Effect of Polyamine Homologation on the Transport and Biological Properties of Heterocyclic Amidines

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Five sets of heterocyclic derivatives of various sizes and complexities coupled by an amidine function to putrescine, spermidine, or spermine were prepared. They were essentially tested to determine the influence of the polyamine chain on their cellular transport. To comment on affinity and on selective transport via the polyamine transport system (PTS), K_i values for polyamine uptake were determined in L1210 cells, and the cytotoxicity and accumulation of the conjugates were determined in CHO and polyamine transport-deficient mutant CHO-MG cells, as well as in L1210 and α-difluoromethylornithine- (DFMO-) treated L1210 cells. Unlike spermine, putrescine and spermidine were clearly identified as selective motifs that enable cellular entry via the PTS. However, this property was clearly limited by the size of substituents: these polyamines were able to ferry a dihydroquinoline system via the PTS but did not impart any selectivity to bulkier substituents.

Introduction

During the past decade, the biosynthetic machinery of natural polyamines (Figure 1) has emerged as a major target for anticancer therapy.[1,2] A variety of inhibitors of polyamine biosynthetic enzymes and, more recently, of polyamine analogues capable of interfering with the functions of naturally occurring polyamines have been designed.[3,4] Some potent antineoplastics are actually in clinical trials. Other strategies have led to the development of drugs conjugated to a polyamine moiety.[4,5] The idea was to use polyamine properties that could potentially benefit the activity or the specificity of drugs. Most designed conjugates tried to take advantages of two features of natural polyamines: (i) their affinity for nucleic acids[6] and (ii) the existence of specific cell polyamine transport systems.[7,8] Other properties of polyamines such as their affinity for ionotropic glutamate and nicotinic acetylcholine receptors, which lead to the design of potent antagonists,[9] will not be discussed here.

Conjugation of polyamines to a variety of nucleic acid targeting drugs, such as DNA intercalators, alkylating agents, or DNA- or RNA-cleavers has been reported.[10−21] Tethering spermine to a DNA-binding agent increases considerably the overall DNA binding affinity as expected from the synergistic activity of two DNA ligands.[11,20,22,23] The conjugation of the aromatic nitrogen mustard chlorambucil to spermidine enhances 10 000-fold its ability at forming interstrand cross-links with naked DNA. However, the activity of these conjugates greatly depends on their architecture: for example, the attachment of a chlorambucil moiety to spermidine improved its in vitro cytotoxicity when the nitrogen mustard functionality was bound to the secondary amine; in contrast, no improvement resulted from the linkage of chlorambucil to the C-5 of spermidine.[24] However, the only spermidine conjugate tested in vivo was only 4-fold more potent than chlorambucil and did not show any improvement of its therapeutic index relative to the unconjugated alkylating agent.[10,25]

Alternatively, the polyamine moiety may confer DNA-binding affinity to compounds devoid of such affinity, as demonstrated for boron- or fluorine-containing *N*-benzylspermidine derivatives designed, respectively, for boron neutron capture therapy and positron emission tomography.[26] The high affinity of polyamines for DNA has been also used to design DNA delivery agents for nonviral gene therapy as recently reviewed.[27]

Targeting the polyamine transport system (PTS) is another reason for designing drug−polyamine conjugates. All cells possess active and energy-dependent PTS, which take up exogenous natural polyamines but also structurally related compounds.[7,8] Because of their high demand for polyamines, tumor cells have higher PTS activity than normal cells.[28−30] Consequently, a drug−polyamine conjugate would accumulate preferentially in the tumor cells if it can be taken up by the PTS. Its accumulation could be boosted by depleting intracellular polyamine pools [e.g., with α-difluoromethylornithine (DFMO), an inhibitor of ornithine decarboxylase], which results in the upregulation of the PTS activity.[8] Thus, vectorization of chemotherapeutic drugs by polyamines, made possible by the broad structural tolerance of the PTS, is expected to enhance cytotoxicity to tumor cells and to diminish secondary effects on normal cells.[4,7] In contrast, another approach aims at inhibiting the PTS, and some polyamine conjugates have been reported to be potent PTS inhibitors.[31−35] Inhibition of polyamine transport with specific antagonists may potentiate the therapeutic action of DFMO under in vivo conditions where exogenous polyamines tend to replenish cellular pools.[3] This inhibitory

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activity has to be considered for the design of polyamine conjugates conceived to be selectively uptaken by the PTS, since such inhibitory activity may severely impair their uptake via the polyamine transporter.[20]

To date, significant work has been accomplished in the characterization of PTS in bacteria, yeast, and, very recently, protozoan parasites.[36−39] However, in mammals, despite recent advances on the mechanism of polyamine transport,[40,41] no proteins have yet been characterized. The lack of molecular information has hampered the rational design of PTS-selective polyamine−drug conjugates, and misconceptions (e.g., confusion between ability to inhibit the uptake of natural polyamines and ability to serve as a substrate) have slowed progress. Investigations on the selectivity of drug−polyamine conjugates toward the PTS require straightforward approaches such as the comparison of their accumulation in wild-type and polyamine-transport-deficient cell lines.[26,42−44] If methods to determine levels are not available, comparison of the cytotoxicity of conjugates on both type of cells may be considered as an alternative.[20,45−49] Because polyamine depletion leads to an enhancement of the PTS activity, the comparison of the accumulation of conjugates in normal and polyamine-depleted cells is also a reliable approach.[26,45,49−51] In contrast, inaccurate conclusions may be drawn from the comparison of cytotoxicity of conjugates on normal and polyamine-depleted cells. Indeed, if polyamine depletion does enhance the cytotoxicity of PTS-selective drugs (due to their more efficient uptake), it also improves the cytotoxicity of certain polyamine-unrelated chemotherapeutic agents. In particular, DFMO-induced polyamine depletion has been shown to potentiate the cytotoxicity of DNA-targeting agents, such as BCNU, an effect that has been assigned to chromatin structure alterations resulting from polyamine loss.[6,52]

Systematic studies appear crucial to identify molecular recognition elements and to delineate the structural tolerance accommodated by the PTS. Two collections of polyamine conjugates were synthesized and screened for affinity for the PTS and uptake via the PTS.

The first collection consisted of several *N*-substituted linear or branched di-, tri-, or tetramine scaffolds with different tether lengths between nitrogen centers. The *N*-substituents were N^1-alkylarenes, with the alkyl spacer ranging from methyl to propyl and the arene nucleus from benzene to pyrene.[47−49,53] These conjugates helped define some key characteristics required for the selective delivery of polyamine−drug conjugates into cells with active polyamine transporters. The triamine homospermidine appeared to be a highly selective vector motif to ferry an arenyl group into cells via the PTS.[48,53] Tetraamines confered high PTS affinity for conjugates but this did not translate into a more efficacious drug.[47] The PTS can accommodate relatively large N^1-substituents such as a pyrene nucleus.[53] The length of the tether connecting the polyamine chain to the arenyl group is critical.[49] These results lead us to define a model that describes a hydrophobic pocket of set dimensions (able to accommodate the N^1-substituent) adjacent to a polyamine-binding site.[49]

The second collection of polyamine conjugates, and the subject of this report, comprised five sets of heterocyclic amidines of various size and complexities conjugated to aminopropanol or to the naturally occurring polyamines putrescine (**1**; PUT), spermidine (**2**; SPD), or spermine (**3**; SPM) (Figure 1). The design of these conjugates was based on previous reports describing amidine derivatives of nonplanar tetracyclic systems (Figure 2): tetrahydroquino[4,3-*b*][1]benzazepine (**4**) and tetrahydrobenzo[*k*]naphthyridine (**5**), with their exocyclic

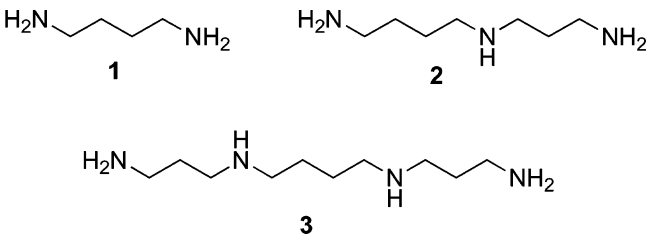


Figure 1. Structures of naturally occurring putrescine (**1**), spermidine (**2**), and spermine (**3**).

nitrogen substituted by alkyl-, hydroxylalkyl- or aminoalkyl-bearing side chains. These conjugates, which exhibited significant in vitro cytotoxicity on ovarian carcinoma cells and in vivo antitumor activity when given in association with DFMO,[54] were found to possess weak but significant DNA-binding affinity, which correlated with their in vitro cytotoxicity.[55] The putrescine conjugates (**4b**, **5b**) that exhibited the greater affinity for DNA were around 10-fold more cytotoxic than their alkyl or hydroxylalkyl homologues. As discussed above, two reasons may explain the potentiation of these conjugates in the presence of DFMO: (i) their putrescine moiety may provide the ability to use the polyamine transport system and then to accumulate to a greater extent in polyamine-depleted cells, and (ii) depletion of polyamines may affect chromatin structure and increase the accessibility of the conjugates to DNA.

To further extend our knowledge of the structure−activity relationships (SAR) of polyamine conjugates with complex systems, we synthesized two sets of the nonplanar tetracyclic systems **4** and **5** conjugated with spermidine **2** (SPD) or spermine **3** (SPM). The substitution of the putrescine side chain by higher polyamines may improve the activity of the amidine derivatives, since these polyamines have higher affinity for the PTS as well as for DNA. Three other sets of polyamine conjugates were obtained by simplification of the initial polycyclic structures **4** and **5**, leading to sets **6** and **7**, and the 3,4-dihydroquinoline derivatives (**8**).

