure described for **3**: mp 56−58 °C; ^{1}H NMR ($CDCl_3$) δ 0.02 (m, 1H), 0.75 (m, 2H), 0.98 (t, 6H), 1.36 (m, 8H), 2.28 (s, 12H), 2.56 (s, 2H), 2.82 (m, 4H), 3.11 (m, 12H), 6.93 (s, 8H); ^{13}C NMR ($CDCl_3$) δ 12.69, 13.82, 20.86, 22.66, 22.83, 24.53, 24.77, 40.03, 44.44, 44.89, 45.20, 131.85, 131.92, 133.15, 133.42, 139.98, 140.00, 140.04, 142.17, 142.37; MS-FAB (*m*/*z*) calcd 1027.46 (M^+), found 1027.44.

3,8,13,18-Tetraaza-10,11-[(*Z*)-1,2-cyclopropyl]eicosane tetrahydrochloride (7) was obtained (84%) from the tetramide **6** following the procedure described for **4**: ^{1}H NMR (D_2O) δ 0.58 (dd, 1H), 1.17 (m, 1H), 1.29 (t, 6H), 1.41 (m, 2H), 1.80 (m, 8H), 2.91 (m, 2H), 3.12 (m, 12H), 3.42 (m, 2H); ^{13}C NMR (D_2O) δ 12.46, 13.36, 14.95, 25.68, 45.73, 49.10, 49.48, 50.15; MS-ESI (*m*/*z*) calcd 299.52 ($M^+ + 1$), found 299.55. Anal. ($C_{17}H_{42}Cl_4N_4$) C, H, N.

3,8,13,18-Tetrakis(mesitylenesulfonamido)-10,11-[(*E*)-1,2-cyclobutyl]-3,8,13,18-tetraazaeicosane (9). Diester **8**[6] (3.0 g, 6.25 mmol) and diamide **2** (6.0 g, 12.5 mmol) were dissolved in dry DMF (50 mL), the solution was stirred and kept at 5 °C during 2 h while NaH (0.6 g) was added and then further stirred at 25 °C during 18 h. The solution was then evaporated to dryness, the residue dissolved in chloroform, the

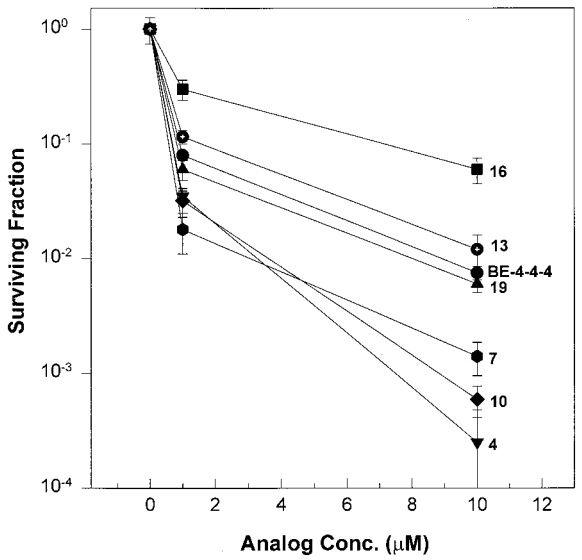


Figure 7. Effects of increasing concentration of BE-4-4-4 (●), **4** (▼), **7** (●), **10** (◆), **13** (⊙), **16** (■), and **19** (▲) on the survival of DuPro cells by CFE assay. Each data point and corresponding error bars are, respectively, an average and the standard deviation of six independent observations.

