Bioorganic & Medicinal Chemistry Letters 20 (2010) 6421–6425

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Synthesis and bioevaluation of aryl-guanidino polyamine conjugates targeting the polyamine transporter

Jianhong Wang [a], Zhiyong Chen [a], Songqiang Xie [b,*], Jin Zhao [a], Chaojie Wang [a,*]

[a] Key Laboratory of Natural Medicine and Immuno-Engineering, Henan University, Kaifeng 475004, China
[b] Institute of Chemistry and Biology, Henan University, Kaifeng 475001, China

ARTICLE INFO

Article history:
Received 10 August 2010
Revised 5 September 2010
Accepted 14 September 2010
Available online 17 September 2010

Keywords:
Polyamine conjugate
Guanidine
Polyamine transporter
Apoptosis

ABSTRACT

Aryl-guanidino polyamine conjugates were prepared to evaluate their recognition for polyamine transporter (PAT) via a-difluoromethylornithine (DFMO) and spermidine (SPD)-treated B16 cell lines. The potent synergistic effects of DFMO on guanidino polyamine conjugates indicated that the presence of DFMO strongly facilitates the transport of conjugates into cells via PAT on cell membrane. The apoptotic mechanisms of triamine conjugates **10** and **1** (with and without guanidine groups) revealed that they induced apoptosis of Hela cells through the mitochondrial pathway associated with lysosomes, while DFMO strongly synergizes the function of **10** without changing the apoptotic route. Taken together, guanidino polyamine conjugates can target PAT for transport as normal polyamine ones, and the presence of guanidine in the polyamine vectors does not seem to alter the cellular targets of the conjugates, which may depend mainly on the pharmacophore.

The biogenic polyamines, spermidine, spermine and putrecine, are small organic molecules essential for regulation of cell growth, proliferation and differentiation. It is well accepted that tumor cells uptake these polyamines via the polyamine transporter (PAT) in greater amounts than do healthy cells, from which generates the idea that some toxic agents coupled with polyamines might have improved anti-cancer effects.[1,2] Some attempts have been made to use the polyamine moiety to ferry cytotoxic agents that might otherwise be poorly transported into tumor cells. Recently, it was found that some synthetic polyamines with the backbone modifications are better recognized by PAT than their native counterparts.[3,4] The polyamine-drug conjugates are, therefore, promising chemical systems for the development of more efficient anti-tumor agents.[5–9]

To date, several characteristic properties of polyamine transport have been determined based on series of biochemical assays.[2] In one aspect, the PAT affinity of polyamine motifs is modulated by their minor structural changes. With anthracene as a cargo, the homospermidine conjugate (**1** in Fig. 1) exhibited excellent PAT recognition and intriguing biological properties.[10] With **1** as a lead compound, further research revealed that the *N*-ethyl substituted homospermidine in conjugate **2** may serve as a less toxic polyamine carrier than the parent homospermidine.[11] Recently we replaced the terminal amino group of diamines[12] or triamines[13] with hydrazine groups, and found that the resultant conjugates such as **3** could harness PAT to enter the cells. These findings prompted us to look for more modified polyamines targeting PAT.

Various polyamine analogues containing terminal guanidino groups have been designed to possess biochemical attributes of antitumor, antitrypanosomal, and antidiarrheal activities well amplified with respect to their parent polyamines.[14] We thus extended our screen to this more basic group, guanidine.[15] During our work, it was reported that the transition from amine to guanidine might be one promising strategy for better antiproliferative activities, and the same compound **4** was synthesized.[16] However, no guanidino triamine conjugates are reported, and little is known about if the guanidino triamine and diamine compounds could utilize PAT for transport. Thus, a series of four guanidino putrecine and homospermidine conjugates were synthesized and their PAT selectivity was evaluated.

