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Synthesis and bioevaluation of *N*-(arylalkyl)-homospermidine conjugates

Songqiang Xie,[a,b] Pengfei Cheng,[c] Guangchao Liu,[d] Yuangfang Ma,[d] Jin Zhao,[a] Mounir Chehtane,[e] Annette R. Khaled,[e] Otto Phanstiel, IV[f,*] and Chaojie Wang[a,c,*]

[a]*Institute of Natural Products & Medicinal Chemistry, Henan University, Henan, Kaifeng 475001, China*
[b]*College of Pharmacy, Henan University, Henan, Kaifeng 475001, China*
[c]*Chemistry Department, Henan University, Henan, Kaifeng 475001, China*
[d]*Medical College, Henan University, Henan, Kaifeng 475001, China*
[e]*Biomolecular Science Center, University of Central Florida, Orlando, FL 32816, USA*
[f]*Department of Chemistry, University of Central Florida, Orlando, FL 32816, USA*

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Abstract—N^1-(Arylalkyl)homospermidines (**1c**–**1f**) and terminally piperazine-substituted homospermidine conjugates (**2a**–**2e**) were synthesized and evaluated for cytotoxicity in mouse leukemia L1210, α-difluoromethylornithine (DFMO)-treated L1210, melanoma B16, spermidine (SPD)-treated B16, and HeLa cell lines. Results demonstrated that homospermidine was a more effective vector than piperazine-substituted homospermidine in ferrying diverse arenes into cells via the polyamine transporter. The leading compound, 9-anthracenemethyl-homospermidine (**1a**), was shown to induce apoptosis in B16 cells and IL-3 dependent FL5.12A pro-B cells. The novel conjugate 4-biphenylmethyl-homospermidine (**1e**) could also induce apoptosis. However, it exhibited different effect on the cell cycle of B16 cells compared to **1a**.

One of the main drawbacks of current cancer chemotherapies is the non-selective delivery of anticancer drugs to both cancerous and normal tissues. The selective delivery of antitumor drugs to targeted cells is a worthwhile endeavor. A success in this area would enhance drug potency and should reduce side effects attributed to non-specific delivery. This goal may be realized through conjugates between antitumor agents and smart vectors, which have elevated affinity for cancer cells.[1] Polyamines are one such potential vector. In particular, polyamines are important growth factors essential for cell proliferation. Many tumor cell types become highly dependent on growth factor stimuli and can either biosynthesize these growth factors internally or import them from exogenous sources via the polyamine transporter (PAT). One hypothesis is that tumors are unable to biosynthesize enough polyamines to sustain their rapid growth rates and rely on the PAT to make up the difference. Structure–activity studies of PAT ligands have demonstrated a wide structural tolerance of this transporter for non-native polyamine derivatives.[2,3]

Indeed, some polyamine-drug conjugates have been reported, which follow this strategy.[4–6] Although the natural polyamines (putrescine, spermidine, and spermine) have been used extensively as vectors, previous efforts revealed that the non-native triamine, homospermidine, was better than its natural counterparts.[7,8] A series of N^1-(arylalkyl)-homospermidine conjugates in which the N^1-substituent was systematically altered from benzyl, naphthylmethyl, anthracenylmethyl to pyrenylmethyl were designed to evaluate the size limitations of the PAT.[9]

These studies confirmed that the PAT could accommodate even the relatively large pyrene group. However, N^1-(anthracen-9-ylmethyl)-homospermidine (**1a**) and N^1-(1-naphthyl-methyl)-homospermidine (**1b**) displayed the best PAT recognition and selectivity in a Chinese hamster ovary (CHO) screen involving CHO wt (PAT-active cells) versus the CHOMG (PAT inactive) mutant cells.[9]

Keywords: Homospermidine; Polyamine conjugate; Synthesis; Apoptosis.
* Corresponding authors. Tel.: +86 136 1981 0550 (C.W.); Tel.: +407 823 5410; fax: +407 823 2252 (O.P.); e-mail addresses: ophansti@mail.ucf.edu; wcjsxq@yahoo.com