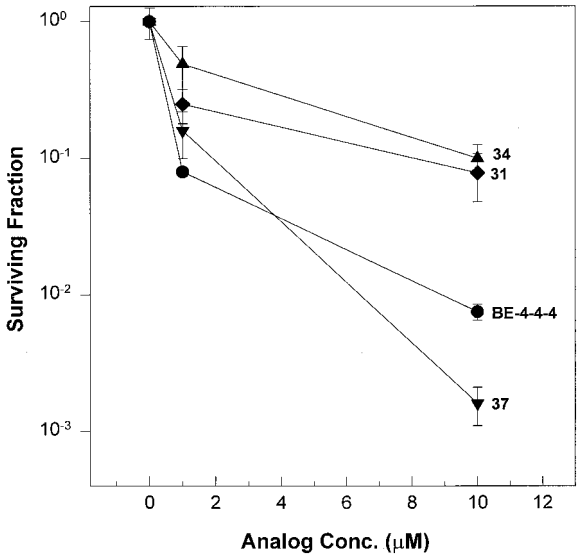


Figure 8. Effects of increasing concentration of BE-4-4-4 (●), **34** (▲), **37** (▼), and **31** (◆) on the survival of DuPro cells by CFE assay. Each data point and corresponding error bars are, respectively, an average and the standard deviation of six independent observations.

organic layer washed first with water, then with concentrated ammonium chloride, dried (Na_2SO_4) and evaporated to dryness. The residue was purified by flash chromatography using hexane−ethyl acetate (from 8:2 to 7:3) as eluant; 5.7 g (87%) of **9** was obtained as a glassy oil: ^{1}H NMR ($CDCl_3$) δ 1.05 (t, 6H), 1.30 (m, 10H), 1.80 (m, 2H), 2.15 (m, 2H), 2.35 (s, 12H), 2.52 (s, 24H), 3.10 (m, 12h), 3.35 (m, 2H), 6.95 (s, 8H); ^{13}C NMR ($CDCl_3$) δ 12.75, 20.89, 22.70, 23.35, 24.38, 24.65, 24.78, 37.62, 40.10, 44.61, 45.40, 50.25, 131.87, 133.51, 139.96, 142.16; MS-FAB (*m*/*z*) calcd 1064.48 (M^+ + Na), found 1064.45.

3,8,13,18-Tetraaza-10,11-[(*E*)-1,2-cyclobutyl]-3,8,13,18-eicosane tetrahydrochloride (10) was obtained (80%) from **9** following the procedure described for **4**; 5.7 g of **9** gave 2.0 g of **10**: mp > 300 °C dec; ^{1}H NMR (D_2O) δ 1.30 (t, 6H), 1.80 (m, 10H), 2.15 (m, 2H), 2.50 (m, 2H), 3.15 (m, 16H); ^{13}C NMR (D_2O) δ 13.28, 13.36, 25.48, 25.58, 37.81, 45.66, 49.03, 49.82, 53.94; MS-ESI (*m*/*z*) calcd 313.54 (M^+ + 1), found 313.56. Anal. ($C_{18}H_{44}Cl_4N_4$) C, H, N.

3,8,13,18-Tetrakis(mesitylenesulfonamido)-10,11-[(*Z*)-1,2-cyclobutyl]-3,8,13,18-tetraazaeicosane (12) was prepared by following the procedure described for **9**. From 2.0 g of the diester **11**[6] (4.1 mmol) and 4.0 g (8.2 mmol) of diamide **2**, 3.4 g (80%) of tetramide **12** was obtained: ^{1}H NMR ($CDCl_3$) δ 0.98 (t, 6H), 1.40 (m, 10H), 1.80 (m, 2H), 2.30 (m, 14H), 2.55 (s, 24H), 3.15 (m, 14H), 6.95 (s, 8H); ^{13}C NMR ($CDCl_3$) δ 12.62, 20.79, 22.70, 24.38, 26.55, 33.85, 39.93, 41.88, 44.31, 45.06, 45.18, 131.78, 132.96, 138.82, 139.80, 141.96, 142.33; MS-FAB (*m*/*z*) calcd 1064.48 (M^++ Na), found 1064.49.

