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Xylylated Dimers of Putrescine and Polyamines: Influence of the Polyamine Backbone on Spermidine Transport Inhibition

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Abstract—Dimeric norspermidine and spermidine derivatives are strong competitive inhibitors of polyamine transport. A xylyl tether was used for the dimerization of various triamines and spermine via a secondary amino group, and of putrescine via an ether or an amino group. Dimerization of putrescine moieties potentiates their ability to compete against spermidine transport to a much greater extent than for triamine dimers.

Polyamines are ubiquitous polycations that are essential for cell proliferation.[1,2] Intracellular polyamine levels are determined by the rate of their biosynthesis and degradation and by the activity of membrane transporters for polyamine uptake and excretion.[3–5] Drugs aimed at depleting the polyamine pools such as inhibitors of the biosynthetic pathway are of great interest in antitumor therapy. Alpha-difluoromethylornithine (DFMO) is an irreversible inhibitor of ornithine decarboxylase, the enzyme responsible for putrescine biosynthesis, and exhibits potent antiproliferative effects in vitro and in some animal models.[6,7] However, clinical cancer trials with DFMO have generally been disappointing.[6,7] This limited efficacy has been partly attributed to the dramatic enhancement of polyamine uptake resulting from inhibition of polyamine biosynthesis.[3,5–8] Indeed, exogenous polyamines at the low levels similar to those found in human plasma can fully restore growth in cells treated with DFMO[9] Moreover, decontamination of the gastro-intestinal tract with antibiotics or feeding a polyamine-deficient diet can markedly improve the antitumor efficacy of DFMO.[10–12] The major contribution of polyamine transport to polyamine homeostasis is further supported by the fact that the antitumor effect of DFMO is potentiated by the inhibition of heparan sulfate assembly,[13] or by genetic inactivation of the polyamine transport system. [14]

These observations led to the concept that inhibition of polyamine transport with specific antagonists might potentiate the therapeutic action of DFMO under in vivo conditions where plasma polyamines tend to replenish cellular pools.[9,13,15] A first generation of competitive transport inhibitors had been designed to antagonize polyamine uptake, including hexapyridinium salts [16] and polymeric polyamine conjugates,[17] but their in vivo efficacy and toxicity were not evaluated. More recently, various spermine monoamides or-sulfonamides, including a lysine-spermine conjugate, have been described as candidate antagonists of polyamine transport with high potency and low toxicity.[18,19] However, metabolic lability of the amide linkage might be an intrinsic limitation to the use of such conjugates for the effective inhibition of polyamine transport in vivo.

We previously showed that a dimeric spermine derivative afforded several-fold stronger competitive inhibition of polyamine transport than its monomeric counterpart,[9] a concept that was recently confirmed for a series of spermine derivatives dimerized via their

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terminal amino group.[20] We later reported the synthesis of homodimers of spermidine and *sym*-norspermidine cross-linked via their central imino group with aliphatic chains of different length and degree of unsaturation, or with a xylyl group.[15] The most potent transport antagonist in the latter series, *N*,*N*,*N*′,*N*′-tetrakis-(3-aminopropyl)-*p*-diamino-xylene (BNSD-X) (Fig. 1) exhibited the unique ability to prevent competitive rescue of DFMO-induced cytostasis by physiological concentrations of exogenous spermidine in several mammalian cell lines.[15] Thus, a *p*-xylyl cross-linker is optimal for generating triamine dimers that potently inhibit polyamine uptake.

The latter studies assessed the effect of the crosslinker structure on the potency of identical triamine dimers as antagonists of polyamine transport. In the present report, we have cross-linked polyamines of various aliphatic chain length and charges with a *p*-xylyl tether to evaluate the role of the polyamine backbone on the potency of transport inhibition by the resulting dimers.

Characterization of the products by ^{1}H and ^{13}C NMR spectrometry was performed as described previously.[15] Bis(tetramethylene)triamine (4-4-TA) and bis(pentamethylene)triamine (5-5-TA) are not commercially available and were prepared by dialkylation of benzylamine **1** with the appropriate bromoalkanenitrile followed by reduction of the resulting nitrile **2** using Raney nickel and then catalytic hydrogenolysis of the benzyl group using palladium on carbon (Scheme 1).[21,22] Primary groups of the various triamines **3** were regioselectively protected with trityl chloride in the presence of diethylamine.[15,23] Spermine **4** was protected using 3 equiv of trityl chloride,[24] thus leaving a single free secondary amino group. The following steps of the synthesis (Scheme 1), including coupling with α,α′-dibromo-*p*-xylene, and acid hydrolysis of the trityl groups were carried out as described.[15] Final purification of the products was carried out by cation exchange chromatography using Dowex 50X8-100 with a HCl gradient (0 to 8 N) as eluent. We thus obtained B-4-4-X **5a**, B-5-5-X **5b**, B-6-6-X **5c**, and BSM-X **6** as their hydrochloride salts [25] following elution from the cation exchange column between 6 and 8 N HCl (**5a**, **5b** and **6**) or between 4 and 6 N (**5c**).

