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Original article

Synthesis, cytotoxicity and apoptosis of naphthalimide polyamine conjugates as antitumor agents

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Abstract

Several naphthalimide polyamine conjugates were synthesized and evaluated for *in vitro* cytotoxicity against human leukemia K562, murine melanoma B16, Chinese hamster ovary CHO cell lines. Both triamine moieties and the length of spacers were crucial in elevating the potency of 1,8-naphthalimide. The typical compounds **5a** and **5d** exhibited excellent cell selectivity to cancer cells through the human hepatoma BEL-7402 and human normal hepatocyte QSG-7701 screens. In addition, **5d** could disturb the cell cycle in B16 cells. The research on caspase activity and cytochrome *c* indicated that **5d** could induce B16 cell apoptosis *via* both the mitochondrial and membrane death receptor pathways, and the Bcl-2 family numbers were involved in the control of apoptosis.

Keywords: Naphthalimide; Polyamine conjugate; Synthesis; Cytotoxicity; Apoptosis

1. Introduction

There are several strategies in the design of antitumor agents based on polyamines. Since polyamines are important for tumor cell growth and function, the biosynthetic pathway of native polyamines (putrecine, spermidine and spermine; **1**–**3** in Fig. 1) has been a popular target for the therapeutic intervention during the last decades [1]. Alkyl polyamine analogues such as biethyl substituted spermine derivatives, which can deplete the intracellular polyamine pool by down-regulating polyamine biosynthesis and uptake, upregulating polyamine catabolism, have exhibited significant antineoplastic activities against several human solid-tumor models [2].

Polyamines are also potential vectors to carry diverse cargoes [3–7]. The idea is that tumor cell types can import both native and structurally modified polyamines from exogenous sources *via* the active polyamine transporter (PAT). Many anticancer drugs are poorly tumor selective, so their therapy causes high incidences of adverse effects, which have prompted the efforts with the aim to improve the tumor selectivity, efficiency and safety. Antitumor agents conjugated with smart polyamine motifs may have elevated affinity for cancer cells, and reach the targeted tissues more specifically. Indeed, various polyamine conjugates have been designed following this strategy [8–10].

Both native and synthetic polyamines have been tried as the vectors. Systematic investigations have helped to define the key characteristics of polyamine conjugates suitable for PAT use such as the cargo size limitation, the number and spacing of charges, and the optimal tether length [11]. Previous reports revealed that N^1-(9-anthracene-methyl)-homospermidine conjugate (**4** in Fig. 1) displayed excellent PAT recognition and selectivity in the Chinese hamster ovary (CHO) and murine melanoma B16 screens [11,12]. We also found that compound

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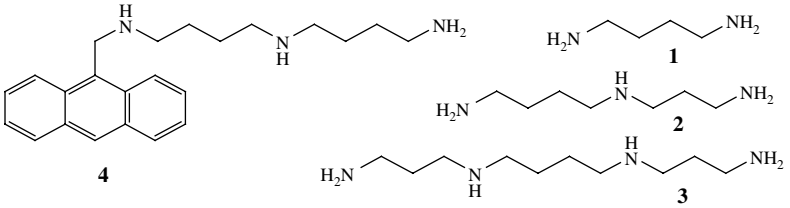


Fig. 1. Structures of putrecine **1**, spermidine **2**, spermine **3** and N^1-(9-anthracenemethyl)-homospermidine **4.**