3,8,13,18-Tetraaza-10,11-[(*Z*)-1,2-cyclobutyl]-3,8,13,18-eicosane tetrahydrochloride (13) was obtained (85%) following the procedure described for **10**: ^{1}H NMR (D_2O) δ 1.30 (t, 6H), 1,80 (m, 10H), 2.20 (m, 2H), 2.90 (m 2H), 3.10 (m, 16H); ^{13}C NMR (D_2O) δ 13.29, 25.22, 25.56, 35.59, 45.66, 49.62, 50.67, 53.94; MS-ESI (*m*/*z*) calcd 313.54 (M^+ + 1), found 313.56. Anal. ($C_{18}H_{44}Cl_4N_4$) C, H, N.

(*E*)-3,8,13,18-Tetrakis(mesitylenesulfonyl)-3,8,13,18-tetraazaeicos-10-ene (15) was obtained (56%) by condensation of the diester **14**[6] and the diamide **2** following the procedure described for **3**: ^{1}H NMR ($CDCl_3$) δ 0.95 (t, J = 7.1 Hz, 6H), 1.34 (m, 8H), 2.29 (s, 24H), 3.09 (m, 12H), 3.72 (d, J = 4.5 Hz, 4H), 5.48 (t, J = 4.3 Hz, 2H), 6.92 (s, 4H), 6.93 (s, 4H); ^{13}C NMR ($CDCl_3$) δ 12.71,20.90, 22.71, 22.76, 24.74, 40.04, 42.21, 44.56, 45.69, 128.45, 131.88, 132.02, 140.16, 142.20, 142.58; MS-FAB (*m*/*z*) calcd 1013.44 (M^+), found 1013.42.

(*E*)-3,8,13,18-Tetraazaeicos-10-ene tetrahydrochloride (16) was obtained (75%) from **15** following the procedure described for **4**: mp >230 °C; ^{1}H NMR (D_2O) δ 1.26 (t, J = 12.5 Hz, 6H), 1.79 (m, 8H), 3.12 (m, 12H), 3.80 (d, J = 7.2, 4H), 6.10 (m, 2H); ^{13}C NMR (D_2O) δ 12.79, 25.10, 45.19, 48.53, 48.62, 50.36, 130.66; MS-ESI (*m*/*z*) calcd 285.49 (M^+ + 1), found 285.50. Anal. ($C_{16}H_{40}Cl_4N_4$) C, H, N.

(*Z*)-3,8,13,18-Tetrakis(mesitylenesulfonyl)-3,8,13,18-tetraazaeicos-10-ene (18) was obtained (55%) by condensation of diester **17**[6] with the diamide **2** following the procedure described for **3**: ^{1}H NMR ($CDCl_3$) δ 0.96 (t, 6H), 1.28 (br, 8H), 2.29 (s, 12H), 2.56 (s, 24H), 3.09 (m, 12H), 3.72 (d, 4H), 5.49 (br, 2H), 6.92 (s, 4H), 6.93 (s, 4H); ^{13}C NMR ($CDCl_3$) δ 12.66, 20.89, 22.71, 24.67, 39.97, 42.13, 44.67, 45.60, 128.40, 131.85, 131.97, 133.00, 133.50, 140.14, 142.30, 142.54; MS-FAB (*m*/*z*) calcd 1013.44 (M^+), 1013.45.

(*Z*)-3,8,13,18-Tetraazaeicos-10-ene tetrahydrochloride (19) was obtained (74%) from **18** following the procedure described for **4**: ^{1}H NMR (D_2O) δ 1.29 (t, 6H), 1.79 (m, 8H), 3.12 (m, 12H), 3.84 (d, 4H), 5.96 (t, J = 4.6 Hz, 2H); ^{13}C NMR (D_2O) δ 12.79, 25.09, 25.23, 45.19, 46.27, 48.54, 48.86, 128.68; MS-ESI (*m*/*z*) calcd 285.49 (M^+ + 1), found 285.49. Anal. ($C_{16}H_{40}Cl_4N_4$) C, H, N.