Our aim was to determine whether these new polyamine conjugates were selective substrates of the PTS and therefore possessed properties required for tumor targeting. For this purpose, their affinity for PTS was determined in L1210 cells as well as their selective cytotoxicity and accumulation in cells with active PTS. In addition, our investigations were extended to other biological properties that may be altered by the presence of the polyamine chain, such as DNA-binding and calmodulin antagonism (both activities that may influence the drug cytotoxicity).

Results and Discussion

Synthesis. Five different heterocycles were coupled by an amidine function to 3-amino-1-propanol or to the natural polyamines (putrescine, spermidine, and spermine), leading to five sets of compounds (Figure 2). Starting from two tetracyclic sets constituted by a 3,4-dihydroquinoline ring fused with a *cis* junction either to a 2,3,4,5-tetrahydro-1*H*-benzazepine (**4**) or to a 1,2,3,4-tetrahydronaphthalene (**5**), three other sets were obtained by a disjunctive approach: sets **6** and **7** and the 3,4-dihydroquinoline set (**8**). Thus, we have prepared two tetracyclic sets either with an endocyclic tertiary amine (**4a**,**b**,**d**) or with an exocyclic secondary amine (**5b**−**d**) and their opened analogues either with a tertiary amine (**6a**−**d**) or with a secondary amine (**7b**−**d**) and a bicyclic set (**8a**−**d**).

The synthesis of compounds **4**−**8** involved the thionation of lactams **12**−**16**, leading to **17**−**21**, which were conjugated by an amidine function to various amines. The starting lactams **12**/

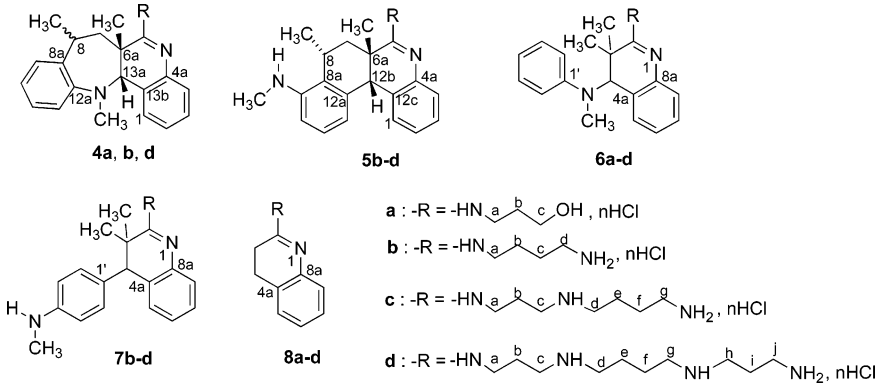
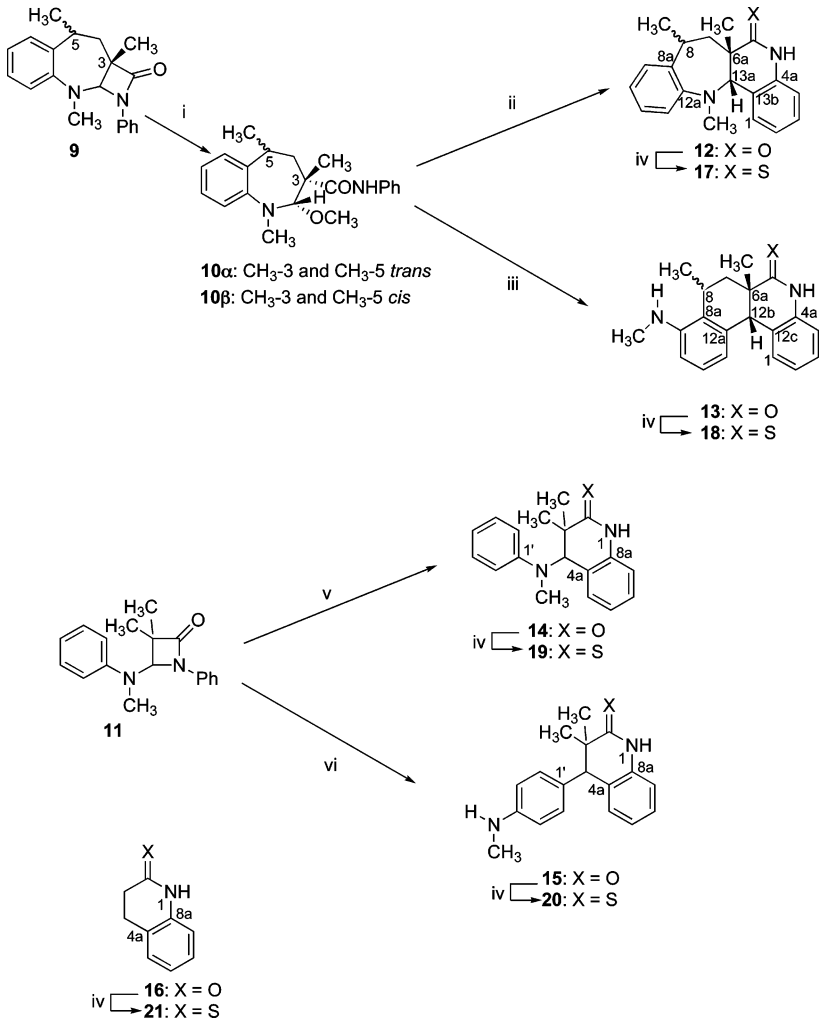


Figure 2. Structure of polyamine−heterocyclic amidine conjugates **4a**−**8d**.

Scheme 1[a]

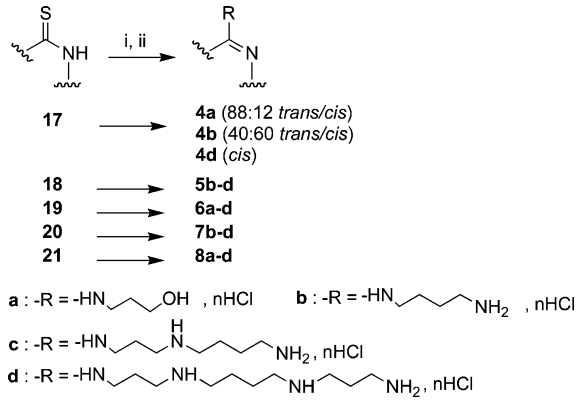


[a] Reagents: (i) CH_3OH/CH_2Cl_2; (ii) 2 M aqueous HCl, $CHCl_3$, 80 °C; (iii) 3 M aqueous HCl, refluxing; (iv) Lawesson's reagent (0.7−4 equiv) in refluxing toluene or dioxane; (v) $(CH_3)_3SiI$, anhydrous CH_2Cl_2, room temperature; (vi) $(CH_3)_3SiI$, anhydrous $CHCl_3$, 70 °C.

13 and **14/15** (Scheme 1) were obtained by rearrangement, in acidic medium, of the corresponding 4-aminoazetidin-2-ones **9** and **11**, respectively.[56,57] The tetracyclic lactams **12** and **13,** bearing three chirality centers (C-6a, C-8, and C-13a or C-12b), were obtained as a 60/40 (*trans*/*cis*) mixture of diastereomers.[57] CH_3-6a and CH_3-8 were in a *trans* relationship in the major isomers and in a *cis* relationship in the minor isomers. Because the chromatographic separation of the diastereomers remained unsuccessful, a chemical method was used to prepare pure or enriched isomers. This method involved the formation of the intermediate aminoethers **10α** and **10*β***,[56] which could be separated by recrystallization in MeOH[54] (Scheme 1). The aminoether **10α** was used for the synthesis of *trans* compounds **5b**−**d** and **4a** (enriched at 88%), and the aminoether **10*β*** was used for the synthesis of *cis* compound **4d** (Figure 2). Compound **4b** was used as a 40/60 (*trans*/*cis*) mixture of diastereomers. In fact, in a previous study, we had shown that the diastereomers of each tetracyclic set (**4b**, **5b**) coupled to the putrescine in pure forms or in mixture had the same order of activity on 3LL carcinoma cells.[54] Lactam **16** was purchased from Aldrich.

Compounds **12**−**16** contained an amide function whose thionation (Scheme 1) was easily achieved according to a

Scheme 2[a]



[a] Reagents: (i) amine or protected amine excess, $HgCl_2$ in refluxing THF; (ii) 2 M HCl gas, ethanol.

classical method: the use of Lawesson's reagent (0.7−4 equiv) in refluxing dioxane or toluene led to the thiolactams **17**−**21** in good yields (46−87%), as was previously reported for **17** and **18**.[54]

The formation of an amidine bond from a thiolactam and an amine is an efficient method,[55,58,59] in which the presence of mercury chloride ($HgCl_2$) favors the completion of the reaction by precipitation of mercury sulfide (HgS) (Scheme 2). In this work, two different cases arose depending on the structure of the amine moiety. The symmetrical polyamine putrescine and spermine (as well as the amino alcohol) could be used without any protective groups: their primary amino group reacted predominantly and a large excess (10−25 equiv) avoided any significant formation of bis-conjugates. Their large water solubility allowed their removal by simple washing of organic layers during the final workup. In contrast, the unsymmetrical spermidine required selective protection to prevent the obtaining of regioisomers. Thus we have prepared N^4,N^8-di-(*tert*-butoxycarbonyl)spermidine according to a known method[60] with the following modification: the reduction of nitrile leading to the formation of the terminal amino group was performed by catalytic hydrogenation (H_2/Ni Raney, EtOH/NH_3) instead of $LiAlH_4$. We used it in smaller excess (5 equiv) for the coupling step to the thiolactams. These spermidine conjugates (**5**−**8c**) were chromatographed and then deprotected in acidic medium (1 M aqueous HCl).

