A Comparison of Chloroambucil- and Xylene-Containing Polyamines Leads to Improved Ligands for Accessing the Polyamine Transport System

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Several disubstituted arylene- and chloroambucil-polyamine conjugates were synthesized and evaluated for their ability to target cells via their polyamine transport system (PAT). As compared to the monosubstituted analogues, the disubstituted arylene systems were superior PAT targeting agents. Using a Chinese hamster ovary (CHO) cell line (PAT active) and its CHO-MG mutant (PAT inactive), the series was screened for their PAT targeting ability. The data were expressed as a CHOMG/CHO IC_{50} ratio. Indeed, the disubstituted systems gave high IC_{50} ratios (e.g., ratio > 2000), which indicated high selectivity for the PAT. The chloroambucil adducts were less toxic than the corresponding arylmethyl compounds. In this regard, having the proper recognition element (i.e., homospermidine) and cytotoxic "cargo" were deemed paramount for successful drug delivery via the PAT.

Introduction

Polyamines are essential growth factors for cells. All cells have methods of manufacturing polyamines from amino acid sources. In addition, cells can import polyamines from outside the cell via a process referred to as the polyamine transporter (PAT[a]).[1] While much is known about polyamine transport in bacteria,[2] yeast,[2] and leishmania,[3] the mammalian polyamine transport system is a measureable, yet poorly described, import process. It is an important cancer target because many cancer cells are unable to produce enough polyamines to sustain their growth rate and rely upon polyamine import to grow.[1,4–10]

Therefore, an opportunity exists to selectively target rapidly dividing cancer cells via their heavy reliance on polyamine import. Drug-polyamine conjugates, which covalently attach a polyamine to a known cytotoxic agent, have been shown to have enhanced cytotoxicity to cancer cells (e.g., mouse melanoma, B-16 cells) over their normal cell counterparts (Mel-A) in vitro.[1,4–10]

In this regard, numerous *N*-alkyl-polyamines have been synthesized with promising anticancer activity.[11] The molecular recognition events involved in polyamine transport have long been known to be sensitive to the distance separating the nitrogen centers as well as to the number of nitrogens present in the molecule.[1,4–11] However, recent systematic studies of N^1-(anthracenylmethyl)polyamines suggested that there are limits to the structural tolerance accommodated by the PAT.[4–8]

Prior work in our laboratories and others has described the structural requirements associated with the delivery of polyamine conjugates via the PAT in mouse leukemia cells (L1210) and CHO cells.[1,4–13] In short, the number of methylene "spacer" units, the size of the N^1 substituent, and the degree of N^1 substitution all influence PAT-mediated delivery.[6,8–10] The linear triamine motifs were identified as excellent vector systems for the PAT.[6,8] In particular, the homospermidine conjugate **1a** (e.g., a 4,4-triamine, Figure 1) had 150-fold higher cytotoxicity in CHO cells than in the mutant cell line, CHO-MG[a], which was PAT-deficient.[6,8,14,15] In addition, a direct correlation was found between the cytotoxicity and the ability of the polyamine conjugate to use the PAT for cellular entry. This strategy was further illustrated, when **1a** was shown to be 10–30 times more selective in killing B16 melanoma cells over Mel A (normal melanocyte) cells.[10] Therefore, the 4,4-triamine motif (homospermidine) was found to impart excellent PAT selectivity in this earlier anthracenylmethyl-polyamine series. We speculated that if homospermidine was an optimal motif for cellular entry, then architectures containing two or more of these recognition elements may have enhanced PAT selectivity and biological properties. Having that in mind, we synthesized the disubstituted compounds **4**−**6** and the tris-homospermidine adduct, **7**.

Beyond the multimeric forms **4**–**7**, we also investigated the effect of appending a different "cytotoxic cargo" to the homospermidine motif. The chlorambucil (CA) architecture was an attractive target for several reasons. First, chlorambucil is a chemotherapeutic, which is given in the treatment of chronic lymphocytic leukemia, low-grade non-Hodgkin's lymphoma, Hodgkin's lymphoma, and ovarian cancer.[16] Second, it is taken up by various tumor cells by simple diffusion and exerts its cytotoxic effects by interaction with DNA.[16] Third, Cohen et al. have reported good results in targeting tumor cells with the chlorambucil-spermidine conjugate **8**.[17] Indeed, **8** showed 10000-fold more activity than chlorambucil at forming interstrand cross-links with naked DNA.[17,18] The increased toxicity of the conjugate may be due to the enhanced DNA binding and/

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[a] PAT, polyamine transporter; CHO, Chinese hamster ovary cells; CHO-MG, Chinese hamster ovary cells polyamine transport deficient mutant; L1210, mouse leukemia cells; HBBS, Hanks balanced salt solution; PBS, Phosphate buffered saline.

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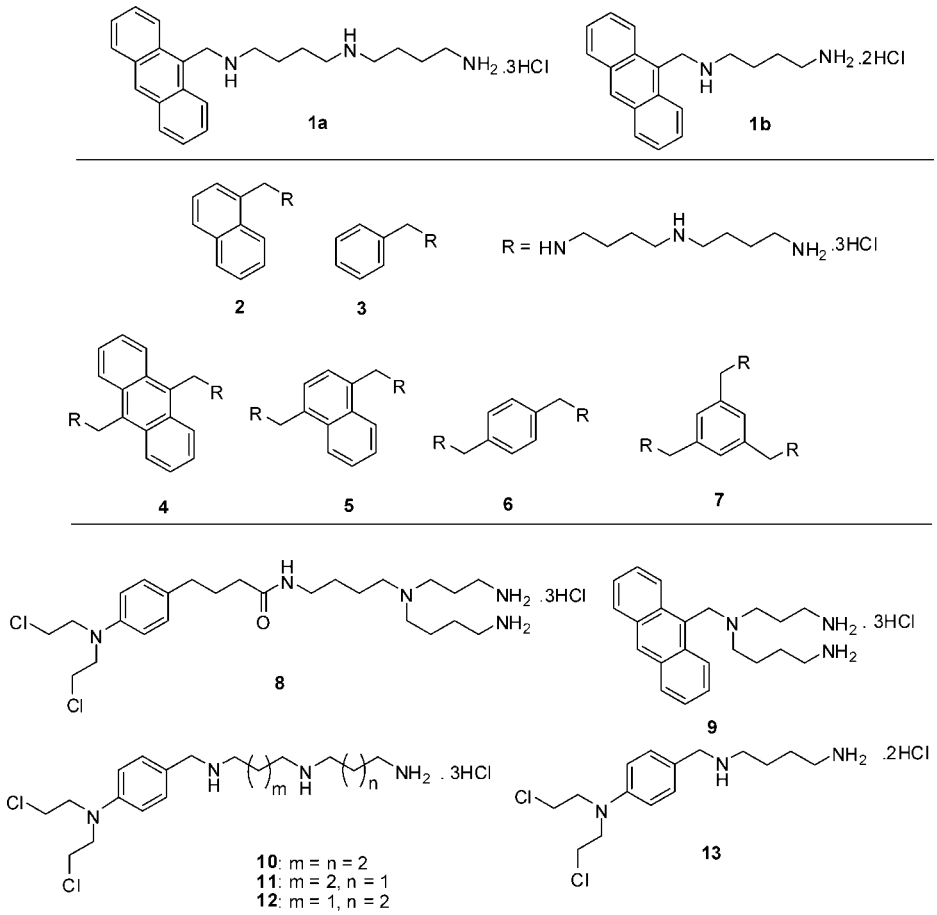
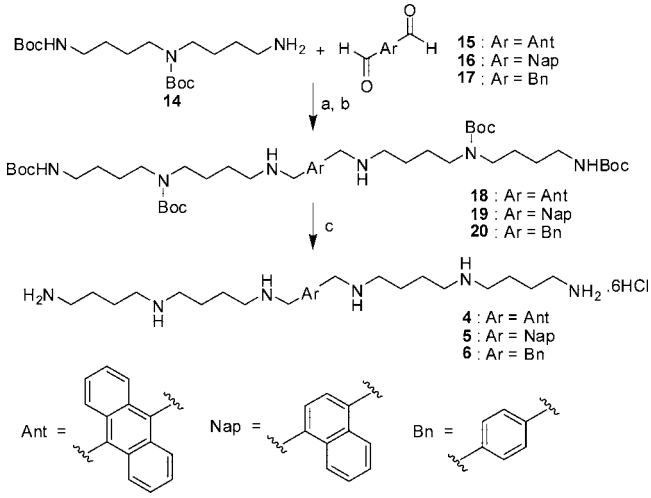


Figure 1. Structures of compounds **1–13**.