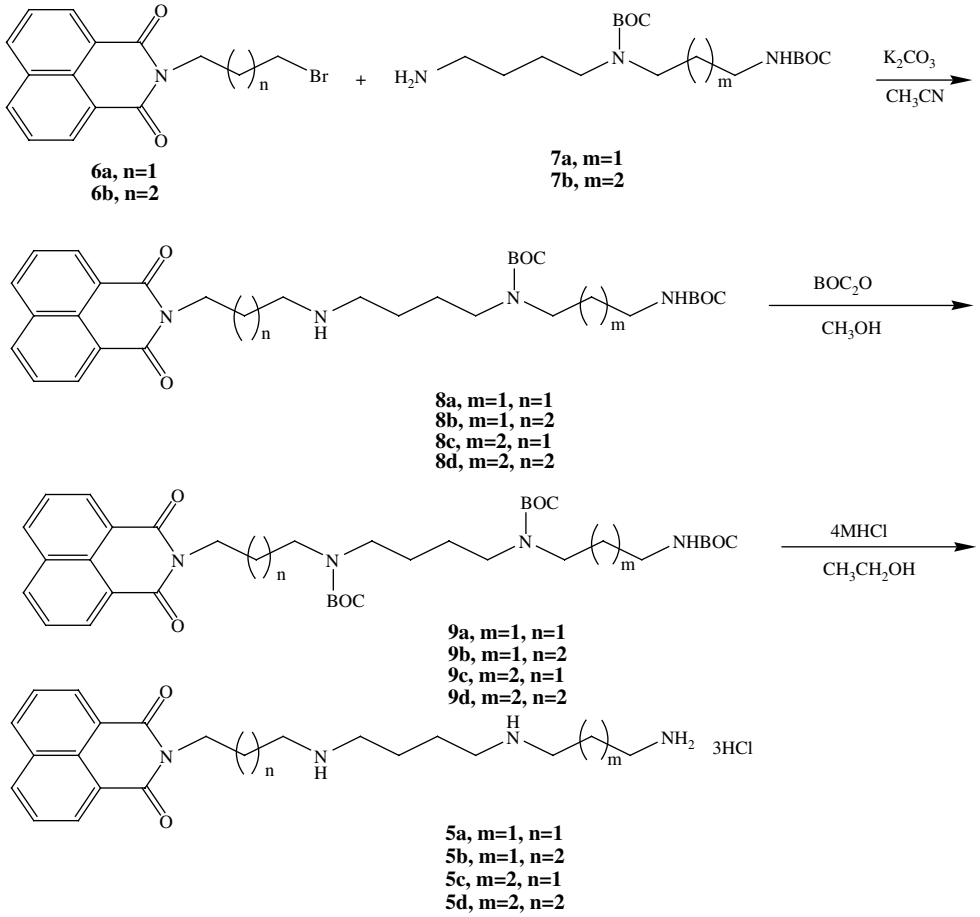
4 could induce apoptosis in several cell lines [13]. These findings stimulated us to search for more promising and applicable cargoes through the tricyclic DNA intercalators instead of anthracene. Naphthalimides which have much structural similarity to anthracene might be ideal candidates.

Naphthalimides, known to be DNA intercalators, have been evaluated extensively as antitumor agents. Some mononaphthalimides (i.e. amonafide) [14] and bisnaphthalimides (i.e. elinafide) [15] have reached clinical trials. However, most of them have been given up for their poor therapeutic efficiency. Mononaphthalimide polyamine conjugates are seldom reported compared to their bisnaphthalimide counterparts [16,17]. In this paper, we report (a) the conjugation of naphthalimide with both natural and synthetic polyamine moieties; (b) *in vitro* anticancer activity and cell selectivity of synthesized conjugates; (c) the mechanism of how the novel conjugate **5d** killed B16 cells.

2. Results and discussion

2.1. Chemistry

The syntheses of the triamine conjugates **5a**–**5d** were achieved by a convenient procedure as shown in Scheme 1. *N*-(3-Bromopropyl)-1,8-naphthalimide **6a** and *N*-(4-bromobutyl)-1,8-naphthalimide **6b** were prepared routinely from 1,8-naphthalimide and 1,4-dibromobutane (or 1,3-diaminopropane) in the presence of potassium carbonate and potassium iodide. The di-Boc protected triamines **7a** and **7b** were prepared according to a modified procedure reported previously [13]. The *N*-alkylation reaction of **7** with **6** in the presence of potassium carbonate in dry acetonitrile at 45 °C generated intermediates **8**, which were difficult for purification because of their unstability [12]. Without separation, *N*-Boc protection of **8** with BOC_2O



Scheme 1. Synthesis of naphthalimide triamine conjugates.

led to the formation of stable intermediates **9**. The yield of these two steps was between 30 and 40%. Subsequently the Boc groups of pure **9** were removed with 4 M HCl at room temperature to provide target compounds **5** as trihydrochloride salts in a yield of 60–80%. The diamine conjugate **12** as dihydrochloride salt was synthesized in a similar procedure as illustrated in Scheme 2. The structures of target compounds were confirmed by ^{1}H NMR, ^{13}C NMR, ESI-MS, and elemental analysis.

2.2. Cytotoxic effects

The *in vitro* cytotoxicities of novel conjugates were assessed by the MTT assay in the presence of aminoguanidine (an inhibitor of amine oxidase) against three cell lines, human leukemia K562, murine melanoma B16 and Chinese hamster ovary CHO. As shown in Table 1, the IC_{50} values of the naphthalimide triamine conjugates **5a**–**5d** were lower than that of its diamine counterpart **12**. These results demonstrated that the presence of triamine motif could elevate the potency of the cargo, naphthalimide, and three free nitrogens in the polyamine moieties were crucial factors for cytotoxicity [12,18,19]. Compounds **5a** and **5d**, which were 7 to 20-fold more potent than **12** against all three cell lines, exhibited best cytotoxic effects compared to **5b** and **5c**. This confirmed that both the synthetic homospermidine (in **5d**) and native spermidine (in **5a**) could serve as efficient vectors to carry more types of tricyclic DNA intercalators instead of anthracene. However, the tether of the novel conjugates (three or four methylene linkage) which is different from that of previously reported conjugate **4** (one methylene) was also very important for the cytotoxicity. Interestingly, **5a** and **5d** contained the spermine skeleton (3, 4, 3) and homospermine skeleton (4, 4, 4), respectively.