All conjugates **4a**−**8d** (Figure 2) were purified by column chromatography and transformed to hydrochlorides by use of 2 M HCl gas in ethanol. They were obtained in acceptable overall yields (19−88%). Purity was checked by combustion analysis (except **4a** and **8a**) and HPLC by a modified version of a method reported for the determination of the natural polyamines in biological samples.[61] Generally, NMR spectra were carried out in $CDCl_3$ on the free base form. Because of a coalescence problem, NMR spectra of **4d**, **5d**, **6d**, **8c**, and **8d** were performed on the hydrochloride salts in D_2O. In addition, the ^{13}C NMR spectrum of **4a** was performed in D_2O.

DNA-Binding Properties. The loss of ethidium fluorescence as a consequence of the displacement of the DNA-bound fluorochrome (at a high [ethidium]:[DNA] molar ratio) as a function of added drug allows a rough estimation of the affinity of drug binding to DNA.[62−64] The drug concentration producing 50% inhibition of fluorescence (CC_{50}) is approximately inversely proportional to its DNA binding constant. CC_{50} values determined for free or conjugated polyamines are listed in Table 1. In a previous report, the nonplanar tetracyclic conjugates **4a**, **4b**, and **5b** have been found to possess weak but significant DNA-binding affinity by thermal denaturation.[55] In perfect agreement with this report, we found that the tetrahydrobenzonaphthyridine **5b** shows a greater overall DNA-binding affinity than its isomeric counterpart **4b** and that the introduction of an

Table 1. Various Biological Properties of the Polyamine−Amidine Conjugates[a]

compd	DNA binding properties, CC_{50}[b] (drug/DNA P)	calmodulin antagonism, IC_{50}[c] (μM)	BSAO oxidation, rel fluorescence intensity[d]	PUT transport inhibition,[e] K_m/K_i (μM)	SPD transport inhibition,[e] K_m/K_i (μM)	SPM transport inhibition,[e] K_m/K_i (μM)
AP			0			
1	>50	>500	3 ± 0.1	19.9		
2	5.5± 0.6	>500	85 ± 4	0.75 ± 0.07	2.45 ± 0.51	
3	0.28 ± 0.01	>500	125 ± 5	0.26 ± 0.03	1.34 ± 0.31	1.06 ± 0.02
4a	>50	>500	0	106 ± 7		
4b	23 ± 1	116 ± 10	0	29.2 ± 5.4		
4d	3.2 ± 0.3	19 ± 4	42 ± 2	0.058 ± 0.006	0.30 ± 0.05	0.24 ± 0.02
5b	7.8 ± 0.1	70 ± 11	0	8.20 ± 1.03		
5c	2.5 ± 0.2	22 ± 5	13 ± 0.4	2.54 ± 0.20		
5d	1.6 ± 0.3	10 ± 6	67 ± 1	0.56 ± 0.06	1.01 ± 0.21	
6a		>500	0	15 ± 1		
6b	5.1 ± 0.1	51 ± 9	5 ± 0.1	20.1 ± 2.3		
6c	2.2 ± 0.1	30 ± 12	17 ± 0.8	6.80 ± 0.52		
6d	1.8 ± 0.2	8 ± 4	53 ± 1	0.052 ± 0.05	0.26 ± 0.04	0.28 ± 0.04
7b	12.4 ± 0.4	373 ± 86	0	>150		
7c	1.8 ± 0.1	64 ± 5	5 ± 0.1	3.41 ± 0.22		
7d	0.64 ± 0.16	23 ± 4	52 ± 1	0.38 ± 0.02	0.20 ± 0.03	
8a	5.5 ± 0.08	>500	0			
8b		>500	0		400 ± 52	
8c	1.19 ± 0.06	>500	46 ±2	0.75 ± 0.05	1.58 ± 0.14	
8d	0.11 ± 0.01	>500	106 ± 1	0.20 ± 0.01	0.38 ± 0.03	

[a] All data are expressed as mean (±SD) from three separate determinations. [b] Conjugate concentration to give 50% decrease in fluorescence of calf thymus DNA-bound ethidium. [c] Concentration of conjugate required to inhibit by 50% c-AMP-dependent calmodulin-activated phosphodiesterase. [d] The hydrogen peroxide generated by the oxidation of the test compound (10 μM) in the presence of bovine serum oxidase was determined by the peroxidase-catalyzed transformation of homovanillic acid into a fluorescent product. [e] Polyamine uptake inhibition was determined in L1210 cells. K_m values for polyamine uptake (shown in boldface type) were determined by Lineweaver−Burk analysis of transport velocity at increasing radiolabeled substrate concentrations. K_i values were calculated from the half-maximal inhibitory concentration (IC_{50}) estimated by iterative curve fitting for sigmoidal equations describing polyamine uptake velocity in the presence of growing concentrations of antagonist.

aminoalkyl side chain (putrescine) favors interaction over a hydroxylalkyl side chain. It has to be noticed that **6a** has a higher affinity for DNA than its closed counterpart **4a** and that the "naked" dihydroquinoline **8a** had a similar activity as **6a**.

The CC_{50} values determined for free polyamines are in agreement with earlier studies[65−68] that have clearly demonstrated a relationship between the number of protonated nitrogens of free polyamines and their affinity for nucleic acids. The affinity for DNA varied as follows: spermine (tetramine) > spermidine (triamine) > putrescine (diamine). A similar relationship was also observed for the conjugated polyamines (**4**−**8**), with the highest affinity observed for spermine conjugates. Increasing the number of amino groups on the side chains in all series enhanced the affinity for double-stranded DNA. As expected from the additive or synergistic activity of two ligands with DNA affinity,[11,20] putrescine and spermidine conjugates were more efficient to release bound ethidium than their free forms. Surprisingly, free spermine remains more efficient than its conjugated forms. It has been suggested that the tetracyclic conjugates **4a**, **4b**, and **5b** bind only weakly to DNA via the minor groove rather than through an intercalative process.[55] Due to the much higher affinity of spermine for DNA, the binding of spermine conjugates may be completely driven by the polyamine side chain and may be constrained to natural "polyamine" binding sites. In this hypothesis, a critical factor may be the bulkiness of the heterocyclic moiety, which may hinder spermine binding to DNA. In agreement with such hypothesis, the DNA-binding affinity of the spermine conjugates varies inversely with substituent size: for example, the affinity for DNA of the tricyclic **6d** was higher than that of the tetracyclic conjugate **4d** but lower than that of the bicyclic conjugates **8d**.

Calmodulin Antagonism. Calmodulin is a ubiquitous Ca^{2+}-binding protein involved in multiple vital cell functions,[69] and calmodulin antagonists are cytotoxic agents.[70] Spermine **3** is a weak calmodulin antagonist,[71] but polyamine conjugates[44] as well as other analogues[72] have been reported to be very potent calmodulin antagonists, a property that could contribute to their cytotoxicity.[4,73] In addition, calmodulin activation has been shown to be involved in the regulation of polyamine uptake, and calmodulin antagonists are known to inhibit polyamine uptake.[74−76] We then investigated the ability of our polyamine conjugates to inhibit calmodulin-activated cyclic nucleotide phosphodiesterase (Table 1).

Unconjugated polyamines **1**, **2**, and **3** had very little effect on calmodulin-activated cyclic nucleotide phosphodiesterase with IC_{50} greater than 0.5 mM. In contrast, all polyamine conjugates of sets **4**−**7**, but not their aminopropanol analogues, inhibited calmodulin-activated cyclic nucleotide phosphodiesterase. Because none of the conjugates had any effect on basal phosphodiesterase activity (data not shown), their inhibitory effect on the calmodulin-activated enzyme is likely due to their interaction with calmodulin. Spermine conjugates were more potent antagonists with IC_{50} below or equal to 23 μM. Spermine is known to bind calmodulin but its binding affinity is in the millimolar range.[71] It has been postulated that these latent properties of spermine may be "amplified" by conjugation with a substituent of significant lipophilicity.[44] In support of this assertion is the observation that polyamines conjugated to the less hydrophobic dihydroquinoline system (set **8**) were devoid of calmodulin antagonism. It is also obvious that the number of positive charges contributes to the calmodulin antagonist properties. The spermine conjugates **4**−**7d** with four positive charges were more potent than the spermidine conjugates with three positive charges (IC_{50} between 20 and 60 μM). In turn, the latter were more potent than the putrescine conjugates with only two positive charges (IC_{50} between 50 and 370 μM). Similarly, the spermine sulfonamides, with one positive charge of the spermine moiety lost by amide bond formation with a sulfonic acid,[44] had similar potency as spermidine conjugates **5**−**7c**. Because of their calmodulin antagonism, some conjugates may limit their own active uptake via the polyamine transport system.

Oxidation by Serum Amine Oxidase. Bovine serum amine oxidase (BSAO, EC 1.4.3.6) catalyzes the oxidation of spermidine and spermine, giving rise to the corresponding aldehydes, ammonia, and hydrogen peroxide.[77,78] While aminopropanol and putrescine derivatives showed no oxidation by BSAO, all spermidine and spermine conjugates were oxidized, as evidenced by the formation of a fluorescent adduct from homovanillic acid in the presence of peroxidase (Table 1). However, the conjugates were poorer substrates than the free corresponding polyamines. Because the oxidation products are cytotoxic, cell culture experiments were performed in the presence of aminoguanidine, an inhibitor of BSAO.