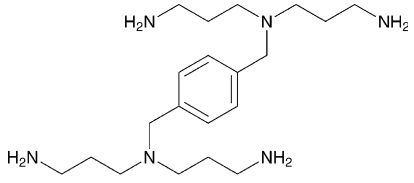
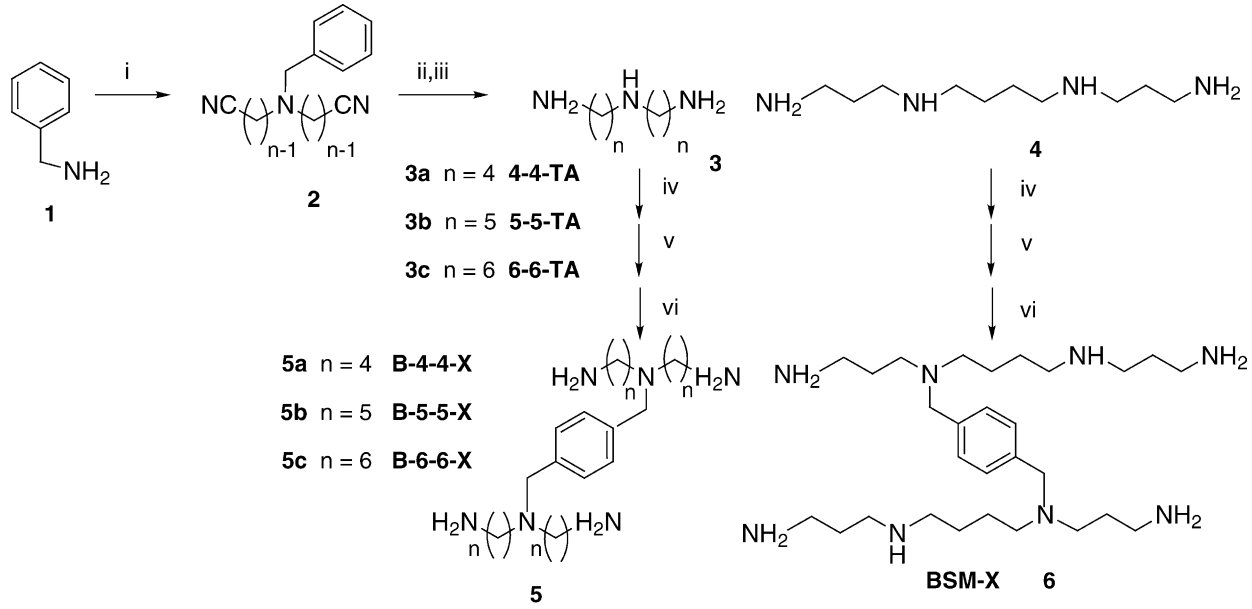


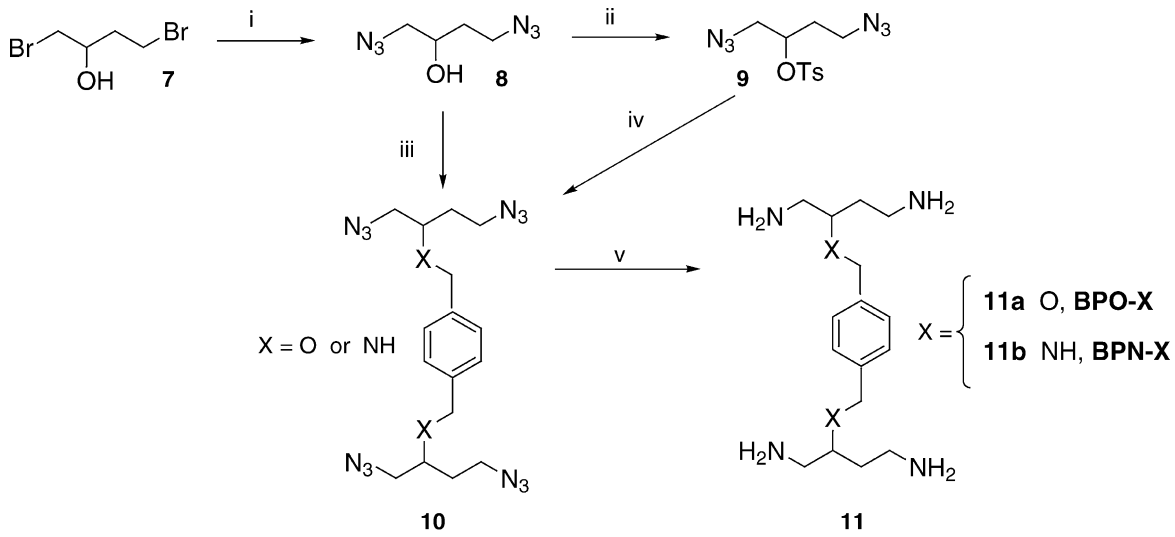
Figure 1. Structure of *N*,*N*,*N*′,*N*′-tetrakis-(3-aminopropyl)-*p*-diaminoxylene (BNSD-X), a prototype for the design of polyamine transport inhibitors.