Scheme 1[a]



[a] Reagents: (a) 25% MeOH/CH_2Cl_2. (b) 50% MeOH/CH_2Cl_2, $NaBH_4$. (c) 4 N HCl/EtOH.

or facilitated uptake via the polyamine transport system (PAT). In compound **8**, chloroambucil is conjugated to spermidine at the N^4 position of spermidine (Figure 1). However, we recently demonstrated that the N^5-alkylated analogue **9** resulted in a complete loss of PAT targeting/selectivity as compared to the N^1-substituted parent **1a**.[10] We speculated that the bioactivity and delivery profile of chlorambucil could be enhanced by introducing this DNA cross-linking motif at the N^1-position of homospermidine. This insight led us to synthesize compounds **10–13** to evaluate this strategy.

This report investigates both the role of the polyamine recognition element and the nature of the appended cytotoxic agent. We found that the disubstituted (polyamine-arylene-polyamine) motifs are excellent PAT targeting agents and have the highest reported CHOMG/CHO IC_{50} ratios (>2000). Moreover, the chloroambucil adducts were much less toxic than the corresponding anthracenylmethyl compounds, (e.g. **1a**). In this regard, having the proper recognition element (homospermidine) and cytotoxic cargo (e.g., xylene) were deemed paramount for successful drug delivery via the PAT.

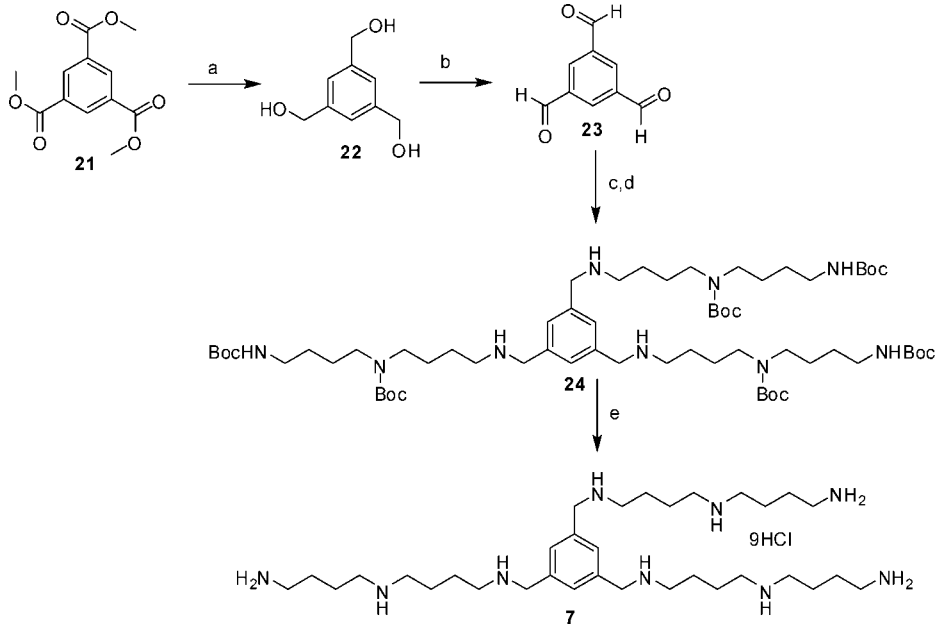
Results and Discussion

Synthesis. New conjugates **4–7** were synthesized to probe the effect of having two or more PAT recognition elements (i.e., homospermidine) within the same molecule. Compound **10** and its controls **11–13** were also synthesized to probe whether homospermidine could enhance the PAT-mediated delivery of the chlorambucil scaffold.

For the synthesis of compounds **4–6**, the diBoc-protected amine **14** was synthesized as reported earlier.[19] As shown in Scheme 1, reductive amination of the respective dialdehydes **15**, **16**, and **17** with amine **14** gave compounds **18**, **19**, and **20**, respectively. Boc removal with 4 N HCl provided the target conjugates, **4–6**.

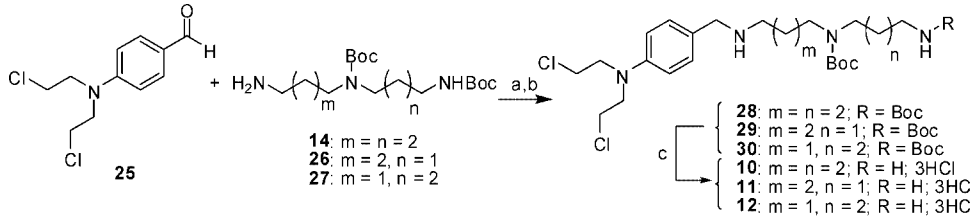
Compound **7** was synthesized from the commercially available benzene-1,3,5-tricarboxylic acid trimethyl ester **21** (Scheme 2), which was reduced to alcohol **22** using $LiAlH_4$ (78% yield). Further oxidation of alcohol **22** using PCC resulted in trialdehyde **23** (51%). Reductive amination of trialdehyde **23** with amine **14** gave the secondary amine **24**. However, the isolation of **24** was problematic due to the coelution of **14**. Therefore,

Scheme 2[a]



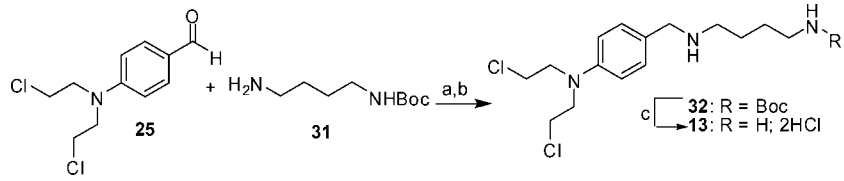
[a] Reagents: (a) $LiAlH_4$/THF. (b) PCC/CH_2Cl_2. (c) 25% MeOH/CH_2Cl_2; **14**. (d) 50% MeOH/CH_2Cl_2/$NaBH_4$. (e) 4 N HCl/EtOH.

Scheme 3[a]



[a] Reagents: (a) 25% MeOH/CH_2Cl_2. (b) 50% MeOH/CH_2Cl_2, $NaBH_4$. (c) 4 N HCl/EtOH.

Scheme 4[a]



[a] Reagents: (a) 25% MeOH/CH_2Cl_2. (b) 50% MeOH/CH_2Cl_2, $NaBH_4$. (c) 4 N HCl/EtOH.

the mixture of **14** and **24** was reacted with BOC-ON to selectively protect the primary amine **14**, which facilitated the purification of compound **24**. Once isolated, the Boc groups of **24** were removed using 4 N HCl to obtain compound **7**, albeit in low overall yield.

As shown in Scheme 3, compounds **10**–**12** were synthesized by reductive amination of aldehyde **25** with their respective di-Boc amines **14**, **26**, and **27** to give the secondary amines **28**, **29**, and **30**, respectively. Removal of the Boc group by 4 N HCl resulted in the compounds **10**−**12**, respectively. Similarly, reductive amination of **25** with putrescine analogue **31** gave the secondary amine **32**, which in turn gave compound **13** (Scheme 4).

Biological Evaluation. Once synthesized, the conjugates (**4**–**7** and **10**–**13**) were screened for cytotoxicity in L1210, CHO, and CHO-MG cells. L1210 cells were selected to enable comparisons with the published IC_{50} and K_i values for a variety of related polyamine substrates.[4–8] Chinese hamster ovary (CHO) cells were chosen along with a mutant cell line (CHO-MG) to comment on selective transport via the PAT.[6–8,12] The results are shown in Tables 1 and 2.