Cell Studies. Three cell lines were chosen for bioassay. L1210 (mouse leukemia) cells were selected to enable comparisons with the published IC_{50} and K_i values known for a variety of polyamine substrates.[53,79−81] CHO cells were chosen along with a mutant cell line (CHO-MG) in order to comment on selective transport via the PTS.[20,47]

Cytotoxicity on L1210. As shown in Table 2, all conjugates displayed moderate or no cytotoxicity on L1210 cells. The introduction of a (poly)aminoalkyl side chain influenced the cytotoxicity of the compounds. Putrescine derivatives **4b**, **5b**, and **6b** were potent agents with IC_{50} below 32 μM and were more active than their aminopropanol counterparts, while **7b** and **8b** were completely devoid of any activity. The introduction of a spermidine side chain was not beneficial since none of the spermidine conjugates had any effect on L1210 cell growth. In contrast, all spermine derivatives were cell growth inhibitors. The position of the methylamino substituent had a critical impact on the cytotoxicity of the compounds. Compounds with the methylamino substituent remote from the amidine residue (**5d**, **7d**; $IC_{50} = 28$ μM) were more potent than compounds **4d** and **6d**. It should be noted that **8d**, a compound devoid of any substituent, had an IC_{50} around 70 μM.

We did not observe any correlation between the cytotoxicity of the drugs and either their DNA binding or their calmodulin antagonism properties. This could be related to their ability to be taken up by cells.

PTS Selectivity. To comment on selective transport via the PTS, we determined for each conjugate: (i) the K_i value (Table 1), which reflects its affinity for the PTS, in a competitive assay with radiolabeled polyamines in L1210 cells; (ii) its growth inhibitory effect (Table 2) and accumulation (Table 3) in CHO and polyamine transport-deficient CHO-MG cells; and (iii) the effect of DFMO on its L1210 cell growth inhibitory effect (Table 2) and cellular accumulation (Table 4).[20,47,48,53]

(i) All polyamine conjugates were determined to be competitive inhibitors of the PTS against at least one of the three natural polyamines **1**−**3**. The affinities of free and substituted polyamines for the PTS in L1210 cells are listed in Table 1. As expected from earlier work,[47,48,53,82] the affinity for the L1210 polyamine transporter increased with the number of nitrogen centers. The affinity varies as follows: tetramines > triamines > diamines > monoamines. The spermine derivatives demonstrated very

Table 2. In Vitro Cell Growth Inhibition[a]

	IC_{50}[b] (μM)					
	L1210		CHO		CHO-MG	
compd	control	+DFMO[c]	control	+DFMO	control	+DFMO
AP	>100					
1	>100	rev[d] (>0.5 μM)	>100	rev	>100	>100
2	>100	rev (>0.1 μM)	>100	rev	>100	>100
3	>100	rev (>0.1 μM)	>100	rev	>100	>100
4a	100					
4b	32	54 ± 4[e]	26 ± 1	45 ± 3[e]	26 ± 3	24 ± 2
4d	78	1.0 ± 0.1[e]	21 ± 1	9.1 ± 1[e]	24 ± 1	27 ±3
5b	28	56 ± 4	31 ± 2	57 ± 4[e]	33 ± 2	30 ± 1
5c	>100	rev (>10 μM)	>100	rev (>2 μM)	>100	>100
5d	22	2.4 ± 0.2[e]	23 ± 2	11 ± 2[e]	29 ± 1	23 ± 2
6a	>100					
6b	15	33 ± 2[e]	13 ± 1	>100	14 ± 1	13 ± 2
6c	>100	rev (>5 μM)	>100	rev (>1 μM)	>100	>100
6d	68	2.3 ± 0.2[e]				
7b	>100	rev (>10 μM)	>100		>100	
7c	>100	rev (>5 μM)	>100		>100	
7d	28	3.1 ± 0.2[e]	23 ± 3	11 ± 1[e]	30 ± 2	23 ± 1
8a	>100					
8b	>100	rev (>5 μM)	>100		>100	
8c	>100	>100	>100		>100	
8d	>100	rev (>0.1 μM)	>100		>100	

[a] All cells were challenged with the conjugates at the time of seeding for L1210 cells and 24 h later for CHO and CHO-MG cells. Cell growth was determined by a MTT assay after 48 h of treatment. [b] Conjugate concentration required to inhibit cell growth by 50% after 48 h; mean value (±%) from three determinations. [c] DFMO (5 mM) was added at the time of the addition of the conjugates. [d] Reversion; compound that reverses the cytostatic effect of DFMO at concentrations above that indicated in parentheses. [e] $p < 0.05$: significantly different from control values without DFMO.

Table 3. Cellular Uptake of Conjugates in CHO and CHO-MG Cells

		cellular uptake[a] (nmol/mg of protein)		
compd	concn (μM)	CHO	CHO-MG	CHO/CHO-MG ratio
4b	25	3.6 ± 0.8	3.3 ± 0.6	1.1
4d	50	0.66 ± 0.10	0.66 ± 0.11	1.0
5b	25	8.1 ± 0.5	7.2 ± 1.3	1.1
5c	50	1.3 ± 0.2	1.2 ± 0.5	1.0
5d	25	2.2 ± 0.1	2.1 ± 0.2	1.0
6b	10	13 ± 2	12 ± 2	1.1
6c	25	2.9 ± 0.1	3.1 ± 0.7	1.0
6d	50	3.3 ± 0.08	3.2 ± 0.3	1.0
7b	50	5.3 ± 0.5	5.9 ± 0.2	0.9
7c	50	1.2 ± 0.3	1.7 ± 0.1	0.7
7d	25	0.72 ± 0.2	0.81 ± 0.3	0.9
8b	5	1.2 ± 0.1	0.26 ± 0.04[b]	4.6
8b	10	2.1 ± 0.5	0.37 ± 0.05[b]	5.7
8c	10	11.0 ± 2.0	1.6 ± 0.2[b]	6.9
8c	25	15.4 ± 1.5	2.2 ± 0.3[b]	7.0
8d	5	0.029 ± 0.004	0.029 ± 0.003	1.0
8d	10	0.029 ± 0.002	0.022 ± 0.004	1.3

[a] Conjugates were added 24 h after seeding; intracellular levels were determined by HPLC on perchloric extracts of cells treated with the conjugates during 24 h; mean values (±SD) from three determinations are given. [b] $p < 0.05$: significantly different from values determined in CHO cells.

high affinity with K_i values in the nanomolar range. The affinity for the PTS was essentially driven by the polyamine moiety.

(ii) Comparison of cytotoxicity in CHO and CHO-MG cells provides an important screen to detect selective conjugate delivery via the PTS.[20,47,48,53] A clear limit to this assay is the cytotoxicity of the drugs and in the present study; we were unable to determine IC_{50} values for seven out of the 13 compounds assayed. Yet none of the six polyamine conjugates (with a measurable growth inhibitory effect) showed any selectivity on CHO. To overcome the cytotoxicity issue, we determined the intracellular concentrations of the conjugates in both cell lines (Table 3). Conjugates of sets **4**−**7** did not show any differential accumulation in CHO vs CHO-MG cells. In contrast, the intracellular concentrations of the putrescine and spermidine conjugates **8b** and **8c** were up to 7 times higher in CHO than CHO-MG cells, indicating that the PTS is involved in their uptake**.** No differential accumulation was observed for their spermine homologue **8d,** which suggests that spermine is not a PTS-selective vector.

Because putrescine and spermidine conjugated to bulkier substituents (e.g., **4**−**7b**,**c**) were not taken up by the PTS, we may conclude that there are limits to the size of substituents which can be accommodated by the PTS. A large substituent would not fit in the hydrophobic pocket of set dimensions adjacent to the PTS polyamine binding site modeled from our recent study on N^1-arylalkylpolyamines containing various aromatic ring systems.[49] Spermidine imparts selectivity of small substituents, such as a benzyl moiety[26] or *N*-methylanthranylic

Table 4. Polyamine and Analogue Levels in L1210 Cells Cultured for 48 h in the Presence of Amidine Derivatives, in the Absence or Presence of DFMO

		polyamine levels[a] (nmol/mg of protein)							
		PUT		SPD		SPM		conjugate	
compd	concn (μM)	−DFMO	+DFMO	−DFMO	+DFMO	−DFMO	+DFMO	−DFMO	+ DFMO
	0	1.6	0.05	22	0.3	8.8	6.5		
4b	20	0.85	0.70[b]	11[c]	0.87[b]	6.3[c]	9.0[b]	13	8.2[d]
4d	10	1.5	0.04	18	0.49	9.7[c]	5.8	0.59	1.5[d]
5b	20	1.6	1.0[b]	14[c]	2.1[b]	8.9	10[b]	23	8.2[d]
5c	25	1.0	0.02	24	1.5[b]	11[c]	8.9[b]	1.2	1.3
5d	10	1.4	0.04	16[c]	0.51	8.2	7.3[b]	0.95	2.1[d]
6c	25	0.65[c]	0.12	21	1.4[b]	10[c]	10[b]	0.64	0.66
6d	10	0.41[c]	0.02	16[c]	0.60[b]	9.5[c]	7.6[b]	1.8	2.6[d]
7b	50	1.9	1.0[b]	20	1.8[b]	8.6	6.6	0.60	0.33[d]
7c	50	1.1	0.08	15[c]	2.4[b]	9.2[c]	8.0[b]	0.77	0.62
7d	10	1.1	0.07	17[c]	0.66[b]	9.4[c]	7.8[b]	0.54	0.74[d]
8b	10	1.8	bdl[e]	21	0.94[b]	12.5[c]	16.5[b]	0.26	0.34[d]
8c	10	bdl	bdl	14.5[c]	3.2[b]	7.1	12.1[b]	4.3	8.2[d]
8d	10	0.33[c]	bdl	8.1[c]	4.2[b]	13.9[c]	13.9[b]	1.5	2.4[d]

[a] Conjugates and DFMO (5 mM) were added at the time of seeding; intracellular levels of polyamines and conjugates were determined by HPLC on perchloric extracts of cells cultured for 48 h. [b] Significantly different between treated and control cells ($p < 0.05$). [c] Significantly different between treated and control cells in the presence of DFMO ($p < 0.05$). [d] Conjugate levels significantly different between cells treated in the absence or in the presence of DFMO ($p < 0.05$). [e] bdl, below detection levels.

acid,[83,84] as well as more bulky tricyclic cargos such as BODIPY, a fluorescent probe,[41,83] or anthracene.[48] It was not surprising that spermidine was also a selective motif to ferry the bicyclic dihydroquinoline system (**8c**). For putrescine, the bulkiness of the substituent is even more limiting. Putrescine is a selective vector to ferry benzyl[26] and dihydroquinoline but not anthracene systems.[53] These observations demonstrate that the limits to the size of substituents depend also on the nature of the polyamine chain.