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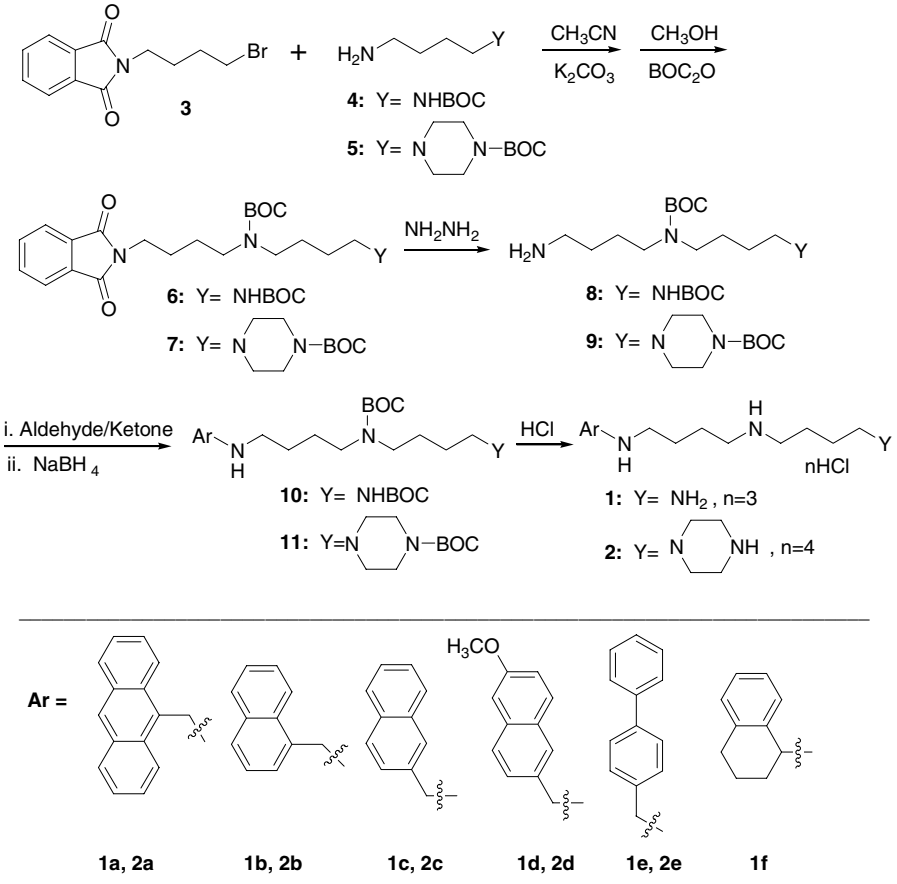
Systematic investigations have helped to define the key properties of polyamine conjugates suitable for PAT use such as the number and spacing of charges and the optimal N^1-tether length.[10,11] Indeed, there were clear size constraints in terms of how large a N^1-substituent the PAT could accommodate. Previously, planar arene–homospermidine conjugates were studied. In this report, more diverse bicyclic arenes were attached to homospermidine to further evaluate its efficacy as a carrier. In addition, the conjugates of a special tetraamine, the terminally piperazine-modified homospermidine, were also synthesized to understand the optimum vector characteristics. Finally, induction of apoptosis by **1a**, the most potent of the homospermidine conjugates tested, was demonstrated by flow cytometry analysis.