Table 1. L1210: K_i and Cytotoxicity Studies

compd	K_i (μM)	L1210 IC_{50} (μM)	L1210 + DFMO IC_{50} (μM)	L1210 + SPD IC_{50} (μM)
1a	1.8 ± 0.1	0.30 ± 0.04	0.09 ± 0.01	0.83 ± 0.09
1b	32.2 ± 4.3	6.30 ± 0.26	9.78 ± 0.42	6.96 ± 0.73[a]
2	3.8 ± 0.5	0.50 ± 0.03	0.43 ± 0.02	ND
3	4.5 ± 0.8	36.3 ± 8.4	ND	ND
4	0.39 ± 0.05	0.78 ± 0.07	0.22 ± 0.08	5.52 ± 0.09
5	0.17 ± 0.02	0.25 ± 0.08	ND	6.42 ± 0.80
6	0.52 ± 0.11	0.16 ± 0.01	0.36 ± 0.03	6.89 ± 0.42
7	0.49 ± 0.02	122.3 ± 8.1	49.3 ± 10.0	102.8 ± 10.9
10	5.97 ± 0.98	35.4 ± 1.6	ND	31.1 ± 2.0
11	12.4 ± 1.2	8.14 ± 0.46	ND	8.13 ± 0.47
12	2.66 ± 0.17	6.53 ± 0.42	ND	7.83 ± 0.35
13	75.0 ± 4.2	2.18 ± 0.15	ND	2.59 ± 0.13
CA	>>150	14.1 ± 0.5	ND	ND

[a] A 100 μM concentration of SPD was used.

L1210 K_i and IC_{50} Studies. The K_i values in Table 1 were determined for [^{14}C]spermidine uptake and reflect the affinity

Table 2. L1210 Cytotoxicity of **1a** in μM in the Absence or Presence of **7**

IC_{50} in the absence of **7**	IC_{50} in the presence of different concentrations of **7**		
1a	**1a** + **7**(5 μM)	**1a** + **7**(10 μM)	**1a** + **7**(20 μM)
0.31 ± 0.02	1.27 ± 0.06	1.06 ± 0.09	1.15 ± 0.08

of the polyamine derivative for the polyamine transport system on the cell surface. The IC_{50} values listed in Table 1 represent the concentration of the polyamine conjugate required to reduce the relative cell growth by 50%. With both parameters, one can determine whether high affinity for the transporter (e.g., low K_i value) translated into high cytotoxicity (e.g., low IC_{50} value).

A priori, one may have expected that conjugates with high PAT affinity would be transported efficiently into the cell by this transporter. However, prior results[10] showed that the K_i values were of minimal value in predicting the cytotoxicity and transport of these systems. Indeed, tetraamine PAT substrates with very low K_i values ($<<1$ μM) have been shown to actually inhibit their own import.[7] In the series of anthracene-triamine conjugates studied, substrates with moderate K_i values ($\sim$2 μM) were efficiently transported into cells via PAT.[6] In summary, the K_i values provided insight as to how alterations of polyamine structure influence the affinity of the polyamine conjugate for the PAT system but were often unreliable predictors of PAT selectivity.

Using competition experiments with radiolabeled spermidine, we were able to rank the binding affinity of the compounds for the PAT. As shown in Table 1, the monosubstituted adduct **1a** had a K_i value of 1.8 μM, whereas the K_i value was lowered to 0.39 μM for the disubstituted moiety **4**. A similar trend was observed in case of the naphthyl (**5**) and benzyl (**6**) derivatives. Inspection of compounds **1a**, **2**, and **3** shows the trend that as the size of the cargo decreases, the K_i value increases (anthracenyl > napthyl > benzyl: 1.8 < 3.8 < 4.5), indicating a lower affinity for PAT. Surprisingly, the same trend was not observed in the case of the disubstituted series (**4**–**6**). Naphthyl derivative **5** showed a lower K_i value than the anthracenyl (**4**) and benzyl (**6**) derivatives. Even the trisubstituted benzyl derivative **7** gave a lower K_i value than the monosubstituted derivatives, suggesting that the di- and trisubstituted analogues **4**−**6** and **7** all had significantly lower K_i values (than the triamines **1a**, **2**, and **3**, respectively) and were better PAT binding agents.

In the case of the chlorambucil derivatives **10**–**13**, high K_i values were observed, suggesting that these compounds have lower binding affinity for PAT.

An interesting trend was apparent in the L1210 IC_{50} values of the compounds **1a**, **2**, and **3** (Table 1). The respective IC_{50} value increased as the size of the cargo is decreased, for example, anthracenyl > naphthyl > benzyl: 0.30 < 0.50 < 36.3 μM. Surprisingly, the opposite trend was observed in compounds **4**–**6**, that is, anthracenyl > naphthyl > benzyl: 0.78 > 0.25 > 0.16 μM.

On the other hand, when each monosubstituted derivative (**1a**, **2**, and **3**) was compared with its corresponding disubstituted derivative (**4**–**6**), no general trend was observed. The IC_{50} increases 2.6 times (0.30 vs 0.78 μM) in the case of the anthracene derivatives (**1a** vs **4**). However, in the case of the naphthyl derivatives (**2** vs **5**), the IC_{50} was reduced in half (0.50 vs 0.25 μM). A significant difference was observed in the case of benzyl derivatives (**3** vs **6**: 36.3 vs 0.16 μM) and was previously rationalized by partial metabolism of **3** to free homospermidine.[8] In contrast, a significantly higher IC_{50} value was observed in the case of trisubstituted benzyl derivative **7**. In general, the disubstituted derivatives showed higher cytotoxicity in L1210 cells than either the trisubstituted analogue **7** or their monosubstituted derivatives, except in the case of **1a**.

The chlorambucil derivatives **10**–**13** were less toxic than **1a** and gave relatively higher L1210 IC_{50} values (Table 1). For example, although both the homospermidine analogue **10** (IC_{50} = 35.4 μM) and **1a** contained the same polyamine "message", **10** was significantly less toxic than the corresponding anthryl analogue **1a** (IC_{50} = 0.30 μM). Compounds **11** and **12** were designed to probe how the orientation of the spermidine motif affected bioactivity. While the K_i value of **12** was less than **11**, the observed IC_{50} values were similar (8.1 and 6.5 μM, respectively). The most toxic analogue of the CA series was derivative **13**, which contained an appended butanediamine motif and was later shown to not use the polyamine transport system for cellular entry.

Cytotoxicity experiments were also performed in murine leukemia cells (L1210, Table 1) with and without the presence of difluoromethylornithine (DFMO), a known inhibitor of ornithine decarboxylase (ODC), the enzyme responsible for polyamine biosynthesis. Typically, when the polyamine biosynthetic pathway is blocked, cells respond by increasing their import of extracellular polyamines via PAT. Therefore, if the conjugates are PAT-selective, one should see a lowering of their respective IC_{50} values in the presence of DFMO, a molecule that facilitates the conjugate's import. Indeed, as shown in Table 1, the IC_{50} values are all lower in the presence of DFMO, with the exception of **1b** and **6**. These observations are consistent with previous studies, wherein we observed an approximate halving of the IC_{50} value when the experiment is conducted in the presence of DFMO. This indicated that the PAT-selective drug was more potent in the presence of the ODC inhibitor, presumably due to its increased cell uptake.

Spermidine rescue experiments were also performed to test whether these drugs were using the PAT for cellular entry. Because spermidine (SPD, 200 μM) is a natural PAT substrate, it should outcompete the PAT-selective drug for cellular entry and "rescue the cell" from the cytotoxic drug. Indeed, exogenous spermidine is an antagonist for drug uptake and provided a significant "import-inhibition" effect with lower amounts of drug entering the cells after 24 h of incubation. This was observed as a general shifting of the cytotoxicity curve (i.e., a plot of % relative viability vs [drug] to the right and a corresponding increased IC_{50} value of the drug in the presence of spermidine). In general, PAT-selective drugs like **1a** resulted in a significant increase in IC_{50} value in the presence of SPD. Indeed, in the presence of SPD, the disubstituted xylene analogues **4**–**6** gave IC_{50} values 7, 26, and 43 times higher than the SPD-free assays (Table 1), respectively. This result indicated that they were excellent PAT ligands. This spermidine protection effect has been observed previously[4,5,12] and can be rationalized here by spermidine's competitive access to cells via the PAT.

In contrast, virtually no SPD protection was observed with **1b** and the chlorambucil derivatives **10**–**13**, suggesting that these drugs did not use the PAT for cellular entry. In this regard, the IC_{50} values remained virtually unchanged even in the presence of excess SPD (200 μM).