1,8-Naphthalimide was also tested as the pharmacophore, and it was the least toxic compound in *in vitro* trials. However, 1,8-naphthalimide is not water soluble; they may enter the cells *via* different pathways.

The introduction of polyamine motif to polyamine conjugates could result in better tumor specificity. One example was that the conjugate **4** exhibited obvious cell selectivity against B16 (1.1 μM) and normal melanocyte Mel-A (8.3 μM) [11]. Therefore we examined the effect of **5a** and **5d** on BEL-7402 and QSG-7701 cells' proliferation by the MTT assay. These cells were chosen because they represent human hepatoma and human normal hepatocyte cells. The IC_{50} values of **5a**, **5d** in Table 2 proved that **5a** and **5d** were much less toxic to the human normal hepatocyte as compared to the hepatoma cells. These results further confirmed that the polyamine moiety might elevate the sensitivity of the conjugates toward cancer cells compared to normal cells. More importantly, **5a** and **5d** are almost untoxic to human normal hepatocyte cells while conjugate **4** still possesses relatively potent toxicity to normal melanocyte Mel-A (8.3 μM). This indicated that naphthalimide might be one cargo superior to anthracene.

Table 1
In vitro activity of compounds **5a**–**5d**, **12**

Compound	IC_{50} (μM)		
	K562	CHO	B16
5a	1.64 ± 0.09	1.21 ± 0.06	0.62 ± 0.03
5b	3.84 ± 0.10	3.43 ± 0.14	2.70 ± 0.16
5c	9.08 ± 0.60	9.09 ± 0.31	5.36 ± 0.09
5d	1.16 ± 0.05	2.58 ± 0.06	0.69 ± 0.01
12	11.37 ± 0.49	20.72 ± 1.90	13.88 ± 0.32
4	0.95 ± 0.13	1.25 ± 0.37	1.40 ± 0.04
1,8-Naphthalimide	>50	>50	>50

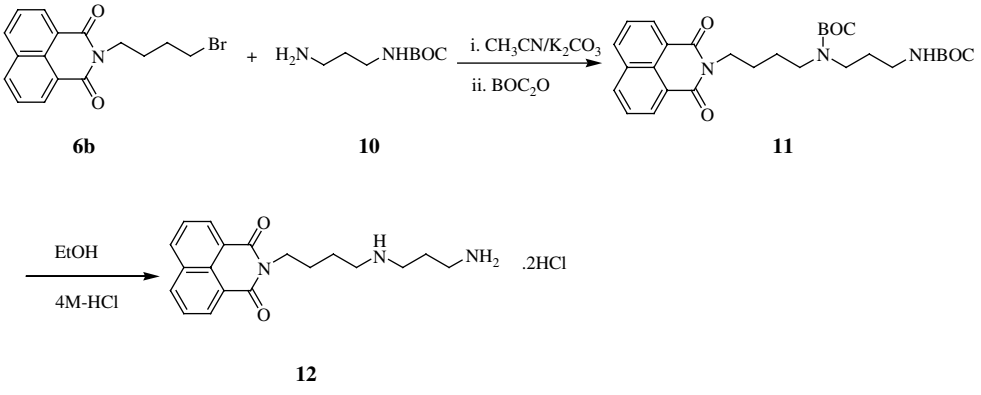
All data are expressed as means ± SD from three separate determinations. IC_{50} values were given only if they were less than 50 μM, which was the maximum concentration tested.

2.3. DNA binding

Naphthalimides are one kind of DNA targeting agents, and compound **4** with the homospermidine backbone could intercalate into calf-thymus (ct) DNA [20]. To evaluate the DNA binding properties of naphthalimide homospermidine conjugate **5d**, the inherent fluorescence of **5d** allowed us to investigate its interaction with ct DNA by FL spectrometry. As shown in Fig. 2, the fluorescence of **5d** was quenched upon addition of DNA. This indicated that DNA is one potential target of **5d** as expected.

2.4. Apoptosis and cell cycle analysis

Many naphthalimides such as amonafide and amonafide analogues induce apoptosis [21]. Previous work demonstrated



Scheme 2. Synthesis of naphthalimide diamine conjugates.

Table 2
In vitro selectivity of **5a**, **5d** toward BEL-7402 and QSG-7701 cells

Compound	IC_{50} (μM)	
	BEL-7402	QSG-7701
5a	1.06 ± 0.10	>50
5d	3.56 ± 0.17	>50

All data represent the means ± S.D. from four independent experiments. IC_{50} values were given only if they were less than 50 μM, which was the maximum concentration tested.

that different homospermidine conjugates could induce apoptosis while they possessed diverse impacts on the cell cycle [13,22]. Flow cytometry DNA analysis revealed that **5d** induced cell cycle perturbation (Fig. 3). Compared to the control (untreated cells), changes in the cell cycle distribution of treated B16 cells were evident. The exposure of B16 cells to 0.5, 1.0, 2.0 μM of **5d** was associated with an enhancement of internucleosomal DNA fragment. The sub-G1 peak and the accumulation of cells in the G2/M phase accompanying reduction in the G0/G1 phase in a dose-dependent manner were also observed after the cells were exposed to **5d**.