3,8,13,18-Tetrakis(mesitylenesulfonyl)-3,8,13,18-tetraazaeicos-10-yne (21) was obtained (59%) by condensation of diester **20**[6] with amide **2** as described for **3**: ^{1}H NMR ($CDCl_3$) δ 0.90 (t, 6H), 1.30 (bs, 8H), 2.20 (s, 12H), 2.45 (s, 24H), 3.05 (m, 12H), 3.75 (s, 4H), 6.87 (s, 8H); ^{13}C NMR ($CDCl_3$) δ 12.70, 20.78, 22.68, 34.65, 39.97, 44.46, 44.99, 78.62, 131.85, 131.98, 132.34, 140.14, 142.13, 142.55; MS-FAB (*m*/*z*) calcd 1011.12 (M^+), found 1011.10.

3,8,13,18-Tetraazaeicos-10-yne tetrahydrochloride (22) was obtained (76%) from **21** following the procedure described for **4**: mp > 280 °C; ^{1}H NMR (D_2O) δ 1.30 (t, 6H), 1.80 (b, 8H), 2.90−3.25 (m, 12H), 4.05 (s, 4H); ^{13}C NMR (D_2O) δ 13.39, 25.64, 39.26, 45.72, 49.20, 81.20; MS-ESI (*m*/*z*) calcd 283.47 (M^+ + 1), found 283.45. Anal. ($C_{16}H_{38}Cl_4N_4$) C, H, N.

10,11-Benzo-3,8,13,18-tetrakis(mesitylenesulfonyl)-3,8,-13,18-tetraazaeicosane (24) was obtained (84%) by condensation of diester **23**[6] with amide **2** as described for **3**: mp 109 °C (from ethyl acetate−hexane); ^{1}H NMR ($CDCl_3$) δ 0.93 (t, 6H), 1.20 (m, 8H), 2.30 (s, s, 12H), 2.65−2.70 (s, s, 24H), 2.90 (m, 8H), 3.10 (t, 6H), 4.25 (s, 4H), 6.90, 6.95 (s, s, 8H), 7.25 (s, 4H); ^{13}C NMR ($CDCl_3$) δ 12.66, 20.84, 22.67, 23.12, 24.71, 39.80, 40.06, 44.32, 44.46, 45.30, 47.28, 128.05, 129.56, 131.78, 132.36, 133.19, 134.27, 139.89, 142.14, 151.27; MS-FAB (*m*/*z*) calcd 1063.50 (M^+), found 1063.49.

10,11-Benzo-3,8,13,18-tetraazaeicosane tetrahydro-

chloride (25) was obtained (56%) from **24** following the procedure described for **4**: mp > 300 °C dec; ^{1}H NMR (D_2O) δ 1.25 (t, 6H), 1.75 (m, 8H), 3.00−3.30 (m, 12H), 4.45 (s, 4H), 7.60 (s, 4H); ^{13}C NMR (D_2O) δ 13.40, 25.74, 25.83, 45.73,49.11, 49.77, 50.72, 133.32, 134.15; MS-ESI (*m*/*z*) calcd 335.55 ($M^+ + 1$), found 335.56. Anal. ($C_{20}H_{42}Cl_4N_4$) C, H, N.

***(Z,Z,Z)*-3,8,13,18-Tetrakis(mesitylenesulfonyl)-3,8,13,-18-tetraazaeicosa-5,10,15-triene (27)** was prepared by condensation of diamide **26**[7] (2.4 g, 5 mmol) with diester **17** (1.07 g, 2.37 mmol) in the presence of 0.155 g of NaH (85%) following the procedure described for **3**. After chromatography 2.5 g (49%) of **27** was obtained: ^{13}C NMR ($CDCl_3$) δ 12.84, 20.93, 22.69, 40.45, 41.49, 42.17, 128.02, 128.58, 129.14, 131.93, 132.06, 140.19, 140.32, 142.24, 142.45; MS-FAB (*m*/*z*) calcd 1009.41 (M^+), found 1009.42.