CHO and CHO-MG Studies. CHO cells were chosen along with a mutant cell line (CHO-MG) to comment on how the synthetic conjugates gain access to cells.[6–8,12] The CHO-MG cell line was polyamine transport-deficient and was isolated after selection for growth resistance to methylglyoxalbis(guanylhydrazone), MGBG, ($CH_3C[=N-NHC(=NH)NH_2]CH[=N-$

NHC(=NH)NH_2]) using a single-step selection after mutagenesis with ethylmethanesulfonate.[14,15]

For the purposes of this study, the CHO-MG cell line represents cells with no PAT activity and provided a model for alternative modes of entry or action, which are independent of PAT. These alternative modes of entry include passive diffusion or utilization of another transporter. The alternative modes of action may also include interactions on the outer surface of the plasma membrane or other membrane receptor interactions.

In contrast, the parent CHO cell line represents a cell type with high PAT activity[14,15] and should be very vulnerable to PAT targeting drugs. Therefore, highly selective PAT ligands should give high (CHO-MG/CHO) IC_{50} ratios.

As reported earlier,[10] dramatic differences in CHO and CHO-MG cytotoxicity (Table 2) were observed with **1a** and **2** (e.g., CHOMG/CHO IC_{50} ratio of **1a**: 148). The benzyl analogue **3** was shown to be partially metabolized into free homospermidine. As such, **3** had an IC_{50} value >1000 μM in both CHO and CHO-MG cell lines.[8]

The disubstituted compounds (anthryl **4**, naphthyl **5**, and benzyl **6**) gave the highest CHO-MG/CHO IC_{50} ratios ever published and were >2222, >833, and 677, respectively. This unprecedented PAT selectivity confirmed that these disubstituted derivatives have extremely high selectivity toward PAT-active CHO cells. Moreover, there was a direct correlation between the derivative's PAT selectivity and the size of the arylene spacer group, wherein the larger anthracenyl ring system in **4** had the highest PAT selectivity.

Unfortunately, the selectivity of the trisubstituted compound **7** was not determined due to its low cytotoxicity in CHO and CHO-MG cells (e.g., CHO IC_{50} >500 μM). However, it may have a high affinity for binding to PAT, especially in light of its low L1210 K_i value (which was similar to **6**) and the expected halving of the L1210 IC_{50} value upon DFMO treatment (Table 1). These observations coupled with the fact that spermidine was unable to rescue L1210 cells from **7** suggests that it may be binding to PAT but not using PAT for its cellular entry. It is reported in the literature that multisubstituted polyamines are PAT inhibitors.[20] Furthermore, to see the PAT inhibitor effect of **7**, another experiment was conducted. L1210 cells were dosed with **1a** in the presence of **7** with the concept that if **7** acts as an inhibitor of PAT, it would rescue the cells from **1a**. Indeed, significant rescue was observed in the presence of **7** with a 4-fold increase in IC_{50} values [IC_{50} **1a**, 0.31 ± 0.02 μM; IC_{50} **1a** + **7** (5 μM), 1.27 ± 0.06 μM]. The L1210 cytotoxicity curves of compound **1a** were determined with different concentrations of **7**. As shown in Table 2, significant rescue was observed at all concentrations of **7** tested (5, 10, and 20 μM). These are statistically significant results. Both the PAT-binding affinity of **7** (low K_i value) and its ability to protect the cells from **1a** make **7** a relatively nontoxic inhibitor of the PAT. Indeed, a nontoxic PAT inhibitor may find use in future biochemical studies of polyamine transport.

The chlorambucil derivatives **10**–**12** were nontoxic to both CHO and CHO-MG cells (IC_{50} > 100 μM), and their respective CHO-MG/CHO IC_{50} ratios were not determined. However, the fact that spermidine was unable to rescue L1210 cells from these drugs suggest that they do not use the PAT for cellular entry. Indeed, **13**, which was the only toxic member of the chlorambucil series, had a CHO-MG/CHO ratio of 0.6, confirming that it was not PAT-selective (Table 3). The low toxicity associated with the chlorambucil derivatives may be due to the instability of the *N*-(2-chloroethyl)amino group at physiological pH. It is reported in the literature that *N*-(2-chloroethyl)amines cyclize at neutral pH to produce aziridines.[21] To investigate this issue, an NMR experiment was conducted in a phosphate buffer at pD 7.4 in D_2O. After 48 h of incubation at 37 °C, the chlorambucil conjugate **10** was 89% degraded by the solvent, which explains the low cytotoxicity of the chlorambucil derivatives. The parent chlorambucil (CA) had a CHO IC_{50} = 111.2 μM and a CHO-MG/CHO ratio of 1.32, which also suggests non-PAT-mediated import.

Table 3. CHO Cytotoxicity Studies

compd	CHOMG IC_{50} (μM)	CHO IC_{50}(μM)	CHOMG/CHO IC_{50} ratio
1a	66.7 ± 4.1	0.45 ± 0.10	148
1b	7.6 ± 0.4	7.7 ± 0.5	1
2	>100	0.6 ± 0.2	>164
3	>1000	>1000	NA[a]
4	>100	0.045 ± 0.003	>2222
5	>100	0.12 ± 0.03	>833
6	50.2 ± 3.8	0.074 ± 0.010	677
7	>500	>500	NA[a]
10	>>100	>>100	1
11	>100	>100	1
12	>100	>100	1
13	17.1 ± 1.5	28.5 ± 1.0	0.6
CA	111.2 ± 4.6	84.5 ± 11.9	1.32

[a] NA, not applicable.

Table 4. IL-3-Dependent FL5.12A B Cells: Apoptosis via Annexin-V Staining (% Apoptosis)[a]

	%				
IL-3 (ng/mL)	UNTR	AG	**1a**	**1b**	**4**
2	3.38	5.17	67.71	>95	5.12
0.1	3.71	3.72	80.41	>95	6.99
0	44.71	79.69	>95	>95	16.39

[a] UNTR (untreated); AG (aminoguanidine). Representative data for the apoptotic effect of compounds **1a**, **1b**, and **4** on FL5.12A cells; experiments were done in triplicate with a margin error of 2%.

IL-3-Dependent Pro-B Cell Line Studies. Having established that the disubstituted compound **4** was extremely PAT-selective, we evaluated it along with **1a** and putrescine analogue **1b** in a cytokine-dependent B cell line. It is known that lymphomas frequently result from molecular abnormalities in the pathways that regulate the proliferation and apoptosis of lymphocytes. The function of polyamine transport in this process is poorly understood. Because polyamines are essential compounds for B cell growth and B cells are known to express PATs,[22] we examined the effect of compounds **1a**, **1b**, and **4** on an IL-3-dependent pro-B cell line, FL5.12A.[23] FL5.12A B cells were cultured for 48 h in three different concentrations of IL-3: 2 ng/mL, which induces cell cycle progression and inhibits apoptosis; 0.1 ng/mL, which causes cell cycle arrest but prevents apoptosis; and 0 ng/mL (absence of IL-3), which causes cell cycle arrest and induces apoptosis. During the last 24 h, compound **1a**, **1b**, or **4** (10 μM) was added in the presence of aminoguanidine (1 mM). Apoptosis was assessed by measuring the binding of annexin-V-FITC to phosphatidylserine exposed on the plasma membrane of apoptotic cells. The initial results are shown in Table 4 and demonstrate that compound **1b** nonspecifically induced apoptosis. Even though compounds **1a** and **4** were PAT-selective, they have different abilities to induce apoptosis. Compound **1a** induced apoptosis in parallel with decreasing amounts of IL-3, while compound **4** did not induce apoptosis.

To examine specific polyamine transport, we performed a spermidine rescue experiment and observed that the apoptotic-inducing abilities of compounds **1a** and **1b** were reduced in the presence of spermidine. The fact that this effect was evident with compound **1b**, which was lethal at this concentration, might

Table 5. IL-3-Dependent FL5.12A B Cells: Spermidine Competition Assay via Annexin-V Staining (% Apoptosis)[a]

	%					
IL-3 (ng/mL)	UNTR	SPD	**1a**	**1a** + SPD	**1b**	**1b** + SPD
2	5.33	5.52	67.71	37.30	>95	47.28
0.1	4.42	6.07	80.41	33.87	>95	35.59
0	55.89	58.16	>95	46.20	>95	35.32

[a] UNTR (untreated); SPD (spermidine). Representative data for the apoptotic effect of compounds **1a** and **1b** on FL5.12A cells in spermidine competition assay; experiments were done in duplicate with a margin error of 5%.