2.5. Effect of *5d* on apoptosis-related proteins

Apoptosis can be triggered by several stimuli and is controlled by two major pathways, namely the mitochondrial pathway and membrane death receptor pathway [23]. In the mitochondrial pathway, mitochondria have a crucial position in apoptosis control. The loss of mitochondrial 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 receptor pathway is characterized by the binding between cell death ligands and cell death receptors and the subsequent activation of caspase-8 and caspase-3. To reveal the molecular mechanism of **5d** induced apoptosis in B16 cells, we observed the effect of **5d** on cytochrome *c* and the activation of caspases. As shown in Fig. 4, the cleavage of caspase-8, caspase-9 and caspase-3 was significantly increased compared with untreated cells in a dose-dependent manner after **5d** treatment. The cytochrome *c* release from mitochondria was enhanced concomitant with the related attenuation of cytochrome *c* in mitochondria.

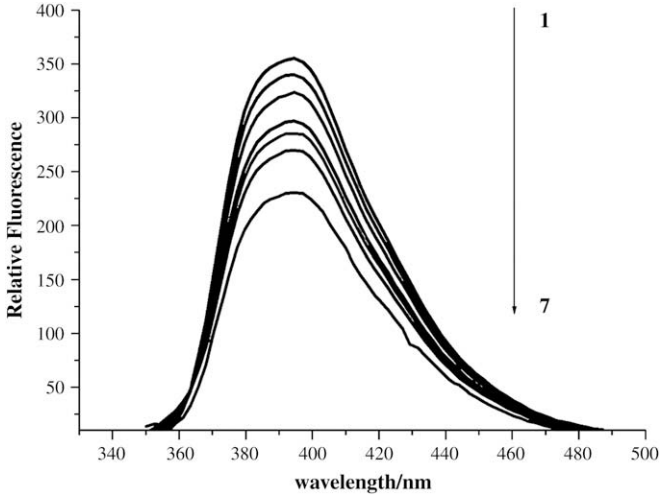


Fig. 2. Fluorescence spectra of compound **5d** with calf-thymus DNA. Numbers 1–7 indicated the DNA concentration: 0, 16.4, 32.9, 54.8, 65.8, 82.2, 109.6 μM, respectively. Compound **5d** applied was 80 μM.

Bcl-2 is an important element during apoptosis mediated by the mitochondrial pathway and has been identified to prevent cytochrome *c* release from the mitochondria. In contrast, Bax can induce the release of cytochrome *c* from the mitochondria [24]. The present report revealed that **5d** induced apoptosis was companied by an increased expression of Bax and a reduced protein level of Bcl-2 in B16 cells after 48 h treatment as shown in Fig. 4.

Therefore, we preliminarily concluded that **5d** induced the apoptosis of B16 cells *via* both the mitochondrial and membrane death receptor pathways, which was different from that of compound **4**. In addition, the Bcl-2 family numbers were involved in the control of apoptosis.

3. Conclusion

A series of naphthalimide polyamine conjugates were synthesized and their *in vitro* antitumor activities were evaluated. The triamine moiety could elevate the potency of naphthalimide more than its diamine counterpart, and the spacer was also one crucial factor in the conjugates. Compounds **5a** and **5d** exhibited best potency against all three tested cells. Importantly, they had excellent cell selectivity in normal vs transformed cells as well. In addition, it was found that **5d** disturbed the cell cycle and induced apoptosis in B16 cells. The effects of **5d** on the activation of caspases and the protein expression of the Bcl-2 family numbers indicated that **5d** might induce apoptosis *via* both the mitochondrial and membrane death receptor pathways. Therefore, **5a** and **5d** are interesting anticancer leads for further synthetic optimization. The structural modification and precise apoptotic signaling pathway involved are ongoing in our laboratory.

4. Experimental protocols

4.1. Synthesis of naphthalimide polyamine conjugates

All chemicals (reagent grade) used were commercially available. Melting points were determined on an X-6 hot stage microscope and are uncorrected. All the ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AV-400 model Spectrometer in D_2O or $CDCl_3$. Chemical shifts for ^{1}H NMR spectra were reported in parts per million to residual solvent protons. ESI-MS spectra were recorded on an ESQUIRE-LC Mass spectrometer. Elemental analyses were performed on a Gmbe VarioEL Elemental instrument and were within 0.4% of the theoretical values.