There are many synthetic protocols to introduce the guanidyl group into the polyamine moieties by the reaction of the corresponding amino group with a suitable guanidyl reagents such as the readily available *N*,*N*′-diBoc protected thiourea or *S*-methylisothiourea.[17] As shown in Scheme 1, *S*-methylisothiouronium sulfate **5** was first Boc protected in the presence of $NaHCO_3$ under quick stirring. Heating the yielded product with putrecine at 50 °C for 1 h generated masked guanidino diamine intermediate **6** through amine addition followed by elimination of volatile methyl mercaptan. The nucleophilic substitution of *N*-(4-bromobutyl)phthalimide by the primary amine **6**, and the subsequent *N*-Boc protection yielded compound **7**. Since compound **7** is

* Corresponding authors. Tel.: +86 13938637212 (S.X.); tel.: +86 13619810550; fax: +86 378 2864665 (C.W.).
E-mail addresses: kfxsq@yahoo.com.cn (S. Xie), wcjsxq@henu.edu.cn (C. Wang).

0960-894X/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2010.09.069

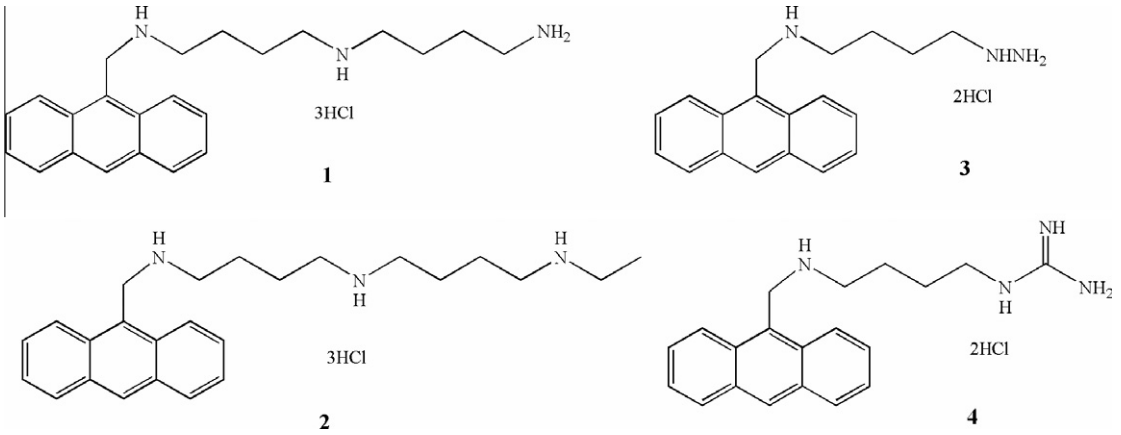
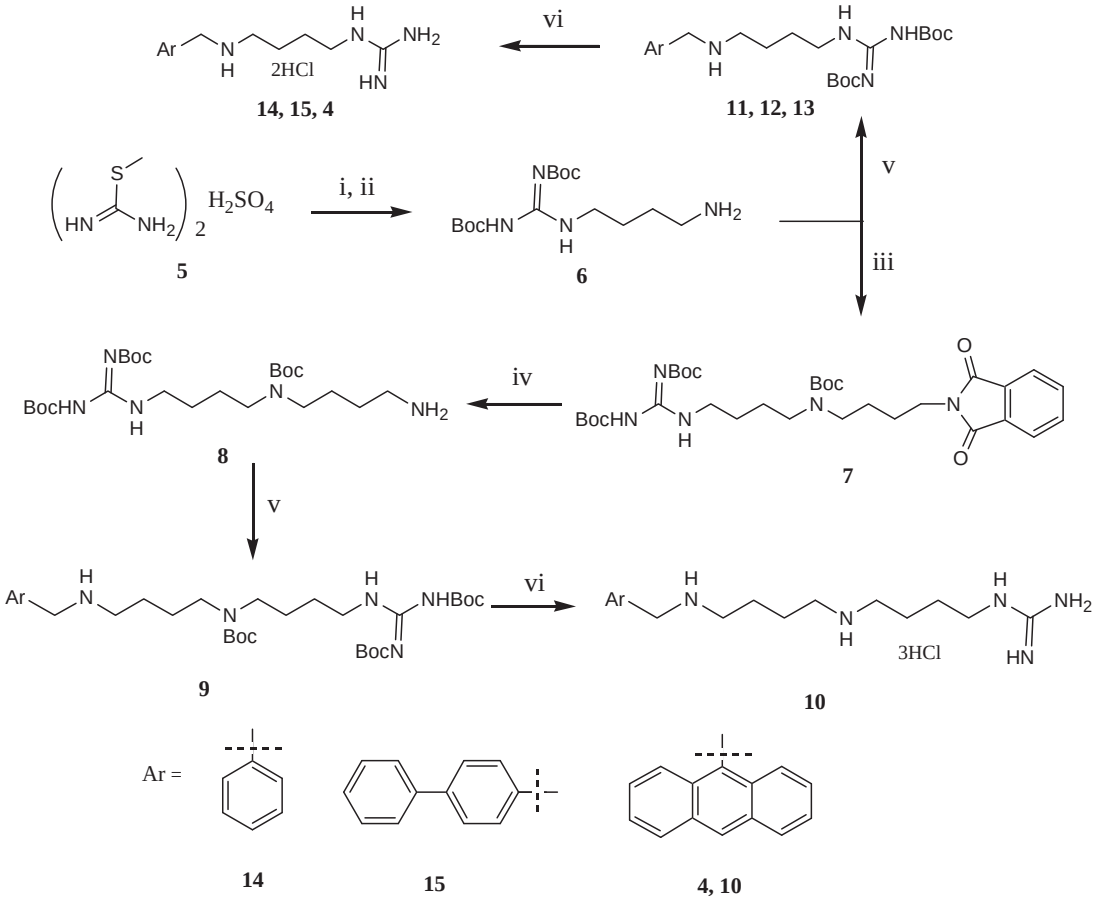