***(Z,Z,Z)*-3,8,13,18-Tetraeicosa-5,10,15-triene tetrahydrochloride (28)** was obtained from **27** following the procedure described for **4**. From 1.45 g of **27**, 0.44 g of **28** was obtained (61%): mp 240 °C; ^{1}H NMR (D_2O) δ 1.31 (J = 7.3 Hz, 6H), 3.14 (q, J = 7.3 Hz, 4H), 3.85 (m, 12H), 5.97 (m, 6H); ^{13}C NMR (D_2O) δ 13.45, 45.42, 46.23, 46.43, 129.15, 129.44, 129.57; MS-ESI (*m*/*z*) calcd 281.46 ($M^+ + 1$), found 281.44. Anal. ($C_{16}H_{36}$-Cl_4N_4) C, H, N.

***(Z,Z)*-3,8,13,18-Tetrakis(mesitylenesulfonyl)-3,8,13,18-tetraazaeicosa-5,10-diene (30)** was prepared by condensation of the diamide **29**[7] (0.68 g, 1.2 mmol) with the diamide **26** (0.57 g, 1.2 mmol) in the presence of NaH (85%, 90 mg) following the procedure described for **3**. After chromatography 0.95 g (79%) of **30** was obtained: ^{1}H NMR ($CDCl_3$) δ 0.96 (t, 3H), 0.99 (t, 3H), 1.34 (m, 4H), 2.29 (s, 12H), 2.55 (s, 24H), 3.10 (m, 8H), 3.70 (m, 8H), 5.49 (m, 4H), 6.92 (s, 8H); ^{13}C NMR ($CDCl_3$) δ 12.67, 12.84, 20.90, 22.67, 22.70, 22.74, 24.69, 39.99, 40.44, 41.49, 42.09, 42.19, 42.28, 44.48, 45.54, 127.98, 128.21, 128.69, 129.20, 131.86, 131.93, 132.00, 132.05, 132.34, 140.02, 140.14, 140.17, 140.29, 142.18, 142.49, 142.59, 142.87; MS-FAB (*m*/*z*) calcd 1011.42 (M^+), found 1011.43.

***(Z,Z)*-3,8,13,18-Tetraeicosa-5,10-diene tetrahydrochloride (31)** was obtained (71%) from **30** following the procedure described for **4**: ^{1}H NMR (D_2O) δ 1.29 (t, 3H), 1.31 (t, 3H), 1.79 (m, 4H), 3.12 (m, 8H), 3.83 (m, 8H), 5.96 (m, 4H); ^{13}C NMR (D_2O) δ 13.34, 13.44, 25.64, 25.77, 45.40, 45.72, 46.22, 46.41, 46.81, 49.07, 49.39, 129.16, 129.29, 129.38, 129.54; MS-ESI (*m*/*z*) calcd 283.47 ($M^+ + 1$), found 283.48. Anal. ($C_{16}H_{38}Cl_4N_4$) C, H, N.

***(Z,Z)*-3,8,13,18-Tetrakis(mesitylenesulfonyl)-3,8,13,18-tetraazaeicosa-5,15-diene (33)** was obtained (42%) by alkylation of the diamide **26** with the diester **32** following the procedure described for the synthesis of **3**: ^{1}H NMR ($CDCl_3$) δ 0.99 (t, 6H), 1.35 (m, 4H), 2.29 (s, 12H), 2.55 (s, 12H), 2.57 (s, 12H), 3.10 (m, 8H), 3.71 (m, 8H), 5.46 (m, 4H), 6.93 (s, 8H); ^{13}C NMR ($CDCl_3$): δ 12.83, 20.91, 22.68, 22.74, 24.67, 40.47, 41.49, 42.06, 45.50, 128.15, 128.87, 131.93, 131.99, 132.80, 140.02, 140.17, 140.28, 142.47, 142.59; MS-FAB (*m*/*z*) calcd 1011.42 (M^+), found 1011.44.