4.1.1. General procedure for the synthesis of *9a–9d*

The Boc protected amine **7a** (or **7b**, **10**) (3 mmol) was dissolved in anhydrous acetonitrile (30 mL), and solid K_2CO_3 (5 mmol) was added. After the mixture was stirred for 15 min

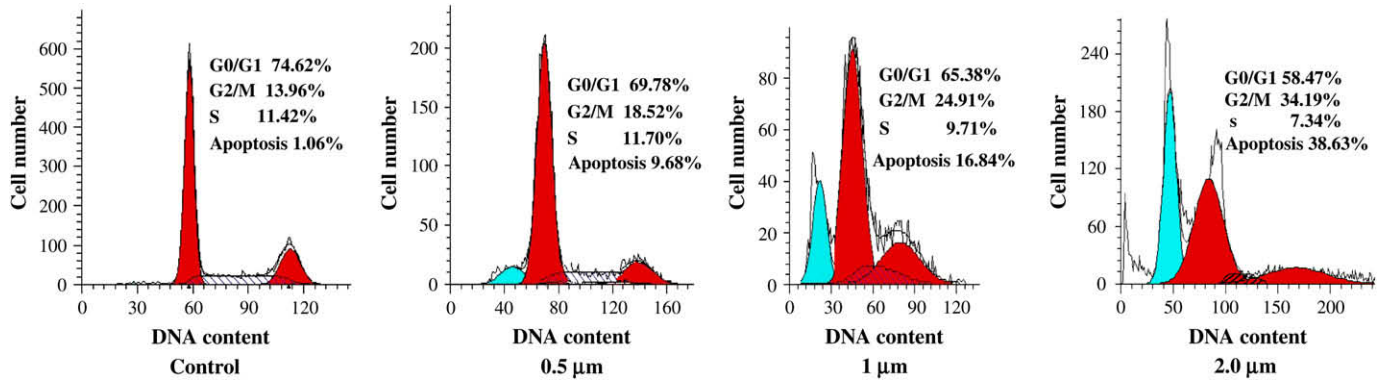


Fig. 3. Inhibition of cell cycle progress in B16 cells treated with **5d** for 48 h. Cells were fixed with ethanol and stained with PI. Cell cycle distribution was analyzed by flow cytometry. First peak represents sub-G1 peak, which was taken as the fraction of the apoptotic cell population.

at ambient temperature, *N*-(3-bromopropyl)-1,8-naphthalimide **6a** (or *N*-(4-bromobutyl)-1,8-naphthalimide **6b**) (2 mmol) in acetonitrile (10 mL) was added dropwise with constant stirring at 45 °C. Then the reaction mixture was stirred overnight. The volatiles were removed under vacuum to give a residue, which was redissolved in $CHCl_3$ (30 mL) and washed with aqueous Na_2CO_3 (10%, w/w). The organic phase was separated, dried over anhydrous Na_2SO_4, filtered, and concentrated.

The above residue (crude **8**) was dissolved in methanol (30 mL), a solution of di-*tert*-butyldicarbonate (3 mmol) in methanol (10 mL) was added dropwise. The mixture was stirred at ambient temperature overnight. The solvents were removed under reduced pressure to give a residue, which was redissolved in $CHCl_3$ (30 mL) and washed with water. The organic phase was separated, dried over anhydrous Na_2SO_4, filtered, and concentrated. The residue was subjected to flash chromatography (25% Petroleum/EtOAc, v/v) to obtain the BOC protected intermediates **9a**–**9d**, or **11**.

*4.1.1.1. 2-{3-[4-(3-Butoxycarbonylaminopropyl-butoxycarbonylamino)butyl-butoxycarbonylamino]propyl}1H-benz-[de]isoquinoline-1,3(2H)-dione (**9a**).* Yield: 40.1%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ_H: 7.76–8.61 (m, 6H, ArH), 4.98–5.02 (br s, 1H, –NHCO), 4.20 (t, $J = 15.2$ Hz, 2H), 3.23 (m, 10H), 1.86 (m, 2H), 1.25–1.49 (m, 33H). ESI-MS *m/z*: 705.4 $(M + Na)^+$.

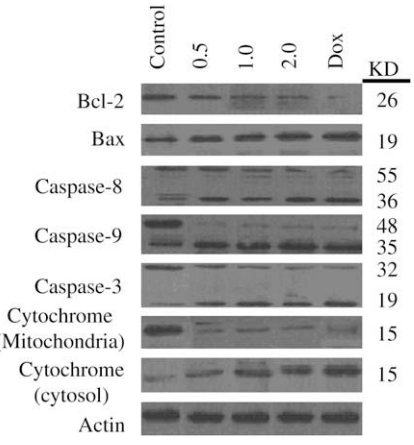


Fig. 4. Expressions of caspase-3, caspase-8, caspase-9, cytochrome *c*, Bcl-2, and Bax in B16 cells treated with **5d** at indicated concentrations. Doxorubicin (Dox) treatment was taken as the positive control. Equal amounts (50 μg/lane) of cellular protein were fractionated on 12% SDS-PAGE gels and transferred to PVDF membranes. Actin was blotted as a control.