Figure 1. The structures of compounds **1**–**4**.



Scheme 1. The synthesis of target compounds. Reagents and conditions: (i) $(Boc)_2O$, CH_2Cl_2/H_2O; (ii) $H_2N(CH_2)_4NH_2$, THF/H_2O; (iii) (a) *N*-(4-bromobutyl)phthalimide, CH_3CN, K_2CO_3; (b) $(Boc)_2O$; (iv) NH_2NH_2, H_2O; (v) (a) ArCHO, $CH_3OH/CHCl_3$; (b) $NaBH_4$, CH_3OH/CH_2Cl_2; (vi) 4NHCl, 1,4-dioxane.

unstable at room temperature, it was treated immediately with excess hydrazine hydrate at room temperature to liberate the key guanylated triamine intermediate **8**.[6]

The targeted guanidino diamine/triamine derivatives were prepared using the known straightforward route.[16] The one-pot reductive amination of **8**/**6** to **9**/**11**–**13** was achieved in two steps by condensation of the primary amine with one equivalent of aryl aldehydes, and subsequently reduced by treatment with excess $NaBH_4$ in CH_2Cl_2/MeOH. Since the purification of **9**/**11**–**13** was crucial for the purity of target compounds, these intermediates were separated by flash column chromatography, and immediately deprotected using 4 N HCl in dioxane to give target compounds (**10**, **14**, **15** and **4**) in high yields as hydrochloride salts.

The in vitro cytotoxicities of the synthesized compounds were evaluated against mouse leukemia L1210, melanoma B16, and HeLa cells by MTT assays.[11] As shown in Table 1, the guanidine and hydrazine-modified polyamine conjugates displayed much less in vitro potency than the reference compound **1**. It is noteworthy that the guanidino diamine conjugate **4** exhibited higher potency than its triamine counterparts **10**, while parent triamine

Table 1
Cytotoxicity results for target compounds in Hela, L1210 and B16 cells

Compound No.	IC_{50} (μM)					
	Hela	L1210	B16	B16 + SPD	B16 + DFMO	(B16)/(B16 + DFMO)
10	⩾50	26.95	49.12	>50(76.47)	11.21	4.38
14	34.36	18.08	36.71	>50(57.23)	12.31	2.98
15	>50	~50	>50	>50	10.84	>5
4	14.37	9.58	>50	30.43	5.77	>8
3	25.22	16.92	12.67	23.58	6.85	1.85
1	1.09	1.04	1.40	2.46	0.97	1.44