(iii) Inhibition of ornithine decarboxylase by α-difluoromethylornithine (DFMO) leads to a significant increase in polyamine uptake as a reaction to the depletion of intracellular polyamine pools.[85,86] Therefore, cytotoxic polyamine conjugates that selectively target the PTS should be more potent on DFMO-treated cells.

In L1210 and CHO cells, but not CHO-MG cells, DFMO increased the potency (lower IC_{50} values) of all spermine conjugates, except the noncytotoxic compound **8d** (Table 2). For spermine conjugates **4**−**7d**, the growth inhibitory curves showed a strong synergistic effect at low concentrations, as shown for conjugate **4d** in Figure 3. As discussed above, this synergistic effect could be due to the intrisinc activity of DFMO. However, the synergistic effect was not observed in DFMO-treated CHO-MG cells. Because CHO-MG cells do not show any alteration in polyamine metabolism and regulatory pathways besides the lack of a functional PTS,[86] the absence of synergistic effect in DFMO-treated CHO-MG cells suggests that the synergy was entirely dependent on an active PTS. The determination of intracellular levels of conjugates in L1210 reveals that polyamine-depleted cells accumulated more efficiently (up to 2 times more) the spermine conjugates (included **8d**) than normal cells (Table 4). In L1210, the potency of the spermine conjugates was similar when the cells were treated in the presence of 200 μM spermidine (data not shown). If the conjugates were using the PTS, spermidine would have been a potent competitor and would have decreased the potency of the drugs.[49] Altogether, these results indicate that in normal cells (referring to cells not treated with DFMO) the spermine conjugates are taken up independently of any PTS. In contrast, in DFMO-treated cells, the synergistic effect was likely the consequence of a supplementary uptake of the conjugates via a PTS. Multiple systems for the uptake of polyamines and related compounds with different characteristics have already been described in a given cell line.[87,88] The synergistic effect observed with the amidine spermine conjugates, which is similar to that reported for amidoacridine−spermine conjugates,[20] could be explained by the presence of a high-affinity PTS expressed only in DFMO-treated cells. Such a PTS would be less stringent than the one expressed in normal L1210 or CHO cells since it will allow the transport of bulky conjugates.

In contrast, all putrescine and spermidine conjugates antagonized the effect of DFMO in L1210 cells as shown in Table 2. They reversed the cytostatic effect of DFMO at low concentrations (e.g., **5b** in Figure 3), and the potency of the cytotoxic conjugates such as **4b**, **5b**, and **6b** was significantly diminished. In L1210 cells treated with the putrescine or spermidine conjugates in the presence of DFMO, intracellular levels of free putrescine and/or spermidine were significantly higher than in DFMO-treated cells not exposed to the drugs (Table 4). One possible explanation for these observations is the release, from the conjugates, of free putrescine or spermidine, which will in turn antagonize the DFMO-induced cytostatic effect. Release of free polyamine from various polyamine conjugates has already been reported, especially in DFMO-treated cells.[26,49,89] However, the reversion of the DFMO-induced cytostatic effect was observed in CHO but not in CHO-MG cells (Table 2, Figure 3), indicating that the reversion was entirely dependent on an active PTS. This strongly suggests that the release of free polyamines from the conjugates occurred outside the cells in the culture medium. However, after incubation for 48 h at 37 °C of putrescine conjugates in culture medium, only traces of free putrescine were detected (data not shown). It is then possible that cultured cells released into the extracellular space an enzyme capable of degrading the conjugates. This remains to be investigated.

The polyamines released from the conjugates were eventually converted into higher polyamines inside the cells, as indicated by the elevated spermine levels in DFMO-treated cells exposed to the putrescine and spermidine conjugates (Table 4). Spermine and spermidine concentrations were also higher in DFMO-treated cells when incubated with spermine conjugates (Table

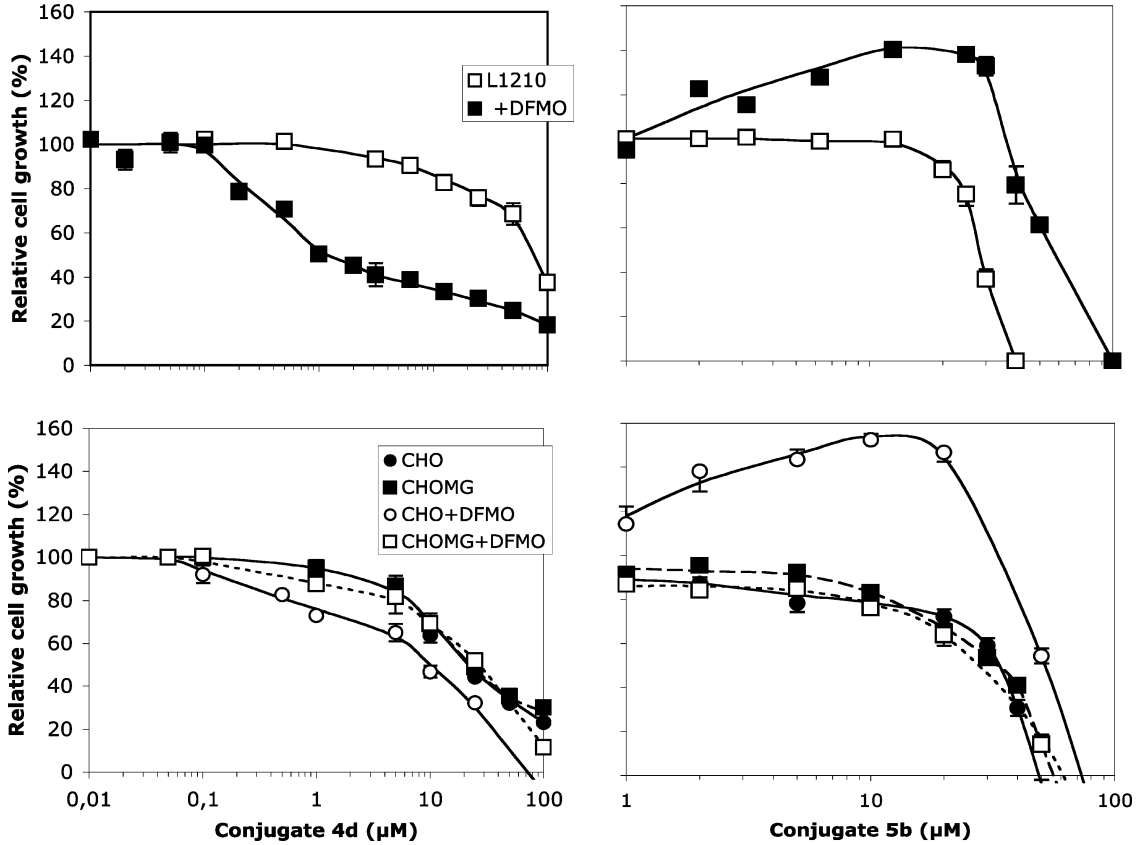


Figure 3. Effect of the conjugates **4d** (left panels) and **5b** (right panels) on L1210 cell growth (upper panels) or CHO and CHO-MG cell growth (lower panels) in the presence or absence of DFMO. Cells were cultured for 48 h with the conjugates in the presence or absence of 5 mM DFMO. Cell growth rates were determined by the MTT assay. The relative cell growth rates were calculated from the value of cell growth of the corresponding control cells cultured in the absence (OD_{540nm} = 1.14, 1.03, and 1.04 for CHO, CHO-MG, and L1210 cells, respectively) or in the presence of DFMO (OD_{540nm} = 0.66, 0.65, and 0.61 for CHO, CHO-MG, and L1210 cells, respectively).

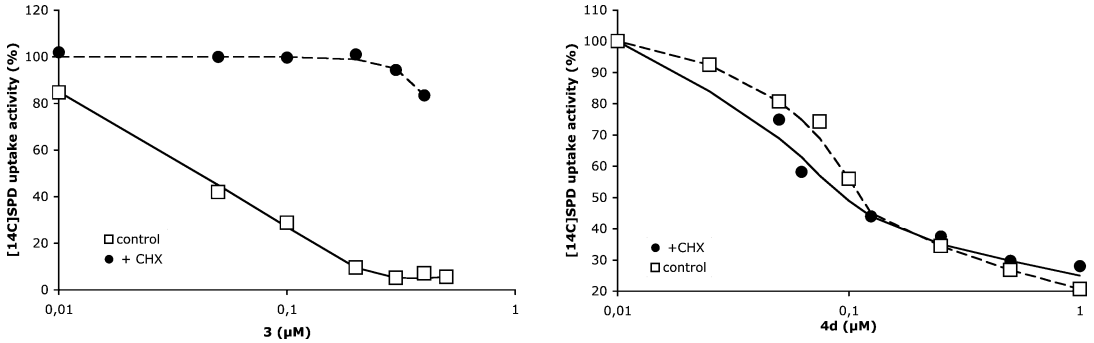


Figure 4. Spermidine uptake activity in CHO cells preincubated with various concentrations of spermine (**3**) or of the spermine conjugate **4d** in the presence or absence of 200 μM cycloheximide (CHX).