Table 6. IL-3-Dependent FL5.12A B Cells: DMSO Shock Treatment Assay via Annexin-V Staining (% Apoptosis)[a]

	%				
IL-3 (ng/mL)	UNTR	DMSO	**4**	**4** + DMSO	**4** + DMSO + SPD
2	8.59	7.54	5.56	44.71	12.89
0.1	4.94	6.29	6.07	56.71	10.20
0	61.85	1.82	8.64	67.53	16.69

[a] UNTR (untreated); DMSO (dimethyl sulfoxide); SPD (spermidine). Representative data for the apoptotic effect of compound **4** on FL5.12A after DMSO shock; experiments were done in duplicate with an error margin of 6%.

be due to the presence of a B cell polyamine transport system, which may also recognize 1,4-diaminobutane (putrescine). Different results in B and CHO cells were observed because their vesicular trafficking patterns are different.[24a] Other PATs have been identified in *Escherichia coli*, which are selective for both putrescine[24b] and spermidine.[24c] PAT-selective compound **1a** also showed rescue in the presence of spermidine, even in cells with different cell cycling behavior (initiated via the corresponding IL-3 dosage). Together, the results from Tables 4 and 5 suggest that targeting PAT in B cells for the delivery of toxic compounds is feasible.

The highly PAT-selective compound **4** did not induce significant apoptosis in FL5.12A cells (Table 4). A provocative model for polyamine transport was recently published by Poulin et al.,[25,26] who described initial transport through a plasma membrane transporter followed by sequestration of polyamine conjugates into polyamine sequestering vesicles. One possibility for the low toxicity of compound **4** is that it remains trapped in vesicles and does not reach its cellular target to initiate the apoptotic response.

To examine this hypothesis, we exposed FL5.12A cells to a brief DMSO shock, to release vesicular contents[27a,b,c] and presumably release compound **4** from the vesicle compartments. After 4 h, we observed rapid cell death only in cells that received compound **4** and underwent DMSO shock (Table 6). Note that DMSO shock alone was antiapoptotic, likely due to its antioxidant effects.[27a,b,c] Additionally, competition with spermidine reversed the toxic effects of compound **4**-treated cells that were shocked with DMSO (Table 6). These results indicate that B cells can be targeted through PAT and that the PAT-selective compounds likely reside in vesicles and must escape from these vesicles to deliver their toxic cargo to cells. Indeed, rapid vesicle sequestration has been observed with compound **1a**[8] and other polyamine derivatives in other mammalian cell types.[25,26] As mentioned previously, B cells and CHO cells have different cell types with different vesicular trafficking, which explains the different results. For example, late endosome trafficking does occur in CHO[28] but does not occur until antigen activation in B cells.[24a]

Conclusions

Di- and trisubstituted polyamine derivatives were synthesized and evaluated for their ability to target the polyamine transport system in L1210 murine leukemia, two CHO cell lines (via CHO-MG/CHO IC_{50} ratios), and a cytokine-dependent pro-B cell line. Both K_i and IC_{50} values were determined in L1210 cells for comparison to previous published polyamine analogues. The disubstituted compounds **4**−**6** represent the next generation and a major advance in terms of PAT-selective agents. These materials have similar cytotoxicities to the original triamine series (**1a**, **2**, and **3**) in L1210 cells but are significantly more selective in terms of entering cells with an active polyamine transport system (e.g., CHO cells and FL5.12A cells). This is represented by their dramatic increase in potency in the CHO wild-type cell line over their monosubstituted counterparts (**1a**, **2**, and **3**).

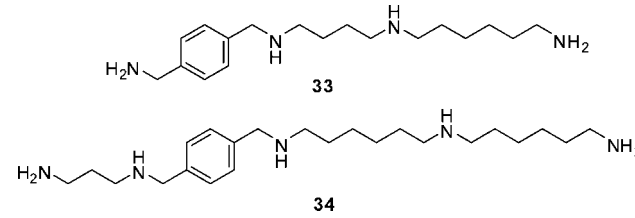


Figure 2. Structured of antizyme inhibitors.

During the preparation of this manuscript, Petros et al.[29] described the synthesis and antizyme-inducing effects of compounds **33** and **34** (Figure 2). Antizyme is an inhibitor of ODC, and its induction leads to reduced polyamine transport and may lead to apoptosis. Because **33** and **34** have similar structures to compounds **4**−**6**, it is possible that these new disubstituted materials are also antizyme-inducing agents. Indeed, high levels of antizyme are consistent with apoptosis, that is, programmed cell death, which has been observed with these compounds (e.g., **1a**). Future efforts will focus on pursuing this antizyme induction hypothesis.

Experimental Section

Materials. Silica gel (32–63 μm) and chemical reagents were purchased from commercial sources and used without further purification. All solvents were distilled prior to use. All reactions were carried out under an N_2 atmosphere. ^1H and ^{13}C spectra were recorded at 300 or 75 MHz, respectively. TLC solvent systems were listed as volume percents, and NH_4OH referred to concentrated aqueous NH_4OH. All tested compounds provided satisfactory elemental analyses. For compounds **4** and **6**, the HRMS data is reported for the double charged species (z = 2) and represent half the theoretical MW (m/z).

Biological Studies. Murine leukemia cells (L1210), CHO, and CHO-MG cells were grown in RPMI medium supplemented with 10% fetal calf serum, glutamine (2 mM), penicillin (100 U/mL), and streptomycin (50 μg/mL). Note that the media must contain L-proline (2 μg/mL) for proper growth of the CHO-MG cells. Cells were grown at 37 °C under a humidified 5% CO_2 atmosphere. Aminoguanidine (2 mM) was added to the culture medium to prevent oxidation of the drugs by the enzyme (bovine serum amine oxidase) present in calf serum. Trypan blue staining was used to determine cell viability before the initiation of a cytotoxicity experiment. L1210 cells in early to mid log phase were used.

IC_{50} Determinations. Cell growth was assayed in sterile 96 well microtiter plates (Becton-Dickinson, Oxnard, CA). L1210 cells were seeded at 6×10^4 cells/mL of medium (100 μL/well). CHO and CHO-MG cells were plated at 1×10^4 cells/mL. Drug solutions (10 μL per well) of appropriate concentration were added at the time of seeding for L1210 cells and after an overnight incubation for the other cells. After exposure to the drug for 48 h, cell growth was determined by measuring formazan formation from 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium using a Titertek Multiskan MCC/340 microplate reader for absorbance (540 nm) measurements.[30]

K_i Procedure. The ability of the conjugates to interact with the polyamine transport system was determined by measuring competition between the conjugates and the radiolabeled spermidine for uptake in L1210 cells. This procedure was used to obtain the data listed in Table 1. Initially, the K_m value of spermidine transport was determined as previously described.[31]

The ability of conjugates to compete for [^{14}C]spermidine uptake was determined in L1210 cells by a 10 min uptake assay in the presence of increasing concentrations of competitor, using 1 μM [^{14}C]spermidine as substrate. K_i values for inhibition of spermidine uptake were determined using the Cheng−Prusoff equation[32] from the IC_{50} value derived by iterative curve fitting of the sigmoidal equation describing the velocity of spermidine uptake in the presence of the respective competitor.[33] L1210 cells were grown and maintained according to established procedures[34] and were washed twice in HBSS prior to the transport assay.

B Cell Culture. FL5.12A cells were a kind gift from James McCubrey, East Carolina University (Greenville, SC). Cells were cultured in RPMI1640, supplemented with 10% heat-inactivated fetal calf serum (FCS), antibiotics (penicillin, 100 units/mL; streptomycin sulfate, 100 μg/mL), and IL-3 (2, 0.1, or 0 ng/mL) at 37 °C, in an atmosphere of 95% air and 5% CO_2 under humidified conditions. Aminoguanidine (1 mM) was added as an inhibitor of amine oxidase derived from FCS and had no effect on the various parameters of the cell measured in this study.