IC_{50} values were from three-independent experiments, and the standard deviations were less than 10%.
IC_{50} values were given only if they were less than 50 μM, which was the maximum concentration tested. The data in parentheses (above 50 μM) were extrapolated ones.

conjugates are generally more toxic than diamine ones in the similar series.[4,6] In the only example of Ohara et al., a similar phenomenon was observed by the comparison of the more active guanidino diamine pyrene conjugate with the corresponding guanidino tetraamine one.[16] It is thus speculated that the diamine conjugates benefit more than their tri- and tetraamine counterparts by the introduction of guanidine.

The growth inhibitory effect was determined in B16 cells coincubated with excess spermidine (SPD) or a-difluoromethylornithine (DFMO) in order to investigate whether these conjugates could enter cells via PAT. DFMO, which depletes intracellular polyamine level by inhibition of ornithine decarboxylase (ODC), could increase uptake of exogenous polyamines. Therefore, cells treated with DFMO should be more susceptible to polyamine derivatives and should provide lower IC_{50} values. As expected, exposure of B16 cells to these conjugates in the presence of DFMO significantly enhanced their growth inhibitory activity, especially for conjugates with polyaromatic groups as cargoes (Table 1). In addition, although DFMO exerts synergistic effects on all these terminal guanidino or hydrazino substituted derivatives, the guanidino polyamine derivatives seem to be more sensitive to DFMO than their parent and hydrazino polyamine conjugates.

Another assessment of the conjugates' ability to target the PAT was conducted by SPD protection experiments. The existence of additional SPD in the system generates a competition with the polyamine conjugates for the PAT protein on the cell membrane, and therefore reduces the uptake of these conjugates which in turn will reduce the cell death. The added SPD led to an obvious decrease in cell death (higher IC_{50}) for hydrazino and guanidino polyamine conjugates (except **4**) as reference compound **1**.

Earlier studies with **1** revealed a direct correlation between conjugate uptake and cytotoxicity.[18] DFMO treatment increased the import of **1** and resulted in a more toxic compound (lower IC_{50} value). In a similar fashion, DFMO should facilitate the entry of these novel conjugates and give lower IC_{50} values in the presence of DFMO. We thus measured the intracellular fluorescence intensity of compound **10** and **1** in Hela cells by high content screening (HCS). As expected, the decreased IC_{50} value (6.43 μM) of **10** against Hela cells in the presence of DFMO corroborated the existence of a strong synergistic effect. In Figure 2, both compounds **10** and **1** displayed upregulated cellular uptake in a time- and dose-dependent manner. However, it seems that the effect of DFMO on the cellular intake of **10** is more sensitive than that of **1**, which is in accordance with the change of IC_{50} values.

Indeed, the PAT of cells can tolerate the terminal modifications of the homospermidine motif from amine to guanidine. However, the data also revealed that the elevated basicity from amine to guanidine groups leads to attenuated potency of the homospermidine conjugate. It is rationally speculated that the high basicity of guanidinium group (pK_a 13.5) in polyamine conjugates may cause their stronger interaction with PAT due to a higher preponderance of protonated species at physiological pH. This strong interaction with PAT may inhibit their entrance into the targeted cells as previously reported tetraamine conjugates,[19] which may be offset by DFMO.

Previous work revealed that **1** could produce cytotoxicity via apoptosis,[18] we thus detected how **10**, with the mere structural difference of guanidine group in homospermidine moiety from **1**, kill cells. Lysosomes are highly dynamic intracellular organelles that involve with the biosynthetic, endocytic, and autophagic pathways. They control the recycling of the majority of cellular macromolecules and organelles through more than 50 acid hydrolases. It has been demonstrated that lysosomal membrane permeabilization (LMP) triggers either necrotic or apoptotic cell death pathways. Tumor cells may be preferentially sensitive to agents that trigger the lysosomal apoptosis pathway, suggesting that a reasonable therapeutic window could be achieved for the LMP-inducing agents. Therefore, LMP-inducing agents have great potential to be developed as novel cancer therapy.[20]