The 1,4-diazidobutanol **8** (Scheme 2) was prepared from 1,4-dibromobutan-2-ol **7** using NaN_3.[25] Dimerization of the putrescine analogue **8** was then performed using α,α′-dibromo-*p*-xylene in the presence of NaH. The tosylate **9** was prepared prior to the coupling reaction with *p*-xylylenediamine. The putrescine dimers BPO-X **11a** and BPN-X **11b** were obtained upon reduction of the azido group with PPh_3.[26–28]

The potency of the polyamines and their xylylated dimers as competitive inhibitors of spermidine transport was evaluated in T-47D human breast cancer cells (Table 1). Consistent with previous reports,[3,8,29] putrescine was a poor inhibitor of spermidine uptake. Likewise,[21,30] *sym*-homospermidine (4-4-TA) was an about 3-fold stronger inhibitor of spermidine uptake than *sym*-norspermidine (3-3-TA), which might be due to the higher pK_3 value of polyamines bearing aminobutylamine groups.[32] The K_i of the triamine 5-5-TA was



Scheme 1. General route of synthesis of xylylated triamine and spermine dimers: (i) $Br(CH_2)_{n-1}CN$ (2 equiv), K_2CO_3, K_i (cat.), *n*-butanol, 115 °C (yield: 70–75%); (ii) H_2/Raney nickel, EtOH, 1 N NaOH (yield: 90–98%); (iii) H_2/$Pd(OH)_2$, EtOH (yield: 95–98%); (iv) TrCl, $N(Et)_3$, $CHCl_3$ (yield: 75%); (v) α,α′-dibromo-*p*-xylene, K_2CO_3, DMF (cat.), acetonitrile, reflux (yield: 90%); (vi) 3 N HCl, reflux (yield: 95%).



Scheme 2. General route of synthesis for putrescine dimers: (i) NaN_3, DMF (yield: 99%); (ii) *p*-TsCl, pyridine, CH_2Cl_2 (yield: 97%); (iii) (a) NaH, DMF; (b) α,α′-dibromo-*p*-xylene (yield: 75–80%); (iv) *p*-xylylenediamine, K_2CO_3, DMF (cat), acetonitrile, reflux (yield: 85%); (v) PPh_3, H_2O, THF (yield: 90–95%).

Table 1. K_i values for the inhibition of spermidine transport by polyamine dimers linked through a xylyl group in T-47D human breast cancer cells

Compd	Substrates or inhibitors	K_m or K_i[a] (μM)
	Putrescine (1,4-diaminobutane)	>500
11a	BPO-X	12.8±0.6[b]
11b	BPN-X	9.3±1.1
	Spermidine (3-4-TA)[c]	4.4±0.9[d]
	sym-Norspermidine (3-3-TA)	9.5±1.3
3a	*sym*-Homospermidine (4-4-TA)	2.7±0.7
3b	5-5-TA	3.4±0.6
3c	6-6-TA	20.9±3.8
	BNSD-X (B-3-3-X)	1.5±0.1[e]
	BSD-X (B-3-4-X)[f]	2.0±0.2[e]
5a	B-4-4-X	2.8±0.5
5b	B-5-5-X	2.8±0.3
5c	B-6-6-X	9.4±0.9
4	Spermine	2.1±0.2
6	BSM-X	1.1±0.1

[a]K_i values were calculated from the half-maximal inhibitory concentration (IC_{50}) estimated by iterative curve fitting for sigmoidal equations describing spermidine uptake velocity in the presence of growing concentrations of antagonist.[15]
[b]Data are presented as the mean±SD of two independent determinations of IC_{50} values, each based on triplicate determinations of uptake velocity at increasing inhibitor concentrations.
[c]In the terminology used here: TA, triamine; B, bis (to indicate dimerization); X, *p*-xylyl moiety; P, putrescine; NSD, *sym*-norspermidine; SD, spermidine; SM, spermine.
[d]The K_m for spermidine uptake was independently determined by Lineweaver–Burke analysis of transport velocity data at increasing substrate concentrations (0.01–300 μM).
[e]From ref 15.
[f]Xylylated dimer of spermidine.[15]

comparable to the K_m of spermidine (3-4-TA), but lower than that found for 6-6-TA.