2). This could be explained by the release of free spermine from the conjugates and its retroconversion into spermidine.

It should be mentioned that the treatment of L1210 cells with DFMO enhanced the accumulation of the putrescine and spermidine conjugates **8b** and **8c**, in agreement with their selectivity for the PTS observed in the CHO/CHO-MG system.

Inhibition of the PTS Activity. Despite its high affinity for the PTS (Table 1), the absence of selectivity of the spermine conjugate **8d** compared to its putrescine and spermidine homologues (**8b** and **8c**, respectively; Table 3) observed in CHO vs CHO-MG cells indicates that the spermine moiety is not a suitable motif for an efficient delivery via the PTS in the absence of DFMO, in agreement with our previous reports.[20,47] Because some spermine conjugates are potent and transitory irreversible inhibitors of the PTS,[20,33,34,90] we envisaged that the spermine conjugates may inhibit their own selective delivery via the PTS.

It is well established that, in response to an elevation of intracellular polyamine pools (e.g., due to the transport of extracellular polyamines), a regulatory pathway aimed at preventing an excessive accumulation of polyamines, that may be detrimental to the cells, is activated. This pathway involves the de novo synthesis of antizyme, a protein responsible for the inhibition and degradation of ODC that also negatively regulates the PTS activity.[8,91,92] As expected, the preincubation of CHO cells with spermine **3** induced a dose-dependent decrease in the spermidine uptake activity (Figure 4). This decrease was prevented when spermine was applied in the presence of 200 μM cycloheximide (CHX), an inhibitor of protein synthesis. This is in agreement with an antizyme-dependent downregulation of the PTS activity. A similar dose-dependent decrease in PTS activity was observed in CHO cells preincubated with **4d**. However, the effect of **4d** was insensitive

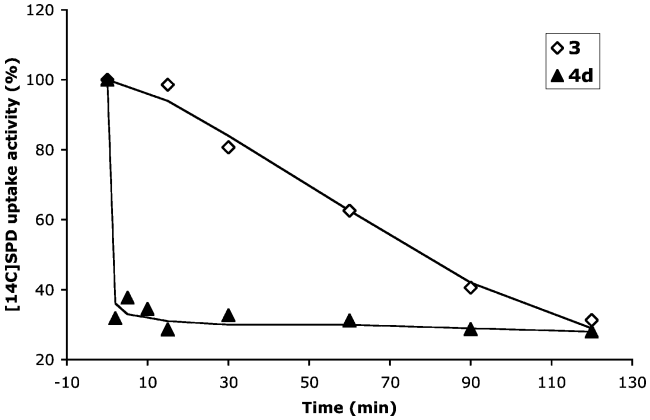


Figure 5. Spermidine uptake activity in CHO cells preincubated with spermine (**3**; 0.2 μM) or with the spermine conjugate **4d** (0.25 μM) as a function of the preincubation time.

to the presence of CHX, as already reported for various spermine conjugates such as acridine−spermine and lysine−spermine.[20,33] These observations eliminate antizyme as an effector in the downregulation of the PTS activity induced by these spermine conjugates. All these spermine conjugates displayed a very high affinity for the PTS as demonstrated by a very low K_i value (<0.1 μM) for spermidine uptake (Table 1).

As discussed earlier, calmodulin, which is involved in the regulation of polyamine uptake, may be envisaged as a potential target of the spermine conjugates. Amidine−spermine conjugates were very potent antagonists with IC_{50} below or equal to 20 μM. Because of their calmodulin antagonism, some conjugates may limit their own active uptake via the polyamine transport system. It has to be noted that **8b** and **8c**, the only two compounds that displayed a preferential accumulation in CHO versus CHO-MG cells, were lacking calmodulin antagonism. However, their spermine homologue **8d** was also devoid of this property but still did not show any selective uptake via the PTS in CHO cells. Calmodulin antagonism may not be the only mechanism limiting the selective uptake of polyamine conjugates.

In CHO cells, the repression of the PTS activity induced by spermine was time-dependent (Figure 5), as it is known to require antizyme synthesis.[8,91,92] In contrast, the repression of the PTS activity induced by spermine conjugates such as **4d** was quite immediate after addition of the drug to the cells. As shown in Figure 5, the drop of the PTS activity was already maximal 2 min after the addition of **4d** on CHO cells. The rapidity of the repression induced by **4d** suggests that the PTS inactivation does not involve cell regulatory pathways but rather implies a very rapid and high-affinity binding of the drug to the transporter itself or to some effector. The proteoglycan Glypican-1, recently identified as a possible vehicle for polyamine uptake in mammalian cells, may be a possible target.[40] Weeks et al.[33] have hypothesized that a slow rate of dissociation of the conjugate from the transporter would prevent the recovery of full transport activity after removal of the conjugates. In agreement with such a supposition, we observed that an anthracene−spermine derivative, a high-affinity PTS ligand, binds rapidly to the cell surface and remains bound even after extensive washing.[47]

Conclusion

Five sets of heterocyclic derivatives of various sizes and complexities coupled by an amidine function to naturally occurring polyamines were synthesized to challenge the architectural and physiochemical constraints allowed by the polyamine transporter of mammalian cells. Our results clearly show that spermidine as well as putrescine is a PTS-selective motifs, but the size of substituents is a limiting factor for the conjugate selectivity. In contrast, spermine, independent of the size of the substituent, was effective in targeting the PTS for cellular entry but only in cells treated with DFMO. Altogether, these findings help further define the key characteristics of polyamine−drug conjugates that can be accommodated by the polyamine transporter.

Experimental Section

Chemistry. Reagent-grade solvents were purchased from chemical companies and used directly without further purification unless otherwise specified. Tetrahydrofuran (THF) was dried under nitrogen by distillation over sodium, and benzophenone and diethyl ether were dried by distillation over $LiAlH_4$. Dry ethanol was stored over 4 Å molecular sieves.

Merck silica gel 60 (70−230 mesh) was used as the solid phase for column chromatography. Thin-layer chromatographies were performed on Merck silica gel 60 F254 (layer thickness 0.22 mm). Solvent systems (expressed in volume percents) and R_f are indicated in the text. The compounds were visualized with UV light, ninhydrin, or iodine. FTIR spectra were recorded on a Perkin-Elmer 16PC instrument (KBr pellets, ν in reciprocal centimeters). NMR spectra were recorded on a Bruker DMX spectrometer at 500 MHz (^{1}H) or 125 MHz (^{13}C) except for **4a**, **5c**, and **6c** for which spectra were recorded on a Bruker AM300 FT spectrometer at 300 MHz (^{1}H) or 75 MHz (^{13}C). Tetramethylsilane (TMS) was used as the internal standard for NMR spectra performed in $CDCl_3$. 3-(Trimethylsilyl)-1-propanesulfonic acid (DSS) was used as the external standard for NMR spectra recorded in D_2O. Broad-band and gated decoupling ^{13}C NMR spectra were recorded, and the assignments were made from chemical shifts, selective decoupling experiments, and coupling constants (^1J and long-range coupling). Values with an asterisk can be inverted.

Electronic impact high-resolution mass spectra (HRMS EI) were recorded on a Varian MAT 311 double-focusing instrument at the CRMPO (Centre Régional de Mesures Physiques de l'Ouest) with a source temperature of 140 °C, an ion accelerating potential of 3 kV, and ionizing electrons of 70 eV and 300 μA. High-resolution mass spectra determined by liquid secondary ion mass spectrometry (HRMS LSIMS) were performed on a ZabSpec Tof Micromass at the CRPMO with a source temperature of 40 °C, an ion (Cs^+) accelerating potential of 8 kV, and mNBA (*meta*-nitrobenzylic alcohol) as matrix.

HPLC analyses were conducted according to versions of a previously described method.[61] This method consists of the separation of amines as ion pairs with *n*-octanesulfonate. Reversed-phase high-performance liquid chromatography (HPLC-1) was performed on a C18 column (Nucleosil 5, C18 AB, 100 mm−5 μm) from Macherey-Nagel (Düren, Germany) on a SpectroPhysics Thermoquest HPLC instrument (for compounds **4a**, **6a**, and **8a**) or on a C18 column (Nucleodur, 150 mm−5 μM) from Macherey-Nagel (Düren, Germany) on a LDC/Milton Roy HPLC instrument (for the other compounds). The solvent system used was the following: buffer A, 0.1 N potassium acetate, pH 4.75, 5% acetonitrile, and 10 mM *n*-octanesulfonate; buffer B, 0.1 N potassium acetate, pH 4.75, 50% acetonitrile, and 10 mM *n*-octanesulfonate; gradient, 0 min (0% B); 1 min (0% B); 15 min (30% B); 27 min (50% B); 31 min (100% B); 38 min (100% B); 39 min (0% B); 45 min (0% B); flow rate, 1 mL/min. A second reversed-phase HPLC (HPLC-2) was performed on a C18 column (Nucleodur, 150 mm−5 μM) from Macherey-Nagel (Düren, Germany) on a LDC/Milton Roy HPLC instrument with MeOH 20/buffer A 80 as solvent system with a 1.5 mL/min flow rate. Absorbance at 254 nm was recorded on a UV 2000 detector (Thermo Separation Products), and fluorescence intensity (excitation at 345 nm; emission at 455 nm) was recorded on a FL2000 fluorescence detector (Thermo Separa-

tion Products) after postcolumn derivatization with *o*-phthalaldehyde/*N*-acetylcysteine as previously described.[61]

Elemental analyses were performed by the Laboratoire de Microanalyses (Faculté de Pharmacie, Université Paris XI, Châtenay-Malabry).