The induction of LMP by **10** was analyzed using the lysosomotropic weak base acridine orange (AO), which prefers to accumulate in normal lysosomes. AO is a metachromatic fluorophore turning red in lysosomes after excitation with blue light. LMP results in the relocation of AO from lysosomes to cytosol, and the fluorescence changes from red to green. Thus, red fluorescence assaying (AO uptake method) was used here to evaluate pronounced lysosomal rupture. As shown in Figure 3A, compounds **10** (10 μM) and DFMO (200 μM) induced moderately decrease in the amount of cells with normal AO red fluorescence, whereas, this effect was significant enhanced after **10** and DFMO co-treatment, indicating significant LMP. To determine the consequence of LMP which will lead to apoptosis or necrosis, we utilized the fluorescence staining using Annexin V-FITC and propidium iodide (PI). PI is a DNA-binding fluorochrome which intercalates in the double-helix. If the cell membrane is normal, PI could not permeate and nucleus staining. Whereas, the membrane of necrotic cells is not integrated, PI could permeate and nucleus staining. When cells are in the state of apoptosis, the phosphatidylserine (PS) will evaginate on the cell surface and Annexin V-FITC will bind with PS.[18] As shown in Figure 3B, the cell numbers of Annexin V-FITC staining significantly increased after **10** and DFMO co-treatment, although DFMO had no effect and **10** had weak effect. Simultaneously, PI staining was not obvious even at **10** and DFMO co-treatment, however, the PI nucleus staining of compound **1** was significant (Fig. 3C). Those results indicated that the antiproliferative effect of **10** and DFMO co-treatment was mainly due to cellular apoptosis, however, compound **1** could trigger apoptosis and necrosis.

Apoptosis is mainly controlled by two major pathways, namely the mitochondrial pathway and membrane death receptor pathway.[21] In mitochondrial pathway, mitochondria have a crucial position in apoptosis control. The loss of mitochondria membrane potential (MMP) induces cytochrome c release from the mitochondria to the cytoplasm, which leads to the activation of caspase-9 and downstream cleavage of caspase-3. The membrane death