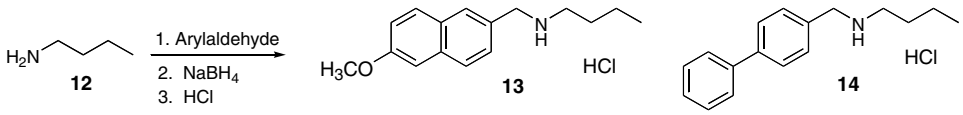
The target compounds (**1a**–**1f** and **2a**–**2e**) were prepared by the Gabriel method according to Scheme 1. The starting materials *N*-(4-bromobutyl)phthalimide (**3**) and mono-Boc-protected 1,4-diaminobutane (**4**) were easily synthesized.[12] The mono-Boc-protected triamine **5** was also prepared by the Gabriel method. The N-alkylation reactions (involving **3** and **4**/**5**) were performed in the presence of K_2CO_3 in dry acetonitrile at 40–50 °C for 8–12 h. Subsequently *N*-Boc protection with BOC_2O led to the formation of intermediates **6** and **7**. Deprotection of the phthalimide groups of **6** and **7** with an excess of hydrazine hydrate in ethanol at room temperature for 12 h gave the di-Boc protected polyamine motifs **8** and **9**, respectively. The yield of these three steps was ~38%.

Condensations of the key polyamine intermediates, **8** or **9**, with different aldehydes or ketones, generated the corresponding imines in situ, which were directly reduced with $NaBH_4$ to give the related amines (**10**, **11**).[13] These two reactions were realized at room temperature for 12 h with methanol and dichloromethane (50%, v/v) as solvents. Because these amine motifs were unstable,[8] their Boc groups were removed immediately with 4N HCl at room temperature to provide target compounds as hydrochloride salts (**1**, **2**) in nearly 75% yield for these three steps.[7] As shown in Scheme 2, two control compounds (**13**, **14**) were prepared from butylamine **12** and the corresponding arylaldehydes. Their structures were confirmed by ^1H NMR, ^{13}C NMR, ESI-MS, and elemental analysis.

Three cell lines, murine leukemia L1210, murine melanoma B16, and human cervical cancer HeLa, were chosen for the preliminary bioevaluation by the MTT cytotoxicity assay in the presence of aminoguanidine (AG, an inhibitor of amine oxidase).[10] In the L1210 experiments, the novel conjugates were administered in the presence and absence of α-difluoromethylornithine (DFMO, 0.5 mM). DFMO is a known irreversible inhibitor of ornithine decarboxylase (ODC)—the enzyme required for the first stage in polyamine biosynthesis.[2] Inhibition of ODC blocks intracellular polyamine



Scheme 1.



Scheme 2.

biosynthesis and leads to a significant increase in polyamine uptake from exogenous sources.[3] Therefore, cells, which are treated with DFMO, should take up more of the toxic polyamine conjugates via the PAT. Therefore, conjugates which selectively target the PAT should give lower IC_{50} values with DFMO-treated cells and provide L1210/(L1210+DMFO) IC_{50} ratios greater than 1.

As shown in Table 1, the IC_{50} values of the homospermidine conjugates **1c**–**1f** were slightly higher than that of reference compounds **1a** and **1b**. However, their IC_{50} values were still within the same order of magnitude and gave a twofold enhancement in cytotoxicity in the presence of DFMO. The novel tetraamine conjugates **2a**–**2e** were less potent than their homospermidine counterparts, especially **2a** and **2b** (L1210 $IC_{50} > 50$ μM). Lastly, the presence of DFMO resulted in increased potency for all conjugates (i.e., gave lower IC_{50} values).

Earlier studies with **1a** revealed a direct correlation between conjugate uptake and cytotoxicity.[10] DFMO treatment increased the import of **1a** and resulted in a more toxic compound (lower IC_{50} value). In a similar fashion, DFMO facilitates the entry of these new conjugates and gives lower IC_{50} values in the presence of DFMO. Therefore, the PAT of L1210 cells can also tolerate structural variances of the N^1-substituent. These results were similar to the previous report in CHO cells.[7]

Interestingly, the anthracene substituent was confirmed to be the most toxic agent of the series for the homospermidine vector (e.g., **1**), but not for the piperazine-modified moiety (i.e., **2**). This suggests that different polyamine vectors may be able to facilitate entry of alternative N^1-substituents.