*4.1.1.2. 2-{4-[4-(3-Butoxycarbonylamino-propyl-butoxycarbonylamino)butyl-butoxycarbonylamino]butyl}1H-benz-[de]isoquinoline-1,3(2H)-dione (**9b**).* Yield: 30.6%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ_H: 7.76–8.61 (m, 6H, ArH), 4.98–5.02 (br s, 1H, –NHCO), 4.20 (t, $J = 15.2$ Hz, 2H), 3.20–3.21 (m, 10H), 1.86 (m, 2H), 1.25–1.42 (m, 35H). ESI-MS *m/z*: 719.4 $(M + Na)^+$.

*4.1.1.3. 2-{3-[4-(4-Butoxycarbonyl aminobutyl-butoxycarbonyl amino)butyl-butoxycarbonyl amino]propyl}1H-benz-[de]isoquinoline-1,3(2H)-dione (**9c**).* Yield: 37.6%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ_H: 7.76–8.61 (m, 6H, ArH), 4.98–5.02 (br s, 1H, –NHCO), 4.01 (t, $J = 15.2$ Hz, 2H), 3.13 (m, 10H), 1.86 (s, 2H), 1.25–150 (m, 35H). ESI-MS *m/z*: 719.5 $(M + Na)^+$.

*4.1.1.4. 2-{4-[4-(4-Butoxycarbonylaminobutyl-butoxycarbonylamino)butyl-butoxycarbonyl-amino]butyl}1H-benz-[de]isoquinoline-1,3(2H)-dione (**9d**).* Yield: 38.9%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ_H: 7.53–8.34 (m, 6H, ArH), 5.00 (br s, 1H, –NHCO), 4.01 (t, $J = 15.2$ Hz, 2H), 3.03 (m, 10H), 1.86 (s, 2H), 1.18–150 (m, 37H). ESI-MS *m/z*: 733.4 $(M + Na)^+$.

*4.1.1.5. 2-[4-(3-Butoxycarbonylaminopropyl-butoxycarbonylamino)butyl]1H-benz-[de]isoquinoline-1,3(2H)-dione (**11**).* Yield: 42.2%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ_H: 7.70–8.34 (m, 6H, ArH), 4.98–5.02 (br s, 1H, –NHCO), 4.01 (t, $J = 15.2$ Hz, 2H), 3.03 (m, 6H), 1.86 (s, 2H), 1.18–150 (m, 22H). ESI-MS *m/z*: 526.4 $(M + Na)^+$.

*4.1.2. General procedure for the preparation of **5a–5d, 12***

The respective *N*-Boc protected amine (**9** or **11**) (1.2 mmol) was dissolved in EtOH (20 mL) and stirred at 0 °C for 10 min. HCl (15 mL, 4 M) was added dropwise at 0 °C. The reaction mixture was stirred at room temperature overnight. The solution typically gave a bright white solid as a precipitate. The solid was filtered, washed several times with absolute ethanol

and dried under vacuum to give the pure target compounds (**5a**–**5d**, **12**).

*4.1.2.1. 2-{3-[4-(3-Aminopropylamino)butylamino]propyl}1H-benz-[de]isoquinoline-1,3(2H)-dione trihydrochloride (**5a**).* Yield: 62.3%; m.p. 236–237 °C. ^{1}H NMR (D_2O, 400 MHz) δ_H: 7.44–7.96 (m, 6H, ArH), 3.89 (t, $J = 6.8$ Hz, 2H, $CONCH_2$), 3.10–3.20 (m, 10H, $5CH_2N$), 1.82–2.13 (m, 8H, $4CH_2$) ppm. ^{13}C NMR (D_2O) δ_C: 164.88, 135.20, 131.33, 130.45, 126.90, 126.27, 119.86, 47.01, 46.95, 45.27, 37.28, 36.54, 24.23, 23.74, 22.79 (2C) ppm. ESI-MS *m/z*: 383.2 $(M + H - 3HCl)^+$. Anal. calcd. for $C_{22}H_{33}Cl_3N_4O_2 \cdot 1.30H_2O$: C 51.28%, H 6.96%, N 10.87%; found: C 50.90%, H 7.07%, N 10.48%.

*4.1.2.2. 2-{4-[4-(3-Aminopropylamino)butylamino]butyl}1H-benz-[de]isoquinoline-1,3(2H)-dione trihydrochloride (**5b**).* Yield: 67.2%; m.p. 237–238 °C. ^{1}H NMR (D_2O, 400 MHz) δ_H: 7.34–7.84 (m, 6H, ArH), 3.71 (t, $J = 6.8$ Hz, 2H, $CONCH_2$), 3.07–3.17 (m, 10H, $5CH_2N$), 1.56–2.10 (m, 10H, $5CH_2$) ppm. ^{13}C NMR (D_2O) δ_C: 164.18, 134.47, 130.64, 129.78, 126.28, 125.56, 119.30, 46.75, 46.50, 46.27, 44.10, 39.15, 35.99, 23.79, 23.29, 22.74, 22.35 (2C) ppm. ESI-MS *m/z*: 397.3 $(M + H - 3HCl)^+$. Anal. calcd. for $C_{23}H_{35}Cl_3N_4O_2 \cdot 1.70H_2O$: C 51.49%, H 7.21%, N 10.44%; found: C 51.33%, H 7.13%, N 10.50%.