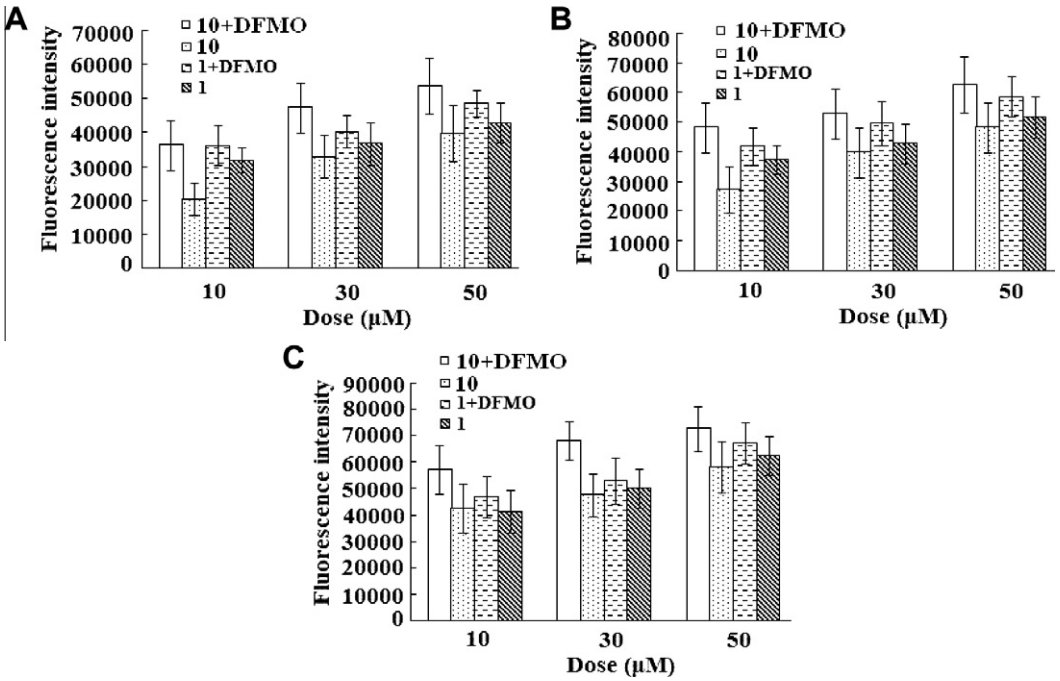


Figure 2. The intracellular fluorescence intensity of **10** and **1** with absent or present DFMO on Hela cells. (A) 15 min; (B) 30 min; (C) 60 min. Each point represents the means ± S.D. from four independent experiments.

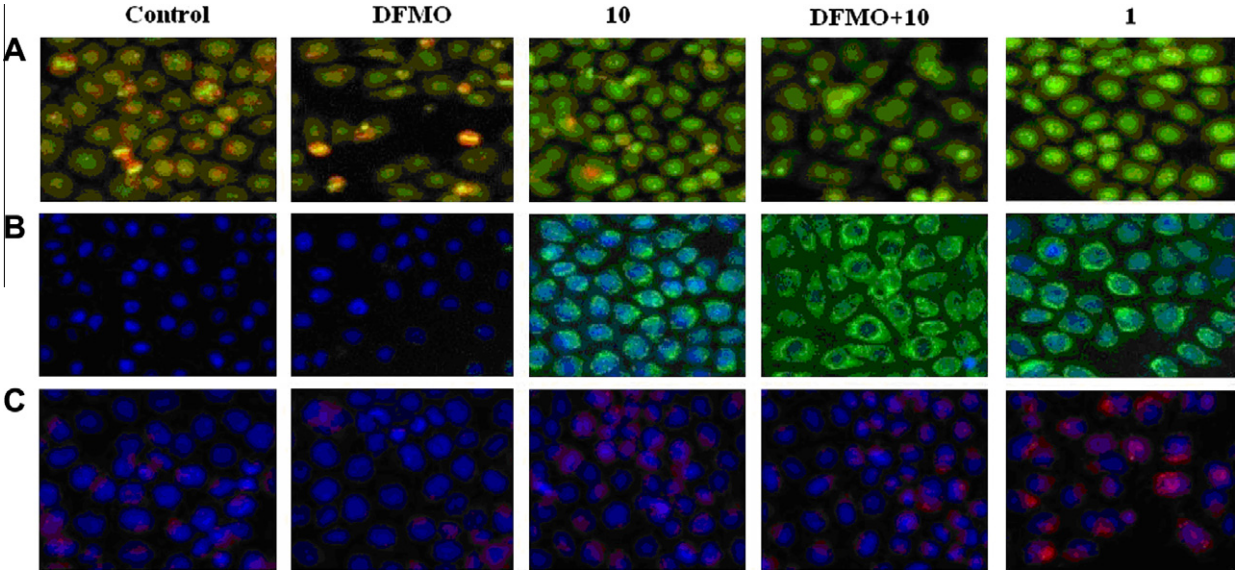


Figure 3. Apoptosis was detected in Hela cells after **10** (10 μM) or **1** (5 μM) treatment with absent or present DFMO (200 μM) for 48 h using high content screening (HCS). (A) Lysosomal membrane stability was determined by assessing AO red/green fluorescence. (B) Apoptosis was detected by Annexin V-FITC and Hoechst33342 double staining. (C) Necrosis was detected by PI and Hoechst33342 double staining (magnification, 20×).

receptor pathway is characterized by the binding between cell death ligands and cell death receptors, and the subsequent activation of caspase-8 and -3.[22] To reveal the precise molecular mechanism of **10** and/or DFMO-induced apoptosis in Hela cells, we observed the effect of **10** and/or DFMO on caspase-3, caspase-8, caspase-9, MMP and cytochrome c. As shown in Figure 4A, **10** or DFMO had no obvious effect on caspase-3, caspase-8 and caspase-9, however, **10** and DFMO co-administration induced caspase-9 and caspase-3 activation but did not affect caspase-8, indicating that Hela cells apoptosis which induced by **10** and DFMO co-administration via mitochondrial pathway, this apoptotic effect was similar to compound **1**. Further, we found that DFMO and **10** co-treatment also induced MMP loss followed by the release of cytochrome *c* with the corresponding decrease in mitochondria (Fig. 4B).