Purity and characterization of compounds were established by a combination of combustion analysis, HPLC, HRMS, and NMR analytical techniques.

The compounds were numbered for the heterocyclic moiety by IUPAC rules and for the polyamine moiety by letters a−n (Figure 2).

Synthesis. Previously reported procedures were used for the synthesis of compounds **12**, **13**, **17**, and **18**;[54] **14** and **15**;[57] **4b**;[54,55] and **5b**.[55]

General Procedure for Thionation. A mixture of lactam (1 equiv) and Lawesson's reagent (0.7 or 1 equiv) was refluxed for 6 h in 60 mL of dry dioxane or dry toluene. After evaporation of solvent under reduced pressure, the residue was purified by column chromatography.

General Procedure A for Coupling Thiolactams to 3-Aminopropan-1-ol, Putrescine, or Spermine. To a refluxing solution of thiolactam (1 equiv) and 3-amino-1-propanol (10 equiv) or putrescine (25 equiv) or spermine (15 equiv) in 2 mL of dry THF was added $HgCl_2$ (1 equiv). The mixture was refluxed for 1 h, during which a black precipitate of HgS was observed. The THF was removed under reduced pressure and the residue was suspended in CH_2Cl_2. The mercury sulfide (HgS) was removed by filtration, and then the organic layer was washed with 0.2 M aqueous $Na_2S_2O_3$ to remove the unreacted $HgCl_2$. After drying over K_2CO_3, the organic layer was evaporated under reduced pressure. The residue was purified by column chromatography.

General Procedure B for Coupling Thiolactams to the Protected Spermidine. To a refluxing solution of N^4,N^8-di-(*tert*-butoxycarbonyl)spermidine[60] (5 equiv) and $HgCl_2$ (1 equiv) in 2 mL of dry THF was added dropwise a solution of thiolactam (1 equiv) in 3 mL of dry THF over 45 min. A black precipitate of HgS was observed. Refluxing was continued for 15 min under stirring. The THF was evaporated under reduced pressure. The Boc protective groups were removed by stirring the residue at 0 °C in 80 mL of 0.5 M aqueous HCl for 3 h. This aqueous solution was then filtered to remove HgS, alkalinized with 32% aqueous NaOH, and extracted with CH_2Cl_2. The organic layer was washed with a 0.2 M aqueous $Na_2S_2O_3$, dried over K_2CO_3, and evaporated under reduced pressure. The resulting crude residue was purified by column chromatography with isopropylamine, CH_3OH, and $CHCl_3$ in a 1/4/4 ratio.

General Procedure for the Preparation of Hydrochlorides. The amino conjugate was stirred in a 2 M solution of HCl gas in ethanol (1.2 equiv/amino group). After evaporation of the ethanol, the residue was triturated in anhydrous ether to give a white hygroscopic solid.

3,3-Dimethyl-4-(methylphenylamino)-3,4-dihydroquinoline-2(1*H*)-thione (19). General procedure for thionation from **14** (1.83 g, 6.52 mmol) and Lawesson's reagent (0.7 equiv) in dry dioxane. Column chromatography with CH_2Cl_2 (a). Yellow-orange solid, 58%; R_f 0.50 (a).

3,3-Dimethyl-4-(4-methylaminophenyl)-3,4-dihydroquinoline-2(1*H*)-thione (20). General procedure fof thionation from **15** (1.24 g, 4.42 mmol) and Lawesson's reagent (1 equiv) in dry dioxane. Column chromatography with CH_2Cl_2 95/Et_2O 5 (b). Yellow-orange solid, 46%; R_f 0.65 (b).

3,4-Dihydroquinoline-2(1*H*)-thione (21). General procedure for thionation from **16** (1 g, 6.80 mmol) and Lawesson's reagent (1 equiv) in dry toluene. Column chromatography with CH_2Cl_2 (c). Yellow-orange solid, 77%; R_f 0.36 (c).

(6aα,8*β*,13aα)-*N*-(6a,8,13,13a-Tetrahydro-6a,8,13-trimethyl-7*H*-quino[4,3-*b*][1]benzazepin-6-yl)-3-aminopropan-1-ol (4a). General procedure A from **17** (0.81 g, 2.4 mmol, 1 equiv) and 3-aminopropan-1-ol. Column chromatography with acetone/NH_4OH (1%) (d). Oil (34%). **Hydrochloride** (90%): R_f 0.57 (d), 0.68 (CH_2Cl_2/EtOH/NH_4OH 70/20/0.5, e), 0.17 (EtOH, f). HPLC-1 t_R 32.35 min, 98.2% pure. HPLC-2 t_R 27.31 min, 98.5% pure.

***N*-(3,3-Dimethyl-4-methylphenylamino-3,4-dihydroquinolin-2-yl)-3-aminopropan-1-ol (6a).** General procedure A from **19** (0.25 g, 0.85 mmol, 1 equiv) and 3-aminopropan-1-ol. Column chromatography with CH_2Cl_2/acetone/NH_4OH 80/20/0.5 (g). Oil; 39%; R_f 0.51 (g). HPLC-1 t_R 31.91 min, 99.5% pure. Anal. ($C_{21}H_{27}N_3O$, HCl, H_2O) C, H, N.

***N*-(3,4-Dihydroquinolin-2-yl)-3-aminopropan-1-ol (8a).** General procedure A from **21** (0.25 g, 1.41 mmol, 1 equiv) and 3-amino-1-propanol. Column chromatography with acetone/NH_4OH 100/0.5 (h). Oil, 52%; R_f 0.35 (h), 0.8 (acetone/NH_4OH 100/0.1, i). HPLC-1 t_R 21.93 min, 98.5% pure. HPLC-2 t_R 11.87 min, 98.5% pure.

***N*-[3,3-Dimethyl-4-(methylphenylamino)-3,4-dihydroquinolin-2-yl]putrescine (6b).** General procedure A from **19** (0.200 g, 0.675 mmol, 1 equiv) and putrescine. Column chromatography with CH_3OH/NH_4OH 90/10 (j). Oil, 72%; R_f 0.43 (j). HPLC-1 t_R 30.19 min, 99.5% pure. Anal. ($C_{22}H_{30}N_4$, 2HCl, H_2O) H, N; C calcd 59.86, found 60.79.

***N*-[3,3-Dimethyl-4-(4-methylaminophenyl)-3,4-dihydroquinolin-2-yl]putrescine (7b).** General procedure A from **20** (0.200 g, 0.675 mmol, 1 equiv) and putrescine. Column chromatography with CH_3OH/NH_4OH 90/10 (j). Oil, 72%; R_f 0.40 (j). HPLC-1 t_R 30.03 min, 98.9% pure. Anal. ($C_{22}H_{30}N_4$, 3HCl) C, H, N.

***N*-(3,4-Dihydroquinolin-2-yl)putrescine (8b).** General procedure A from **21** (0.250 g, 1.53 mmol, 1 equiv) and putrescine. Column chromatography with CH_3OH/NH_4OH 90/10 (j). Oil, 57%; R_f 0.21 (j). HPLC-1 t_R 24.14 min, 100% pure. Anal. ($C_{13}H_{19}N_3$, 2HCl, 1.2H_2O) C, H, N.

(6aα,8*β*,12bα)-N^1-(6a,7,8,12b-Tetrahydro-6a,8-dimethyl-9-methylaminobenzo[*k*]phenanthridin-6-yl)spermidine (5c). General procedure B from thiolactam **18** (0.4 g, 0.124 mmol, 1 equiv) and N^4,N^8-di-(*tert*-butoxycarbonyl)spermidine. Oil, 65%; R_f 0.29 (isopropylamine/CH_3OH/$CHCl_3$ 1/4/4, k). HPLC-1 t_R 30.66 min, 99.7% pure. Anal. ($C_{27}H_{39}N_5$, 4HCl, 0.9H_2O) C, H, N.

***N*^{1}-(3,3-Dimethyl-4-methylphenylamino-3,4-dihydroquinolin-2-yl)spermidine (6c).** General procedure B from **19** (0.35 g, 1.18 mmol, 1 equiv) and N^4,N^8-di-(*tert*-butoxycarbonyl)spermidine. Oil, 40%; R_f 0.36 (k). HPLC-1 t_R 30.41 min, 98.4% pure. Anal. ($C_{25}H_{37}N_5$, 3HCl, 1.3H_2O) C, H, N.

***N*^{1}-[3,3-Dimethyl-4-(4-methylaminophenyl)-3,4-dihydroquinolin-2-yl]spermidine (7c).** General procedure B from **20** (0.25 g, 0.844 mmol, 1 equiv) and N^4,N^8-di-(*tert*-butoxycarbonyl)spermidine. Oil, 52%; R_f 0.32 (k). HPLC-1 t_R 30.45 min, 99.7% pure. Anal. ($C_{25}H_{37}N_5$, 4HCl, 0.9H_2O) H, N; C calcd 52.71, found 53.46.

***N*^{1}-(3,4-Dihydroquinolin-2-yl)spermidine (8c).** General procedure B from **21** (0.2 g, 1.52 mmol, 1 equiv) and N^4,N^8-di-(*tert*-butoxycarbonyl)spermidine. Oil, 25%; R_f 0.23 (k). HPLC-1 t_R 28.48 min, 99.5% pure. Anal. ($C_{16}H_{26}N_4$, 3HCl, 1.2H_2O) H, N; C calcd 48.26, found 48.92.