*4.1.2.3. 2-{3-[4-(4-Aminobutylamino)butylamino]propyl}1H-benz-[de]isoquinoline-1,3(2H)-dione trihydrochloride (**5c**).* Yield: 61.1%; m.p. 236.5–237.5 °C. ^{1}H NMR (D_2O, 400 MHz) δ_H: 7.27–7.78 (m, 6H, ArH), 3.72 (t, $J = 6.4$ Hz, 2H, $CONCH_2$), 2.89–2.95 (m, 10H, $5CH_2N$), 1.63–1.85 (m, 10H, $5CH_2$) ppm. ^{13}C NMR (D_2O) δ_C: 164.80, 135.16, 131.27, 130.38, 126.85, 126.19, 119.78, 46.94, 46.88, 46.83, 45.24, 38.75, 37.24, 24.19, 23.88, 22.78, 22.73 (2C) ppm. ESI-MS *m/z*: 397.3 $(M + H - 3HCl)^+$. Anal. calcd. for $C_{23}H_{35}Cl_3N_4O_2 \cdot 1.10H_2O$: C 52.55%, H 7.14%, N 10.66%; found: C 52.70%, H 7.40%, N 10.56%.

*4.1.2.4. 2-{4-[4-(4-Aminobutylamino)butylamino]butyl}1H-benz-[de]isoquinoline-1,3(2H)-dione trihydrochloride (**5d**).* Yield: 79.8%; m.p. 245–246 °C. ^{1}H NMR (D_2O, 400 MHz) δ_H: 7.20–7.67 (m, 6H, ArH), 3.59 (t, 2H, $J = 4.8$ Hz, $CONCH_2$), 3.00–3.09 (m, 10H, $5CH_2N$), 1.52–1.77 (m, 12H, $6CH_2$) ppm. ^{13}C NMR (D_2O) δ_C: 163.85, 134.34, 130.47, 129.52, 126.17, 125.21, 118.96, 46.63, 46.40, 46.36, 46.33, 39.04, 38.27, 23.63, 23.39, 22.67, 22.30, 22.34 (2C) ppm. ESI-MS *m/z*: 411.3 $(M + H - 3HCl)^+$. Anal. calcd. for $C_{24}H_{37}Cl_3N_4O_2 \cdot 1.20H_2O$: C 53.23%, H 7.33%, N 10.35%; found: C 53.08%, H 7.39%, N 10.23%.

*4.1.2.5. 2-[4-(3-Aminopropylamino)butyl]1H-benz-[de]isoquinoline-1,3(2H)-dione dihydrochloride (**12**).* Yield: 66.1%; m.p. 237–239 °C. ^{1}H NMR (D_2O, 400 MHz) δ_H: 7.22–7.74 (m, 6H, ArH), 3.57 (t, $J = 15.2$ Hz, 2H), 3.03–3.05 (m, 6H), 1.81 (m, 2H), 1.45–1.48 (m, 2H), 1.38–1.39 (m, 2H) ppm. ^{13}C NMR (D_2O) δ_C: 164.75, 135.02, 131.19, 130.35, 126.54, 126.13, 119.88, 47.35, 44.51, 39.60, 36.60, 24.21, 23.78, 23.18 ppm. ESI-MS *m/z*: 326.1 $(M + H - 2HCl)^+$. Anal. calcd. for $C_{19}H_{25}Cl_2N_3O_2 \cdot 1.00H_2O$: C 54.81%, H 6.54%, N 10.09%; found: 54.72%, H 6.47%, N 10.08%.

4.2. Biological materials and methods

All chemicals used in bioassay were purchased from Sigma, unless otherwise indicated. RPMI 1640 and fetal calf serum (FCS) were purchased from Gibco. Calf-thymus DNA was purchased from the Sigma Biotechnology Company. Primary antibodies against caspase-3, caspase-8, caspase-9, cytochrome *c*, Bcl-2, Bax as well as peroxidase-conjugated goat antimouse or antirabbit secondary antibody were purchased from Santa Cruz biotechnology. Stock solution (10 mM) was prepared in DMSO and diluted to various concentrations with serum-free culture medium.

4.2.1. Cell culture

Cell lines, K562, B16, CHO, BEL-7402 and QSG-7701 were obtained from American Type Culture Collection (ATCC). Cells were cultured in RPMI 1640, supplemented with 10% heat-inactivated fetal calf serum (FCS) or 20% heat-inactivated FCS for QSG-7701 cells, antibiotics (penicillin, 100 units/mL; streptomycin sulfate, 100 μg/mL) at 37 °C, in an atmosphere of 95% air and 5% CO_2 under humidified conditions. Aminoguanidine (1 mM) was added as an inhibitor of amine oxidase derived from FCS and had no effect on the various parameters of the cell measured in this study.