Dimerization of the triamines with a xylyl cross-linker improved the inhibitory potency of spermidine (3-4-TA), *sym*-norspermidine (3-3-TA) and 6-6-TA to compete against spermidine transport, whereas no significant effect was observed for 4-4-TA or 5-5-TA. Likewise, the spermine dimer BSM-X had a 2-fold lower apparent K_i for spermidine uptake inhibition than spermine. Quite remarkably, dimerization of putrescine via a *p*-xylylenediamino or a *p*-xylylenediether cross-linker improved its transport inhibitory potency by >50-fold relative to the diamine.

Thus, increasing the number of cationic centers generated by dimerizing two diamines dramatically improves the ability of the resulting polyamine to interact with the polyamine carrier. Despite the charge separation due to the presence of the *p*-xylyl cross-linker, the tetramine BPO-X had a transport inhibitory potency in the order of that observed for the hexamine B-6-6-X. Moreover, the presence of two extra charges in the BPN-X putrescine-like dimer did not substantially potentiate spermidine uptake inhibition over that noted for BPO-X, suggesting that the presence of four cationic centers is nearly sufficient for an optimal interaction with the polyamine carrier. Of special interest is the fact that compounds BPN-X and BPO-X each contain two chiral centers, and therefore each compound exists as a *meso* compound with three different diastereoisomers. The separation and assessment of the various diastereoisomers of these dimers should provide further insight in the structural requirements for optimal interaction of xylylated polyamine transport antagonists of this series with the polyamine carrier.

On the other hand, the extent to which spermidine uptake inhibition is potentiated by dimerizing the triamines depends on the number of methylene groups connecting the amino groups. As demonstrated here, linear[30,31,33] or N^4-substituted homospermidine chains[33,34] better interact with the polyamine carrier than their spermidine or *sym*-norspermidine counterparts. Surprisingly, however, an aminopropyl moiety confers an optimal ability to dimeric xylylated polyamines for competing against spermidine uptake, as suggested by the fact that the lowest K_i values were

observed for BNSD-X and BSM-X, which both bear four aminopropyl groups, and BSD-X, in which two aminopropyl groups can face each other on the opposite ends of the cross-linker (Table 1).

Covalent linking of the central amino group of triamines to a benzyl strongly decreases the efficiency of polyamine transport inhibition.[30–34] Therefore, to account for the structure–activity relationships of the putrescine dimers, the decrease in transport antagonism that is conferred to any single polyamine chain by introducing the *p*-xylyl cross-linker must be counteracted by additional binding interactions afforded by the presence of the second polyamine chain. Accordingly, a cross-linker of optimal length and structure such as the *p*-xylyl chain[15] could be viewed as a 'hinge' allowing the simultaneous and cooperative interaction of both diamine or polyamine chains of the dimers. Moreover, the efficiency of the latter interaction should be a function of the electrostatic repulsion between the two positively charged chains, and of additional conformations made possible by the formation of intramolecular hydrogen bonds and/or hydrophobic interactions. Thus, the second polyamine chain introduced by dimerization of a triamine possessing suboptimal methylene backbone lengths between cationic centers, such as the 6-6-TA, might promote a more efficient binding to the polyamine carrier through such additional interactions, as in the case of the putrescine-like dimers. Likewise, novel conformational arrangements of the aminopropyl chains resulting from the dimerization of spermine, spermidine, and *sym*-norspermidine could be responsible for the improved interaction with the spermidine transporter observed for BSM-X, BSD-X, and BNSD-X, respectively. Evidence for a role of intramolecular hydrogen bonds in the efficiency of triamine binding with the polyamine transporter has already received experimental support. [8,32]

Acknowledgements

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^{13}C NMR ($CDCl_3$) δ 137.9, 127.6, 78.6, 70.7, 44.7, 38.5, 35.6; **BPN-X:** ^1H NMR (D_2O) δ 7.58 (m, 4H, aromatic-H); 4.2 (m, 4H, 2×CH_2Ph); 3.8 (m, 2H, 2×CH); 3.3 (m, 4H, 2×CH_2N); 3.2 (m, 4H, 2×CH_2N); 2.1–2.4 (m, 4H, 2×CH_2).

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