As shown in Table 2, the homospermidine conjugates **1c**–**1f** had similar cytotoxicities as the naphthyl derivative **1b** in both the B16 and HeLa cell lines, while **1a** remained the most potent agent. The performance of **2a**–**2e** was mixed. All the homospermidine conjugates (**1a**–**1e**) possessed higher activity than their respective tetraamine counterparts (**2a**–**2e**). In this regard, the homospermidine motif provided the better vector motif.

Table 1. Bioactivity of polyamine conjugates in L1210 cells in vitro

Compound	L1210 IC_{50} (μM)	L1210+DFMO IC_{50} (μM)	L1210/(L1210+DFMO) IC_{50} ratio
1a	4.80 ± 0.11	3.60 ± 0.13	1.33
1b	8.04 ± 0.17	5.08 ± 0.22	1.58
1c	16.94 ± 0.32	8.13 ± 0.23	2.08
1d	15.06 ± 0.16	5.60 ± 0.13	2.69
1e	9.98 ± 0.38	4.86 ± 0.09	2.05
1f	14.77 ± 0.23	9.08 ± 0.75	1.63
2a	>50	17.89 ± 0.30	>2.5
2b	>50	37.88 ± 0.52	>1
2c	35.77 ± 0.42	23.35 ± 0.59	1.53
2d	15.09 ± 0.20	8.57 ± 0.25	1.76
2e	20.26 ± 0.58	7.48 ± 0.33	2.71

IC_{50} values from three independent experiments were given as means ± SD. IC_{50} values were given only if they were less than 50 μM, which was the maximum concentration tested.

The SPD experiments revealed that the added SPD led to an obvious decrease in cell death for all conjugates (higher IC_{50} values). With the exception of **2d** and **2e**, significant rescue was observed. Overall, compounds **1a** and **2c** were the most sensitive to the presence of SPD. Since the SPD additive provided significant cellular rescue, the uptake of these polyamine conjugates is most likely via the PAT.

Two control compounds, **13** and **14**, were synthesized in order to evaluate the role of the polyamine motif. These materials do not contain the "smart" homospermidine motif, but are still water soluble and contain the corresponding aryl group. As such they represent appropriate water-soluble aryl controls.[10] Using Table 2, IC_{50} comparisons of methoxy-naphthyl **13** with **1d** (and 4-phenyl-benzyl **14** with **1e**) in B16 and HeLa cells proved that conjugation of an aryl architecture onto the homospermidine vector dramatically enhanced its cytotoxicity.

Compounds **1a** and **1e** exhibited potent activity (against several tumor cell lines) from this series of homospermidine conjugates, but their precise anti-tumor mechanism has not been elucidated. Other studies have indicated that the alkyl polyamine analogues could produce cytotoxicity via apoptosis.[14] Since, **1a** has been shown to be very selective in killing the B16 cell line,[10] we investigated whether **1a** and **1e** could induce apoptosis in B16 cells.

The preliminary flow cytometry DNA analysis revealed that **1a** induced G0/G1 phase cell cycle arrest with an accompanying decrease in S phase. A typical feature of cells undergoing apoptosis is the occurrence of a sub-G1 cell population by flow cytometry analysis of DNA content. After treatment with **1a**, the sub-G1 cell population was observed clearly in a dose-dependent manner, as shown in Figure 1. However, **1e** induced S phase cell cycle arrest with an accompanying decrease in G_0/G_1 phase and G_2/M phase. Though the cytotoxic activity of **1e** was weaker than that of **1a**, the sub-G1 cell population was remarkably increased compared to **1a** at the same dose, as shown in Figure 2. These data suggest that **1a** and **1e** are able to induce apoptosis in B16 cells, and the related mechanism may be different.