4.2.2. MTT assay

Chemosensitivity was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Briefly, exponentially growing K562 cells were seeded into 96-well plates at 4000 cells/well and treated with indicated concentrations of samples for 48 h, and then 10 μL of MTT (10 mg/mL) was added. After incubation for 4 h at 37 °C, the purple formazan crystals (i.e. a reduced form of MTT) generated from viable cells were dissolved by adding 100 μL 10% SDS (Sodium dodecyl sulfate) in each well. The absorbance of each well was then read at 570 nm.

In addition, exponentially growing B16, CHO, BEL-7402 and QSG-7701 were seeded into 96-well plates at 5000 cells/well and allowed to attach to plate bottom overnight. The cells were treated with the indicated concentration of samples for 48 h, and then 100 μL of MTT (1 mg/mL) was added. After incubation for 4 h at 37 °C, the MTT solution was removed and the remaining formazan crystals were dissolved with 150 μL DMSO in each well. The absorbance of each well was then read at 570 nm.

4.2.3. DNA binding

Solutions of ct DNA in 20 mM Tris–HCl buffer (pH 7.4) gave a ratio of UV absorbance at 260 and 280 nm of 1.81:1, indicating that the DNA was sufficiently free of protein. The concentration of ct DNA (2.74×10^{-4} M) was determined spectrophotometrically assuming that the molar absorption is 6600 mol L^{-1} cm^{-1} (260 nm).

Solution of **5d** (2 mL, 2.00×10^{-4} mol/L in Tris–HCl (pH = 7.4)) was mixed with 0.0, 0.30, 0.60, 1.00, 1.20, 1.50, 2.00 mL of ct DNA (2.74×10^{-4} M). The mixture was diluted to 5 mL with Tris–HCl (pH = 7.4). Thus, two groups of samples were prepared in the concentration of ct DNA at 0.0, 16.4, 32.9, 54.8, 65.8, 82.2, 109.6 μM. One contained only compound **5d** (80 μM) as a control, the other contained different concentrations of ct DNA but had the same concentration of **5d**. All the above solutions were shaken for 10 min. at room temperature. Fluorescence wavelengths and intensity areas of samples were measured at following conditions: excitation: 356 nm, emission: 320–500 nm.

4.2.4. Apoptosis and cell cycle analysis

Apoptosis was quantified by assessing the fraction of cells with sub-G1 DNA content by flow cytometry. The B16 cells were seeded in 25 cm^2 flasks, and then pre-incubated in RPMI 1640 supplemented with 0.2% FCS for 24 h which induced cell cycle synchronization. The synchronous cells were treated with indicated concentrations of **5d**. After incubated for 48 h, cells were washed twice with ice-cold PBS, fixed and permeabilized with ice-cold 70% ethanol at −20 °C overnight. The cells were treated with 50 μg/ml RNase A at room temperature for 30 min after washed with ice-cold PBS, and finally stained with 50 μg/ml propidium iodide (PI) in the dark at 4 °C for 30 min. The distribution of cell cycle phases with different DNA contents was read in a flow cytometer. Ten thousand events were acquired in each sample.

4.2.5. Western blot

The B16 cells, treated with different concentrations of **5d** for 48 h, were harvested by trypsinization and washed with PBS. Cytosolic and mitochondrial fractions were prepared as described [25]. Briefly, cells were resuspended in 300 μL buffer (2 mM HEPES–KOH (pH 7.5), 10 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 250 mM sucrose and protease inhibitor). After homogenization, unbroken cells, large plasma membrane pieces and nuclei were removed by centrifugation at 1000×*g* for 10 min. The supernatant was subjected to centrifugation at 10,000×*g* for 20 min. The pellet fraction containing mitochondria was resuspended in 500 μL buffer (10 mM Tris–acetate (pH 8.0), 0.5% NP-40, 5 mM $CaCl_2$). The supernatant was further centrifuged at 50,000×*g* for 2 h to generate cytosol. The detection of cytochrome *c* in cytosolic and the mitochondrial fractions was analyzed by western blot. Total cellular protein was isolated using the protein extraction buffer (containing 150 mM NaCl, 10 mM Tris (pH 7.2), 5 mM EDTA, 0.1% Triton-100, 5% glycerol and 2% SDS). Protein concentrations were determined using the protein assay kit. Equal amounts of proteins (50 μg/lane) were fractionated using 12% SDS-PAGE and transferred to PVDF membranes. The membranes were incubated with primary antibodies against caspase-3, caspase-8, caspase-9, cytochrome *c*, Bcl-2 and Bax (1:5000). After washed with PBS, the membranes were incubated with peroxidase-conjugated goat antimouse or antirabbit secondary antibody (1:3000), followed by enhanced chemiluminescence staining through the enhanced chemiluminescence system. Actin was used to normalize protein loading.

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