(6aα,8α,13aα)-N^1-(6a,8,13,13a-Tetrahydro-6a,8,13-trimethyl-7*H*-quino[4,3-*b*][1]benzazepin-6-yl)spermine (4d). General procedure A from **17** (0.4 g, 1.24 mmol, 1 equiv) and spermine. Column chromatography with isopropylamine/CH_3OH/$CHCl_3$ 1/4/4 (k). Oil (50%). **Hydrochloride** (90%): R_f 0.27 in isopropylamine/CH_3OH/$CHCl_3$ 2/4/4 (l). HPLC-1 t_R 31.23 min, 100% pure. Anal. ($C_{30}H_{46}N_6$, 4HCl, 1.2H_2O) C, H, N.

(6aα,8*β*,12bα)-N^1-(6a,7,8,12b-Tetrahydro-6a,8-dimethyl-9-methylaminobenzo[*k*]phenanthridin-6-yl)spermine (5d). General procedure A from **18** (0.450 g, 1.39 mmol, 1 equiv) and spermine. Column chromatography with isopropylamine/CH_3OH/$CHCl_3$ 1/4/4 (k). Oil (31%). **Hydrochloride** (91%) R_f 0.11 (k), 0.16 (l). HPLC-1 t_R 30.59 min, 99.6% pure. Anal. ($C_{30}H_{46}N_6$, 5HCl, H_2O) H, N; C calcd 52.15, found 52.66.

***N*^{1}-(3,3-Dimethyl-4-methylphenylamino-3,4-dihydroquinolin-2-yl)spermine (6d).** General procedure A from **19** (0.4 g, 1.35 mmol, 1 equiv) and spermine. Column chromatography with isopropylamine/CH_3OH/$CHCl_3$ 2/4/4 (l). Oil, 48%; R_f 0.23 (l). HPLC-1 t_R 30.46 min, 100% pure. Anal. ($C_{28}H_{44}N_6$, 4HCl, 2H_2O) C, H, N.

***N*^{1}-[3,3-Dimethyl-4-(4-methylaminophenyl)-3,4-dihydroquinolin-2-yl]spermine (7d).** General procedure A from **20** (0.23 g, 0.777 mmol, 1 equiv) and spermine. Column chromatography with isopropylamine/CH_3OH/$CHCl_3$ 2/4/4 (l). Oil, 69%; R_f 0.24 (l). HPLC-1 t_R 30.58 min, 98.8% pure. Anal. ($C_{28}H_{44}N_6$, 5HCl, 0.6H_2O) H, N; C calcd 51.12, found 51.58.

***N*^{1}-(3,4-Dihydroquinolin-2-yl)spermine (8d).** General procedure A from **21** (0.23 g, 1.41 mmol, 1 equiv) and spermine. Column chromatography with isopropylamine/CH_3OH/$CHCl_3$ 2/4/4 (l). Oil, 59%; R_f 0.11 (l). HPLC-1 t_R 29.62 min, 99.1% pure. Anal. ($C_{19}H_{33}N_5$, 4HCl, 1.3H_2O) C, H, N.

Biological Studies. Unless otherwise stated, usual laboratory chemicals were purchased from Merck (Darmstadt, Germany) or Sigma (St Louis, MO). DFMO was obtained from Ilex Oncology (San Antonio, TX). Purified bovine serum oxidase was a generous gift from Professor B. Mondovi (University La Sapienza, Rome, Italy). [^{14}C]Putrescine dihydrochloride, [^{14}C]spermidine trihydrochloride, [^{14}C]spermine tetrahydrochloride, and [8-^{3}H]adenosine-3′,5′ cyclic phosphate were obtained from Amersham (Les Ulis, France). A SPEX Fluorolog 2 spectrofluorometer (Jobin Yvon, Longjumeau, France) was used for fluorescence measurements. All data are given as mean values of three or more experiments. Comparisons between means were made by Student's *t*-test, with the assumption of significance at $p < 0.05$.

Ethidium Bromide Displacement from Calf Thymus DNA. The release of ethidium bromide (EB) from the EB−DNA complex was monitored fluorometrically following the procedure described by Basu et al.[64] EB−DNA complex was formed by adding calf thymus DNA (0.2 A_{260} unit) to 3 mL of a solution of EB (2 μM in 50 mM NaCl and 1 mM sodium cacodylate, pH 7.0). The complex was titrated by adding small volumes of concentrated solutions of the test compounds at room temperature. Fluorescence ((λ_{exc} 546 nm; λ_{em} 595 nm) was monitored in 10 mm path length quartz cuvettes. The fluorescence values were corrected for the dilution of the complex caused by the addition of compound. CC_{50} values were the concentration of compounds that release 50% of the bound EB.

Assay of Calmodulin-Activated cAMP-Dependent Phosphodiesterase. The assay of PDE activity was performed as previously described.[44] Briefly, 0.2 milliunit of PDE (crude preparation from bovine heart containing calmodulin and Ca^{2+}, or an activator-deficient PDE from bovine heart) was incubated for 20 min at 37 °C in the presence of 0.1 mM [8-^{3}H]cAMP (10 μCi, Amersham, Les Ulis, France) and various concentrations of the drug to be tested in 30 mM Tris-HCl, 3 mM $MgSO_4$, and 0.6 mM AMP. The enzyme reaction was stopped by adding 50 μL of an aqueous solution of $ZnSO_4$ (21.5 mM) to the 100 μL reaction mixture. The addition of 50 μL of a solution of 17.7 mM $Ba(OH)_2$ precipitated the AMP together with $BaSO_4$. Radioactivity was counted in a 150 μL aliquot of the supernatant.

Oxidation by Bovine Serum Amine Oxidase. Purified bovine serum amine oxidase was used for the determination of the substrate properties of the compounds. Hydrogen peroxide formation was determined at 10 μM of each compound using the horseradish peroxidase-catalyzed oxidation of homovanillic acid into a fluorescent product, following a published procedure.[93]

Cell Culture. Murine leukemia L1210, CHO, and CHO-MG cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, glutamine (2 mM), penicillin (100 units/mL), streptomycin (50 μg/mL) (Eurobio, Les Ulis, France). L-Proline (2 μg/mL) was added to the culture medium for 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 the oxidation of the drugs by the serum amine oxidase present in the calf serum.

In Vitro Evaluation of Drug Cytotoxicity/Cytostasy. The effect of the amidine derivatives on L1210 cell growth was assayed in sterile 96-well microtiter plates (Becton Dickinson, Oxnard, CA). L1210 cells were seeded at 5×10^4 cells/mL of medium (100 μL/well). Single CHO and CHO-MG cells harvested by trypsinization were plated at 2×10^3 cells/mL. Drug solutions (5 μL/well) of appropriate concentration were added at the time of seeding for L1210 cells and after an overnight incubation for CHO and CHO-MG cells. In some experiments, 5 mM DFMO was added in the culture medium at the time of drug addition. 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 by use of a Titertek Multiskan MCC/340 microplate reader (Labsystems, Cergy-Pontoise, France) for absorbance (540 nm) measurements.[94]

Polyamine Uptake Inhibition in L1210 and CHO Cells. The ability of the amidine derivatives to compete with radiolabeled putrescine, spermidine, or spermine for uptake was determined in L1210 cells by a 10 min uptake assay at 37 °C in the presence of increasing concentrations of competitor, with 9.4 μM [^{14}C]putrescine, 1 μM [^{14}C]spermidine, or 1 μM [^{14}C]spermine as substrate. The assay mixture contained 2×10^6 L1210 cells and was performed in a final volume of 0.6 mL of Hanks' balanced salt solution supplemented with 20 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HBSS−HEPES). Initially, the K_m values of putrescine, spermidine, and spermine transport were determined as previously described.[95] K_i values were determined by use of the Cheng−Prusoff equation[96] from the IC_{50} value derived by iterative curve fitting of the sigmoidal equation describing the velocity of polyamine uptake in the presence of the respective competitor.[32,97]

The ability of the amidine derivatives to inhibit the activity of the PTS was assayed in CHO cells. CHO cells were grown in 12-well plates. After three washings in prewarmed PBS, 1 mL of a prewarmed solution of HBSS−HEPES buffer containing various concentrations of drug to be tested was added in each well. In some experiments, 200 μM CHX was added. After incubation at 37 °C (2−120 min), the medium was eliminated, and the wells were washed with prewarmed buffer. The reaction was initiated by the addition of 1 μM [^{14}C]spermidine in 500 μL of buffer. After an incubation at 37 °C for 5 min, the cells were washed three times with ice-cold PBS and lysed in 0.1 N NaOH. Aliquots were used for protein determination[98] and for scintillation counting.

Cellular Uptake. For determination of the cellular uptake of the derivatives, cells were seeded in culture flasks at 4×10^4 cells/mL for L1210 cells and at 2×10^5 cells/mL for CHO and CHO-MG cells. Drugs were added at the time of seeding for L1210 and 24 h after seeding for CHO and CHO-MG cells. Cells were harvested 24 h (CHO and CHO-MG) or 48 h (L1210) after drug addition. Harvested cells were washed 3 times in cold 0.14 M NaCl. Cell pellets were disrupted by sonication in 1 mL of 0.2 N $HClO_4$. After a night at 4 °C, homogenates were centrifuged at 15 000 rpm for 30 min. Supernatants were used for HPLC determination of polyamine and derivatives as described above. Pellets were dissolved in 0.1 N NaOH and used for protein determination.

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Supporting Information Available: Spectroscopic and purity data for target compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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