Generally speaking, the SPD/DFMO experiments corroborated that the guanidino polyamine conjugates could target PAT for drug delivery as parent polyamine conjugates. However, the less active guanidino triamine conjugate compared to corresponding diamine derivatives, which is different from previous reports, indicated that the presence of guanidine in conjugates may inhibit their own transport via PAT. In this case, the co-administration of DFMO is critical for better cancer therapy.[2] The apoptotic mechanism revealed that both the normal and guanidino polyamine conjugates

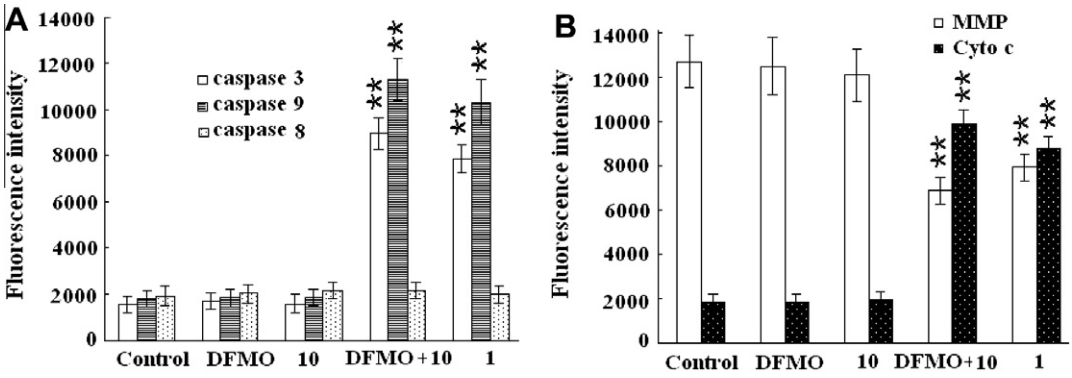


Figure 4. The change of MMP and Cyto C, caspase-3, caspase-8 and caspase-9 were detected using Rh123 staining and immunofluorescence assay by HCS, respectively. Each point represents the means ± S.D. from four independent experiments. Compared with control $^{*}p$ <0.05, $^{**}p$ <0.01.

trigger apoptosis through the lysosomes-associated mitochondrial pathway, supported by the upregulation of caspases 3 and 9. DFMO strongly synergized the functions of **10**, but did not alter the apoptotic pathway, which corroborated that DFMO only facilitated the transport of **10** into cells, and could not change the cellular targets of the conjugates for cytotoxicity.

It is also intriguing that the guanidino putrecene-anthracene conjugate **4** displayed strong DFMO effect and reversed SPD effect. Because the natural polyamines in body often reduce the antitumor efficacy of polyamine-based drugs, the elevated potency of this drug in the presence of SPD and DFMO favors its further study as one lead compound.

Acknowledgments

This work was supported by National Science Foundations of China (20872027, 90913001) and Henan Natural Science Foundation (082102270002).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.09.069.