Compound **1a** was also tested in a growth-factor-dependent B lymphocyte line. B lymphocytes are known to express active polyamine transporters as they enter and

Table 2. Cytotoxicity of homospermidine derivatives in B16 and HeLa cells in vitro

Compound	B16 IC_{50} (μM)	B16+SPD IC_{50} (μM)	(B16+SPD)/B16 IC_{50} ratio	HeLa IC_{50} (μM)
1a	0.31 ± 0.02	2.06 ± 0.03	6.6	1.10 ± 0.06
1b	2.33 ± 0.13	5.86 ± 0.13	2.5	1.38 ± 0.08
1c	1.66 ± 0.15	6.29 ± 0.48	3.8	1.90 ± 0.21
1d	4.88 ± 0.17	17.41 ± 0.49	3.6	1.20 ± 0.08
1e	1.22 ± 0.06	2.87 ± 0.22	2.4	3.61 ± 0.31
1f	6.96 ± 0.14	12.07 ± 0.20	1.7	—
2a	6.07 ± 0.19	12.22 ± 0.43	2.0	9.58 ± 1.15
2b	5.46 ± 0.20	12.50 ± 0.16	2.3	3.60 ± 0.50
2c	2.89 ± 0.13	25.88 ± 2.08	9.0	3.70 ± 0.45
2d	>50	>50	—	11.35 ± 0.29
2e	>50	>50	—	26.82 ± 2.46
13	15.01 ± 0.20	—	—	12.54 ± 0.69
14	10.84 ± 1.09	—	—	10.87 ± 1.50

IC_{50} values from three independent experiments were given as means ± SD. IC_{50} values were given only if they were less than 50 μM, which was the maximum concentration tested.

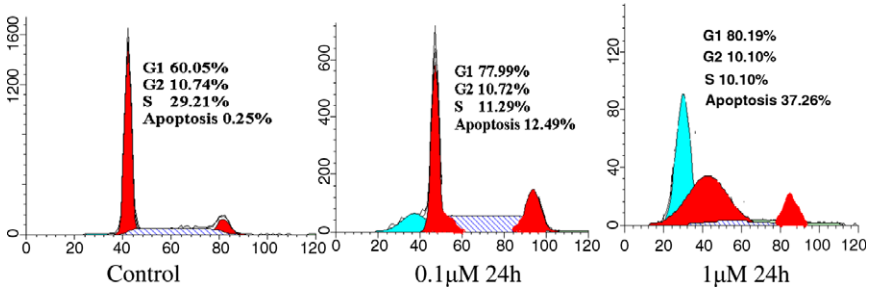


Figure 1. The effect of **1a** on cell cycle and apoptosis rate in B16 cells.

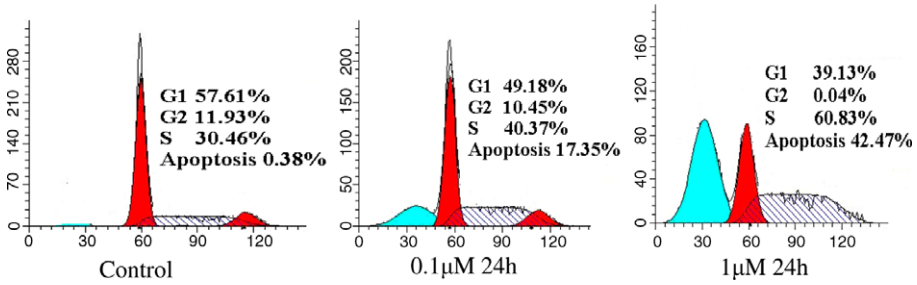


Figure 2. The effect of **1e** on cell cycle and apoptosis rate in B16 cells.