***(Z,Z)*-3,8,13,18-Tetraeicosa-5,15-diene tetrahydrochloride (34)** was obtained (80%) from **33** following the procedure described for **4**: ^{1}H NMR (D_2O) δ 1.31 (t, 6H), 1.81 (m, 4H), 3.13 (m, 8H), 3.83 (m, 8H), 5.96 (m, 4H); ^{13}C NMR (D_2O) δ 13.43, 25.77, 45.39, 46.19, 46.78, 49.35, 129.12, 129.41; MS-ESI (*m*/*z*) calcd 283.47 ($M^+ + 1$), found 283.48. Anal. ($C_{16}H_{38}$-Cl_4N_4) C, H, N.

***(Z)*-3,8,13,18-Tetrakis(mesitylenesulfonyl)-3,8,13,18-tetraazaeicos-5-ene (36)** was obtained (79%) by condensation of the iodoalkyl diamide **35**[7] with diamide **2** following the procedure described for the synthesis of **3**: ^{1}H NMR ($CDCl_3$) δ 0.97 (t, 3H), 1.00(t, 3H), 1.43 (m, 8H), 2.29 (s, 12H), 2.54 (s, 3H), 2.56 (s, 12H), 2.57 (s, 9H), 3.07 (m, 12H), 4.12 (m, 4H), 5.48 (m, 2H), 6.93 (s, 8H); ^{13}C NMR ($CDCl_3$) δ 12.69, 12.70, 20.91, 22.70, 22.71, 22.76, 22.91, 24.46, 24.70, 39.98, 40.48, 41.49, 41.99, 44.49, 44.93, 45.43, 128.18, 128.90, 131.88, 131.94, 132.04, 139.98, 140.12, 140.16, 142.21, 142.29, 142.48; MS-FAB (*m*/*z*) calcd 1013.44 (M^+), found 1013.44.

***(Z)*-3,8,13,18-Tetraazaeicos-5-ene tetrahydrochloride (37)** was obtained (73%) from **36** following the procedure described for **4**: ^{1}H NMR (D_2O) δ 1.29 (t, 3H), 1.31 (t, 3H), 1.79 (m, 8H), 3.12 (m, 12H), 3.83 (m, 4H), 5.96 (t, 2H); ^{13}C NMR (D_2O) δ 13.33, 13.43, 25.64, 25.78, 45.37, 45.71, 46.19, 46.77, 49.08, 49.34, 49.69, 129.20, 129.34; MS-ESI (*m*/*z*) calcd 285.49 ($M^+ + 1$), found 285.50. Anal. ($C_{16}H_{40}Cl_4N_4$) C, H, N.

1,4-Bis(mesitylenesulfonyloxy)butane (32). 1,4-Butanediol (4.5 g, 50 mmol) and benzyltriethylammonium bromide (0.67 g, 2.5 mmol) were dissolved in a mixture of 45 mL of 50% KOH and 30 mL dioxane. The solution was kept at 5 °C while a solution of 26 g (120 mmol) mesitylenesulfonyl chloride in 40 mL dioxane was added in small portions. After completing the addition, stirring was kept for 4 h at 5 °C, water (200 mL) was added and the mixture further stirred at 25 °C for 18 h. The precipitate was filtered, dried, and crystallized from ethyl acetate−hexane: 14.6 g (64%); mp 108 °C; ^{1}H NMR ($CDCl_3$) δ 1.75 (m, 4H), 2.30 (s, 6H), 2.55 (s, 12H), 3.95 (m, 4H), 7.02 (s, 4H); ^{13}C NMR ($CDCl_3$) δ 20.86, 22.39, 25.11, 68.26, 130.50, 131.65, 139.64, 143.22; MS-MALDI (*m*/*z*) calcd 477.59 (M^+ + Na), found 477.60.