References and notes

1. Palmer, A. J.; Wallace, H. M. *Amino Acids* **2010**, *38*, 415.
2. Xie, S.-Q.; Wang, J.-H.; Zhang, Y.-H.; Wang, C.-J. *Expert Opin. Drug Deliv.* **2010**, *7*, 1049.
3. Gardner, R. A.; Delcros, J. G.; Konate, F.; Breitbeil, F., III; Martin, B.; Sigman, M.; Phanstiel, O. *J. Med. Chem.* **2004**, *47*, 6055.
4. Wang, C.; Delcros, J.-G.; Biggerstaff, J.; Phanstiel, O. *J. Med. Chem.* **2003**, *46*, 2663.
5. Phanstiel, O.; Kaur, N.; Delcros, J.-G. *Amino Acids* **2007**, *33*, 305.
6. Tian, Z. Y.; Xie, S. Q.; Du, Y. W.; Ma, Y. F.; Zhao, J.; Gao, W. Y.; Wang, C. J. *Eur. J. Med. Chem.* **2009**, *44*, 393.
7. Tian, Z. Y.; Xie, S. Q.; Mei, Z. H.; Zhao, J.; Gao, W. Y.; Wang, C. J. *Org. Biomol. Chem.* **2009**, *7*, 4651.
8. Kaur, N.; Delcros, J.-G.; Imran, J.; Khaled, A.; Chehtane, M.; Tschammer, N.; Martin, B.; Phanstiel, O. *J. Med. Chem.* **2008**, *51*, 1393.
9. Barret, J. M.; Kruczynski, A.; Vispé, S.; Annereau, J. P.; Brel, V.; Guminski, Y.; Delcros, J.-G.; Lansiaux, A.; Guilbaud, N.; Imbert, T.; Bailly, C. *Cancer Res.* **2008**, *68*, 9845.
10. Xie, S. Q.; Liu, G. C.; Ma, Y. F.; Cheng, P. F.; Wu, Y. L.; Wang, M. W.; Ji, B. S.; Zhao, J.; Wang, C. J. *Toxicol. In Vitro* **2008**, *22*, 352.
11. Wang, J.; Xie, S.; Li, Y.; Guo, Y.; Ma, Y.; Zhao, J.; Phanstiel, O.; Wang, C. *Bioorg. Med. Chem.* **2008**, *16*, 7005.
12. Zhou, J. J.; Huang, H.; Xie, S. Q.; Wang, Y. X.; Zhao, J.; Wang, C. J. *Chin. Chem. Lett.* **2008**, *19*, 99.
13. Zhou, J.J. Master thesis of Henan University, 2007.
14. (a) Pallan, P. S.; Ganesh, K. N. *Biochem. Biophys. Res. Commun.* **1996**, *222*, 416; (b) Constantinos, M.; Garnelis, T.; Pantazaka, E.; Papaioannou, D. *Tetrahedron Lett.* **2004**, *45*, 8815; (c) Dardonville, C.; Brun, R. *J. Med. Chem.* **2004**, *47*, 2296; (d) Manetti, F.; Cona, A.; Angeli, L.; Mugnaini, C.; Raffi, F.; Capone, C.; Dreassi, E.; Zizzari, A. T.; Tisi, A.; Federico, R.; Botta, Maurizio. *J. Med. Chem.* **2009**, *52*, 4774.
15. Chen, Z. Y. Master thesis of Henan University, 2007.
16. Ohara, K.; Smietana, M.; Restouin, A.; Mollard, S.; Borg, J. P.; Collette, Y.; Vasseur, J. J. *J. Med. Chem.* **2007**, *50*, 6465.
17. Guo, Z.-X.; Cammidge, A. N.; Horwell, D. C. *Synth. Commun.* **2000**, *30*, 2933.
18. Xie, S.-Q.; Wang, J.-H.; Ma, H.-X.; Cheng, P.-F.; Zhao, J.; Wang, C.-J. *Toxicology* **2009**, *263*, 127.
19. Wang, C.; Delcros, J. G.; Cannon, L.; Konate, F.; Carias, H.; Biggerstaff, J.; Gardner, R. A.; Phanstiel, O. *J. Med. Chem.* **2003**, *46*, 5129.
20. (a) Boya, P.; Kroemer, G. *Oncogene* **2008**, *27*, 6434; (b) Chen, Z.; Liang, X.; Zhang, H.; Xie, H.; Liu, J.; Xu, Y.; Zhu, W.; Wang, Y.; Wang, X.; Tan, S.; Kuang, D.; Qian, X. *J. Med. Chem.* **2010**, *53*, 2589.
21. Goldschneider, D.; Mehlen, P. *Oncogene* **2010**, *29*, 1865.
22. Grimm, S.; Noteborn, M. *Trends Mol. Med.* **2010**, *16*, 88.