Annexin-V Staining. Apoptosis was assessed using the Annexin-V-FITC Apoptosis Detection Kit I (BD Biosciences). FL5.12A cells were cultured in 25 cm^2 flasks and preincubated for 24 h with different concentrations of IL-3 in 24 well plates (5×10^6 cell/well) and then treated with 10 μM concentrations of compounds **1a**, **1b**, and **4** along with a 10 μM concentration of aminoguanidine. After 24 h of incubation, cells were washed twice with ice-cold PBS and then stained with Annexin-V using the Annexin-V-FITC Apoptosis Detection Kit I following the manufacturer's protocol (BD Biosciences). FITC fluorescence was detected using a BD FACSCalibur flow cytometer. Ten thousand events were acquired in each sample. The percents listed in Tables 3−5 are % annexin V-stained cells out of the total population. This directly corresponded to % of cells in apoptosis.

Spermidine Competition Assay in B Cells. FL5.12A cells were cultured in 25 cm^2 flasks and then incubated in 24 well plates (5×10^6 cell/well) with different concentrations of IL-3 for 24 h; the cells were treated with the indicated concentration of compounds **1a**, **1b**, and **4** overnight in the presence or absence of SPD (100 μM), and cell apoptosis was assessed by annexin-V staining as described before.

DMSO Shock Assay in B Cells. FL5.12A cells were cultured in 25 cm^2 flasks and then incubated in 24 well plates (5×10^6 cell/well) with different concentrations of IL-3 for 24 h; cells were then treated with compound **4** overnight in the presence or absence of SPD (100 μM) and then were washed and incubated in 10% DMSO for 2 min, washed twice with Hank's media, and incubated for 2 h at 37 °C. Apoptosis was assessed with annexin-V-FITC staining as previously described.

General Procedure for Removal of BOC Group. The respective solution of the BOC-protected compound dissolved in absolute ethanol (13 mL) was stirred at 0 °C for 10 min. A 4 N HCl solution (22 mL) was added dropwise to the reaction mixture and stirred at 0 °C for 20 min and then at room temperature overnight. The solution was concentrated in vacuo to give the respective amine HCl salt as a solid.

***N*-(4-Amino-butyl)-*N*′-(10-{[4-(4-amino-butylamino)butylamino]-methyl}anthracen-9-ylmethyl)butane-1,4-diamine, Hydrochloride Salt, 4.** Yellow solid; yield, 95%. ^{1}H NMR (D_2O): δ 8.19 (d, 4H), 7.79 (d, 4H), 4.83 (s, 4H), 3.30 (t, 4H), 3.11 (m, 12H), 1.79 (m, 16H). ^{13}C NMR (D_2O): δ 129.7, 127.7, 124.7, 124.0, 47.7, 47.2, 47.1, 42.8, 39.0, 24.2, 23.2, 23.1. HRMS (FAB) calcd for $C_{32}H_{52}N_6 \cdot 6HCl$ [(M + 2H − 6HCl)/2]$^+$, 261.2199; found, 261.2199.

***N*-(4-Amino-butyl)-*N*′-(4-{[4-(4-amino-butylamino)butylamino]-methyl}naphthalen-1-ylmethyl)butane-1,4-diamine, Hydrochloride Salt, 5.** Yellow solid; yield, 94%. ^{1}H NMR (D_2O): δ 8.20 (m, 2H), 7.78 (m, 2H), 7.72 (s, 2H), 4.83 (s, 4H), 3.28 (t, 4H), 3.11 (t, 8H), 3.04 (t, 4H), 1.85–1.73 (m, 16H). ^{13}C NMR (D_2O): δ 131.3, 129.3, 128.6, 128.0, 123.8, 48.2, 47.3, 47.2, 47.1, 39.0, 24.2, 23.2, 23.1. HRMS (FAB) calcd for $C_{28}H_{50}N_6 \cdot 6HCl$ (M + H − 6HCl)$^+$, 471.4170; found, 471.4155.

***N*-(4-Amino-butyl)-*N*′-(4-{[4-(4-amino-butylamino)butylamino]-methyl}benzyl)butane-1,4-diamine, Hydrochloride Salt, 6.** White solid; yield, 93%. ^{1}H NMR (D_2O): δ 7.59 (m, 4H), 4.30 (s, 4H), 3.17 (t, 4H), 3.11 (t, 8H), 3.05 (t, 4H), 1.78 (m, 16H). ^{13}C NMR (D_2O): δ 132.4, 130.9, 64.3, 51.1, 47.4, 47.3, 47.1, 39.3, 24.5, 23.4, 23.3. HRMS (FAB) calcd for $C_{24}H_{48}N_6 \cdot 6HCl$ [(M + 2H − 6HCl)/2]$^+$, 211.2043; found, 211.2043.

***N*-(4-Amino-butyl)-*N*-(3,5-bis-{[4-(4-amino-butylamino)butylamino]-methyl}benzyl)butane-1,4-diamine, Hydrochloride Salt, 7.** White soild; yield, 97%. ^{1}H NMR (300 MHz, D_2O): δ 7.64 (s, 3H, aromatic), 4.32 (s, 6H, CH_2), 3.18 (t, 6H, CH_2), 3.07 (m, 18H, CH_2), 1.77 (m, 24H, CH_2). ^{13}C NMR (D_2O): δ 135.6, 135.1, 53.2, 49.8, 49.7, 41.7, 26.9, 25.8, 25.7. HRMS (FAB) *m*/*z* calcd for $C_{33}H_{78}N_9Cl_9$ (M + H − 9HCl)$^+$, 592.5749; found, 592.5749.

***N*-(4-Amino-butyl)-*N*′-{4-[bis-(2-chloro-ethyl)amino]benzyl}-butane-1,4-diamine, Hydrochloride Salt, 10.** White solid; yield, 96%. ^{1}H NMR (300 MHz, D_2O): δ 7.41 (d, 2H, aromatic), 7.05 (d, 2H, aromatic), 4.16 (s, 2H, CH_2), 3.87 (t, 4H, CH_2), 3.72 (t, 4H, CH_2), 3.06 (m, 8H, CH_2), 1.75 (m, 8H, CH_2). ^{13}C NMR (D_2O): δ 131.7, 114.7, 53.7, 50.7, 47.1, 47.1, 46.1, 42.1, 40.4, 39.0, 24.2, 23.1, 23.0. HRMS (FAB) *m*/*z* calcd for $C_{19}H_{37}N_4Cl_5$ (M + H − 3HCl)$^+$, 389.2233; found, 389.2235.

***N*-(3-Amino-propyl)-*N*′-{4-[bis-(2-chloro-ethyl)amino]benzyl}-butane-1,4-diamine, Hydrochloride Salt, 11.** White solid; yield, 93%. ^{1}H NMR (300 MHz, D_2O): δ 7.47 (d, 2H, aromatic), 7.17 (d, 2H, aromatic), 4.20 (s, 2H, CH_2), 3.92 (t, 4H, CH_2), 3.70 (t, 4H, CH_2), 3.19–3.05 (m, 8H, CH_2), 2.09 (q, 2H, CH_2), 1.78 (m, 4H, CH_2). ^{13}C NMR (D_2O): δ 143.2, 132.2, 131.9, 124.5, 116.8, 55.1, 50.6, 47.3, 46.3, 44.8, 39.8, 36.8, 24.0, 23.1, 23.0. HRMS (FAB) *m*/*z* calcd for $C_{18}H_{35}N_4Cl_5$ (M + H − 3HCl)$^+$, 375.2080; found, 375.2069.

***N*^{1}-(3-{4-[Bis-(2-chloro-ethyl)amino]benzylamino}propyl)butane-1,4-diamine, Hydrochloride Salt, 12.** White solid; yield, 91%. ^{1}H NMR (300 MHz, D_2O): δ 7.44 (d, 2H, aromatic), 7.11 (d, 2H, aromatic), 4.21 (s, 2H, CH_2), 3.89 (t, 4H, CH_2), 3.71 (t, 4H, CH_2), 3.19–3.07 (m, 6H, CH_2), 3.03 (t, 2H, CH_2), 2.12 (q, 2H, CH_2), 1.76 (m, 4H, CH_2). ^{13}C NMR (D_2O): δ 144.6, 131.8, 122.4, 115.5, 54.2, 50.9, 47.3, 44.7, 43.9, 40.2, 39.0, 24.2, 23.0, 22.9. HRMS (FAB) *m*/*z* calcd for $C_{18}H_{35}N_4Cl_5$ (M + H − 3HCl)$^+$, 375.2077; found, 375.2066.