progress through the cell cycle.[15] The IL-3-dependent pro-B cell line, FL5.12A,[16] was cultured for 24 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, which causes cell cycle arrest and induces apoptosis. Compound **1a** (10 μM) was added in the presence of aminoguanidine (AG) (1 mM). Twenty-four hours after addition of the compounds, apoptosis was detected by measuring the binding of Annexin-V-FITC. In apoptotic cells, the membrane phospholipid, phosphatidylserine (PS), moves from the inner to the outer layer of the plasma membrane, exposing PS to the extracellular environment. Annexin-V, conjugated to the fluorochrome, FITC, has a high binding affinity for PS and thus serves a marker for apoptotic cells that can be detected by flow cytometric analysis. As shown in Figure 3, treatment with AG did not induce apoptosis in the presence of IL-3 (Fig. 3a and b); while, loss of IL-3 did (Fig. 3c). Treatment with compound **1a** caused increasing cell death, which correlated to the loss of the IL-3 growth factor. In the presence of 2 ng/mL IL-3, 62% of cells were apoptotic (Fig. 3d), in the presence of 0.1 ng/mL IL-3 (Fig. 3e), 75% of the cells were apoptotic, and in the absence of IL-3, 99% of the cells were apoptotic (Fig. 3f). This suggests that, in both actively cycling (IL-3 = 2 ng/mL) and non-cycling (IL-3 = 0.1 ng/mL) B cells, compound **1a** is a potent inducer of cell death.

In summary, the homospermidine motif enhanced the delivery of various N^1-cargoes of the proper size[10] into the target cells via the PAT. This was confirmed via spermidine rescue experiments. In contrast, the piperazine-containing polyamine performed like a common tetraamine, and did not show much promise as an effi-

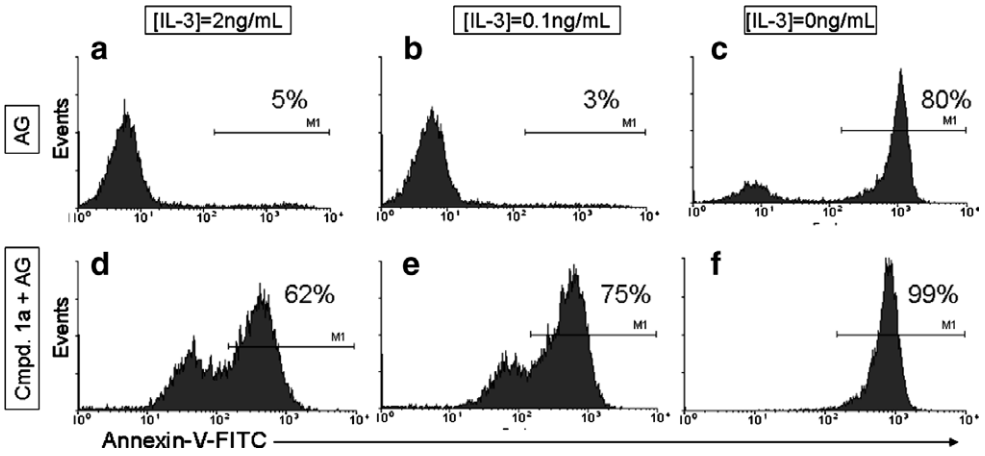


Figure 3. The effect of **1a** on the apoptosis of IL-3 dependent FL5.12A pro-B cells. (a–c) FL5.12A cells were cultured with IL-3 (2, 0.1, and 0 ng/ml) for 24 h. AG (1 mM) was added and cells cultured for an additional 24 h. (d–f) FL5.12A cells were cultured with IL-3 (2, 0.1, and 0 ng/ml) for 24 h. Compound **1a** (10 μM) and AG (1 mM) were added and cells cultured for an additional 24 h. Percent apoptosis correlated with the percent of cells binding Annexin-V-FITC, as detected by flow cytometry using a BD FACSCalibur flow cytometer.

cient drug vector. The most potent homospermidine conjugate **1a**, was shown to induce apoptosis of B16 cells and IL-3-dependent FL5.12A pro-B cells. The novel conjugate **1e** could also induce apoptosis and exhibited different effect on the cell cycle of B16 cells compared to **1a**. In conclusion, arylmethyl-polyamine conjugates were shown to have potent anti-tumor activity and induce apoptosis in both actively cycling and non-cycling cells. The precise apoptotic signaling pathway involved will be the subject of a future report.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.06.009.

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