Biology. Materials: DuPro cells were obtained from M. Eileen Dolan of the University of Chicago, Chicago, IL. All other cell lines used in this study were obtained from the American Type Culture Collection (Rockville, MD). Tissue culture medium was obtained from Fisher Scientific (Itasca, IL) and fetal bovine serum was obtained from Gemini Bioproducts, Inc. (Calabasas, CA). All other reagents were analytical grade. Deionized double-distilled water was used in all studies.

Tissue culture:[17] Cells were seeded into 75-cm^2 culture flasks with 15 mL of Eagle's minimal essential medium supplemented with 10% fetal calf serum and nonessential amino acids. The flasks were incubated in a humidified 95% air/5% CO_2 atmosphere. The cells were grown for at least 24 h to ensure that they were in the log phase of growth. They were then treated with the tetramines. Cells were harvested by treatment for 5 min with STV (saline A, 0.05% trypsin, 0.02% EDTA) at 37 °C. The flasks were rapped on a lab bench, pipetted several times and aliquots of the cell suspension were withdrawn and counted using a Coulter particle counter that was standardized for each cell line using a hemacytometer.

Polyamine analysis:[24] Approximately 1 × 10^6 cells were taken from harvested samples and centrifuged at 1000 rpm at 4 °C for 5 min. The cells were washed twice by resuspending them in chilled Dulbecco's isotonic phosphate buffer (pH 7.4) and centrifuged at 1000 rpm at 4 °C. The supernatant was decanted and 250 μL of 2% perchloric acid was added to the cell pellet. The cells were then sonicated and the lysates were kept at 4 °C for at least 1 h. After centrifugation at 8000*g* for 5 min, the supernatant was removed for analysis. An appropriate volume of the supernatant (50−100 μL) was fluorescence-labeled by derivatization with dansyl chloride following procedures published elsewhere.[24] Each sample was loaded onto a C-18 high-performance liquid chromatography column and separated at the analytical laboratory of the University of Wisconsin Comprehensive Cancer Center (UWCCC) using a previously published procedure.[24] Peaks were detected and quantitated using a Shimadzu HPLC fluorescence monitor coupled to a Spectra-Physics peak integrator. Because polyamine levels vary with environmental conditions, control cultures were sampled for each experiment.

MTT assay:[6] Trypsinized cell suspensions were diluted to seed a 80-μL suspension of 500 cells into each well of a 96-well Corning microtiter plate. The plates were incubated overnight at 37 °C in a humidified 95% air/5% CO_2 atmosphere. 20 μL of appropriately diluted stock solutions of each drug was added to the middle 8 columns of the microtiter plates. Each drug concentration was run in quadruplicate. The outer columns of the plates were used for buffer controls. Cells were incubated with the drug for 6 days. 25 μL of a 5 mg/mL solution of 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each well and the plates were incubated at 37 °C for 4 h. Cells were then lysed by incubating at 37 °C overnight with 100 μL of lysis buffer (500 mL of the lysis buffer contained: 100 g lauryl sulfate (SDS), 250 mL of

N,*N*-dimethylformamide in 2 mL of glacial acetic acid, pH 4.8). The color developed was read at room temperature at 570 nm in a E-max precision microplate reader (Molecular Devices Corp., Sunnyvale, CA) and data were analyzed using manufacturer supplied cell survival software.

CFE assay:[17] All of the cell lines that were used in this assay have previously been optimized with respect to the necessary incubation times for observable colony formation. Both floating and attached cells were harvested and centrifuged at 1000 rpm for 10 min at 4 °C. The pelleted cells were resuspended and replated in quadruplicate at appropriate dilution into 60-mm plastic Petri dishes. The Petri dishes were prepared not more than 24 h in advance with 4 mL of supplemented Eagle's minimum essential medium containing 5−10% fetal bovine serum (standardized for each cell line). Cells were incubated for the previously standardized number of days in a 95% air/5% CO_2 atmosphere. The plates were stained with 0.125% crystal violet in methanol and counted. Results are expressed as a surviving fraction of an appropriate control.

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