***N*^{1}-{4-[Bis-(2-chloro-ethyl)amino]benzyl}butane-1,4-diamine, Hydrochloride Salt, 13.** White solid; yield, 96%. ^{1}H NMR (300 MHz, D_2O): δ 7.50 (d, 2H, aromatic), 7.21 (d, 2H, aromatic), 4.22 (s, 2H, CH_2), 3.93 (t, 4H, CH_2), 3.71 (t, 4H, CH_2), 3.11 (t, 2H, CH_2), 3.04 (t, 2H, CH_2), 1.77 (m, 4H, CH_2). ^{13}C NMR (D_2O): δ 142.9, 131.9, 124.8, 117.0, 55.2, 50.6, 46.4, 39.7, 39.0, 24.3, 23.0. HRMS (FAB) *m*/*z* calcd for $C_{15}H_{27}N_3Cl_4$ (M + H − 2HCl)$^+$, 318.1501; found, 318.1495.

General Procedure for Reductive Amination in the Synthesis of Disubstituted Derivatives. To a stirred solution of **14** (2.5 mmol) in 25% MeOH/CH_2Cl_2 (20 mL) was added a solution of respective dicarbaldehyde (1 mmol) in 25% MeOH/CH_2Cl_2 (15 mL) under N_2. The mixture was stirred at room temperature overnight until the imine formation was complete [as monitored by the disappearance of the ^{1}H NMR ($CDCl_3$) signal of aldehyde]. The solvent was removed in vacuo, the solid residue was dissolved in 50% MeOH/CH_2Cl_2 (40 mL), and the solution was cooled to 0 °C. $NaBH_4$ (6 equiv) was added in small portions to the solution, and the mixture was stirred at rt overnight. The solvent was removed in vacuo, and the solid residue was dissolved in CH_2Cl_2 (50 mL) and washed with 10% aqueous Na_2CO_3 solution (3 × 30 mL). The CH_2Cl_2 layer was separated, dried over anhydrous Na_2SO_4, filtered, and removed in vacuo to give an oily residue. The oil was purified by flash column chromatography to yield the product.

(4-*tert*-Butoxycarbonylamino-butyl)-(4-{[10-({4-[*tert*-butoxycarbonyl-(4-*tert*-butoxycarbonylamino-butyl)amino]butylamino}methyl)anthracen-9-ylmethyl]amino}butyl)carbamic Acid *tert*-Bu-

tyl Ester, 18. Pale yellow viscous oil (92%), $R_f = 0.3$ (5% MeOH/0.5% NH_4OH/CH_2Cl_2). ^1H NMR ($CDCl_3$): δ 8.36 (d, 4H), 7.50 (d, 4H), 4.82 (br m, 2H), 4.68 (s, 4H), 3.13 (m, 12H), 2.87 (t, 4H), 1.65–1.30 (m, 52H). ^{13}C NMR ($CDCl_3$): δ 155.9, 155.4, 131.9, 129.9, 125.6, 124.8, 79.1, 78.9, 53.5, 50.3, 46.9, 46.7, 46.0, 40.2, 28.6, 28.5, 27.5. HRMS (FAB) *m/z* calcd for $C_{52}H_{84}N_6O_8$ $(M + H)^+$, 921.6423; found, 921.6414.

(4-*tert*-Butoxycarbonylamino-butyl)-(4-{[4-({4-[*tert*-butoxycarbonyl-(4-*tert*-butoxycarbonylamino-butyl)amino]butylamino}-methyl)naphthalen-1-ylmethyl]amino}butyl)carbamic Acid *tert*-Butyl Ester, 19. Pale yellow viscous oil (60%), $R_f = 0.3$ (7% MeOH/0.5% NH_4OH/CH_2Cl_2). ^1H NMR ($CDCl_3$): δ 8.12 (m, 2H), 7.50 (m, 2H), 7.38 (s, 2H), 4.87 (br m, 2H), 4.19 (s, 4H), 3.10 (m, 12H), 2.73 (t, 4H), 1.65–1.35 (m, 52H). ^{13}C NMR ($CDCl_3$): δ 155.9, 155.4, 135.3, 132.0, 125.7, 125.4, 124.2, 79.1, 51.7, 49.7, 46.9, 46.7, 40.2, 28.5, 28.5, 27.4, 26.7, 26.1, 25.8. HRMS (FAB) *m/z* calcd for $C_{48}H_{82}N_6O_8$ $(M + H)^+$, 871.6267; found, 871.6211.

(4-*tert*-Butoxycarbonylamino-butyl)-{4-[4-({4-[*tert*-butoxycarbonyl-(4-*tert*-butoxycarbonylamino-butyl)amino]butylamino}-methyl)benzylamino]butyl}carbamic Acid *tert*-Butyl Ester, 20. Pale yellow viscous oil (54%), $R_f = 0.38$ (6% MeOH/0.5% NH_4OH/CH_2Cl_2). ^1H NMR ($CDCl_3$): δ 7.17 (m, 4H), 4.83 (br m, 2H), 3.68 (s, 4H), 3.06 (m, 12H), 2.55 (m, 4H), 1.60–1.21 (m, 52H). ^{13}C NMR ($CDCl_3$): δ 155.9, 155.4, 138.8, 128.1, 126.8, 79.1, 78.8, 64.3, 53.5, 49.0, 46.9, 46.7, 40.2, 28.5, 28.5, 27.5, 27.3, 26.6, 26.0, 25.6. HRMS (FAB) *m/z* calcd for $C_{44}H_{80}N_6O_8$ $(M + H)^+$, 821.6110; found, 821.6083.

1,3,5-Tris(hydroxymethyl)benzene, 22. Trimethyl-1,3,5-benzenetricarboxylate **21** (2 g, 7.9 mmol, Acros Chemicals) in dry THF (30 mL) was added through a pressure-equalized addition funnel into a 250 mL flask containing $LiAlH_4$ (0.90 g, 23.6 mmol) in dry THF (65 mL) at 0 °C under a N_2 atmosphere. The mixture was allowed to warm to room temperature and stirred for 4 h. The reaction was quenched by the slow addition of a 1:1 mixture of Celite and $KHSO_4$. The suspension was filtered, and the Celite was washed with MeOH (100 mL). The solvent was removed under reduced pressure, and triol **22** was obtained in 78% yield (1.05 g). ^1H NMR of the product matched that of the authentic material. ^1H NMR (300 MHz, DMSO): δ 7.18 (s, 3H), 5.17 (br s, 3H), 4.50 (s, 6H).

1,3,5-Triformyl Benzene, 23. 1,3,5-Tris(hydroxymethyl)benzene **22** (1.05 g, 6.25 mmol) was suspended in CH_2Cl_2 (25 mL), and solid pyridinium chlorochromate (PCC, 5.98 g, 27.74 mmol) was added. After 30 min of stirring, the reaction mixture was diluted with acetone (10 mL) and was allowed to stir for 3 h. The precipitated chromium salts were filtered off and washed with CH_2Cl_2. The organic phase was washed with a saturated solution of aqueous Na_2CO_3 three times, then separated and dried over anhydrous Na_2SO_4, filtered, and concentrated to provide a solid. Column chromatography (100% CH_2Cl_2) afforded **23** as white crystals (0.51 g, 51%). ^1H NMR (300 MHz, $CDCl_3$): δ 10.21 (s, 3H, CHO), 8.66 (s, 3H, aromatic).

{4-[3,5-Bis-({4-[*tert*-butoxycarbonyl-(4-*tert*-butoxycarbonyl-amino-butyl)amino]butylamino}methyl)benzylamino]butyl}-(4-*tert*-butoxycarbonylamino-butyl)carbamic Acid *tert*-Butyl Ester, 24. 1,3,5-Triformyl benzene **23** (0.180 g, 1.11 mmol) was dissolved in 25% $MeOH/CH_2Cl_2$ (10 mL). A solution of Boc-protected homospermidine **14** (1.44 g, 4.011 mmol) in 25% $MeOH/CH_2Cl_2$ (10 mL) was added via an addition funnel. The reaction mixture was stirred overnight under a N_2 atmosphere. Loss of the starting material was monitored via ^1H NMR spectroscopy and the disappearance of the aldehyde proton at 10.21 ppm. Upon conversion of the starting material, the solvent was removed in vacuo and the crude material was redissolved in a solution of 50% MeOH/CH_2Cl_2. To this new solution was added $NaBH_4$ (0.45 g, 11.9 mmol) at 0 °C. The solution was stirred overnight under a N_2 atmosphere. The solvent was removed in vacuo, and flash column chromatography (1% NH_4OH/5% CH_3OH/CH_2Cl_2) provided a mixture of the coeluting BOC-protected homospermidine **14** and the desired product **24** (1.08 g). To aid in the chromatographic separation of **24** and **14**, another reaction was carried out. The mixture was dissolved in THF (45 mL) and stirred for 20 min at 0 °C. A solution of 2-(*tert*-butoxycarbonyloyimino)-2-phenylacetonitrile (BOC-ON, 0.23 g, 0.93 mmol) was added dropwise with constant stirring. After the addition was complete, the reaction was stirred for 2 h at 0 °C under a N_2 atmosphere. Upon completion, the solution was concentrated in vacuo, and the residue was redissolved in CH_2Cl_2 and washed with a saturated aqueous Na_2CO_3. The organic layer was separated, dried over anhydrous Na_2SO_4, filtered, and concentrated. Flash column chromatography of the residue gave pure **24** as a colorless oil (79 mg). Yield, 6%; $R_f = 0.35$ (1% NH_4OH/6.5% CH_3OH/CH_2Cl_2). ^1H NMR (300 MHz, $CDCl_3$): δ 7.14 (s, 3H, aromatic), 4.69 (s, 3H, NH), 3.75 (s, 6H, CH_2), 3.15 (m, 18H, CH_2), 2.65 (t, 6H, CH_2), 1.65- 1.15 (m, 78H, CH_2, CH_3). ^{13}C NMR ($CDCl_3$): δ 156.0, 155.6, 140.5, 126.7, 79.3, 79.2, 54.1, 49.5, 47.1, 46.9, 40.4, 28.7, 28.6, 27.6, 27.5, 26.8, 26.1, 25.8. HRMS (FAB) *m/z* calcd for $C_{63}H_{117}O_{12}N_9$ $(M + H)^+$, 1192.8894; found, 1192.9008.

General Procedure for Reductive Amination in the Synthesis of Chlorambucil Derivatives. To a stirred solution of the respective Boc-protected amine (1.5 mmol) in 25% $MeOH/CH_2Cl_2$ (20 mL) was added a solution of commercially available aldehyde **25** (1 mmol) in 25% $MeOH/CH_2Cl_2$ (15 mL) under N_2. The mixture was stirred at room temperature overnight until the imine formation was complete [as monitored by the disappearance of the ^1H NMR ($CDCl_3$) aldehyde signal]. The solvent was removed in vacuo, the solid residue was dissolved in 50% $MeOH/CH_2Cl_2$ (40 mL), and the solution was cooled to 0 °C. $NaBH_4$ (3 equiv) was added in small portions to the solution, and the mixture was stirred at rt overnight. The solvent was removed in vacuo, and the solid residue was dissolved in CH_2Cl_2 (50 mL) and washed with 10% aqueous Na_2CO_3 solution (3 × 30 mL). The CH_2Cl_2 layer was separated, dried over anhydrous Na_2SO_4, filtered, and removed in vacuo to give an oily residue. The oil was purified by flash column chromatography to yield the product.

(4-{4-[Bis-(2-chloro-ethyl)amino]benzylamino}butyl)-(4-*tert*-butoxycarbonyl-amino-butyl)carbamic Acid *tert*-Butyl Ester, 28. Pale yellow viscous oil (91%); $R_f = 0.5$ (6% MeOH/0.5% NH_4OH/CH_2Cl_2). ^1H NMR ($CDCl_3$): δ 7.19 (d, 2H), 6.63 (d, 2H), 5.05 (brs, 1H), 3.69 (m, 6H), 3.61 (m, 4H), 3.12 (m, 6H), 2.62 (t, 2H), 1.55–1.30 (m, 26H). ^{13}C NMR ($CDCl_3$): δ 155.8, 155.3, 144.8, 129.5, 129.1, 111.8, 79.0, 78.8, 53.5, 53.1, 48.9, 46.9, 40.5, 40.2, 28.5, 28.4, 27.4, 27.2, 26.4, 26.1, 25.2. HRMS (FAB) *m/z* calcd for $C_{29}H_{50}N_4O_4Cl_2$ $(M + H)^+$, 589.3282; found, 589.3277.

(4-{4-[Bis-(2-chloro-ethyl)amino]benzylamino}butyl)-(3-*tert* butoxycarbonylamino-propyl)carbamic Acid *tert*-Butyl Eester, 29. Pale yellow viscous oil (90%); $R_f = 0.3$ (5% MeOH/0.4% NH_4OH/CH_2Cl_2). ^1H NMR ($CDCl_3$): δ 7.18 (d, 2H), 6.63 (d, 2H), 3.74–3.35 (m, 10H), 3.24–3.04 (m, 6H), 2.61 (t, 2H), 1.64 (m, 2H), 1.52–1.41 (m, 22H). ^{13}C NMR ($CDCl_3$): δ 155.9, 144.9, 129.6, 128.7, 111.8, 79.4, 78.8, 53.5, 53.1, 50.0, 48.8, 46.9, 44.3, 43.7, 40.5, 37.4, 28.4, 28.2, 27.1, 26.4, 26.2. HRMS (FAB) *m/z* calcd for $C_{28}H_{48}N_4O_4Cl_2$ $(M + H)^+$, 575.3130; found, 575.3092.

(3-{4-[Bis-(2-chloro-ethyl)amino]benzylamino}propyl)-(4-*tert*-butoxycarbonyl-amino-butyl)carbamic Acid *tert*-Butyl Ester, 30. Pale yellow viscous oil (93%); $R_f = 0.3$ (5% MeOH/0.4% NH_4OH/CH_2Cl_2). ^1H NMR ($CDCl_3$): δ 7.18 (d, 2H), 6.61 (d, 2H), 4.88 (brs, 1H), 3.73–3.58 (m, 10H), 3.24–3.06 (m, 6H), 2.59 (t, 2H), 1.68 (q, 2H), 1.53–1.41 (m, 22H). ^{13}C NMR ($CDCl_3$): δ 155.8, 155.4, 144.8, 129.6, 129.0, 111.8, 79.1, 78.8, 53.5, 53.2, 46.5, 46.2, 45.1, 44.4, 40.5, 40.2, 28.5, 28.4, 27.4, 25.9, 25.5.

(4-{4-[Bis-(2-chloro-ethyl)amino]benzylamino}butyl)carbamic Acid *tert*-Butyl Ester, 32. Pale yellow viscous oil (69%); $R_f = 0.3$ (5% MeOH/0.3% NH_4OH/CH_2Cl_2). ^1H NMR ($CDCl_3$): δ 7.17 (d, 2H), 6.61 (d, 2H), 5.17 (br s, 1H), 3.72–3.54 (m, 10H), 3.07 (m, 2H), 2.61 (t, 2H), 1.52–1.35 (m, 13H). ^{13}C NMR ($CDCl_3$): δ 155.8, 144.8, 129.5, 129.0, 111.8, 78.7, 53.4, 53.1, 48.7, 40.5, 28.5, 27.9, 27.3. HRMS (FAB) *m/z* calcd for $C_{20}H_{33}N_3O_2Cl_2$ $(M + H)^+$, 418.2026; found, 418.2006.

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Supporting Information Available: Elemental analyses and ^{1}H and ^{13}C spectra for compounds **4**–**7**, **10**–**13**, **18**–**20**, **24**, **28**, **29**, and **32**. This material is available free of charge via the Internet at http://pubs.acs.org.

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